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Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3221231 A1 2022/12/01

(21) 3 221 231

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) **Date de dépôt PCT/PCT Filing Date:** 2022/05/23
(87) **Date publication PCT/PCT Publication Date:** 2022/12/01
(85) **Entrée phase nationale/National Entry:** 2023/11/22
(86) **N° demande PCT/PCT Application No.:** US 2022/030557
(87) **N° publication PCT/PCT Publication No.:** 2022/251120
(30) **Priorité/Priority:** 2021/05/24 (US63/192,296)

(51) **Cl.Int./Int.Cl.** ***C07K 14/725*** (2006.01), ***A61K 39/00*** (2006.01), ***A61P 35/00*** (2006.01), ***C07K 14/705*** (2006.01)

(71) **Demandeur/Applicant:**
KITE PHARMA, INC., US

(72) **Inventeurs/Inventors:**
MENG, KEVIN P., US;
MURAKAMI, JODI, US;
HAILE, SAMUEL T., US;
LIAO, EDWARD H., US

(74) **Agent:** NORTON ROSE FULBRIGHT CANADA LLP/S.E.N.C.R.L., S.R.L.

(54) **Titre : RECEPTEUR ANTIGENIQUE CHIMERIQUE**
(54) **Title: CHIMERIC ANTIGEN RECEPTOR**

(57) **Abrégé/Abstract:**
Provided are chimeric antigen receptors (CARs) comprising an NGK2D ecto domain. Provided are compositions, cells and cell therapies comprising the same. Further provided are methods of treatment.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
01 December 2022 (01.12.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/251120 A3

(51) International Patent Classification:
C07K 14/725 (2006.01) *A61K 39/00* (2006.01)
C07K 14/705 (2006.01) *A61P 35/00* (2006.01)

(21) International Application Number:
PCT/US2022/030557

(22) International Filing Date:
23 May 2022 (23.05.2022)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/192,296 24 May 2021 (24.05.2021) US

(71) Applicant: **KITE PHARMA, INC.** [US/US]; 2400 Broadway, Santa Monica, California 90404 (US).

(72) Inventors: **MENG, Kevin P.**; c/o Kite Pharma, Inc., 2400 Broadway, Santa Monica, California 90404 (US). **MURAKAMI, Jodi**; c/o Kite Pharma, Inc., 2400 Broadway, Santa Monica, California 90404 (US). **HAILE, Samuel T.**; c/o Kite Pharma, Inc., 2400 Broadway, Santa Monica, California 90404 (US). **LIAO, Edward H.**; c/o Kite Pharma, Inc., 2400 Broadway, Santa Monica, California 90404 (US).

(74) Agent: **SANNY, Tony** et al.; c/o Kite Pharma, Inc., 2400 Broadway, Santa Monica, California 90404 (US).

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

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

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(88) Date of publication of the international search report:
16 February 2023 (16.02.2023)

WO 2022/251120 A3

(54) Title: NKG2D-BASED CHIMERIC ANTGEN RECEPTOR

(57) Abstract: Provided are chimeric antigen receptors (CARs) comprising an NGK2D ecto domain. Provided are compositions, cells and cell therapies comprising the same. Further provided are methods of treatment.

CHIMERIC ANTIGEN RECEPTOR

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Patent Application No. 63/192,296, filed 24 May 2021 and titled "Chimeric Antigen Receptor," the entirety of which is incorporated by reference herein.

TECHNICAL FIELD

[0002] The present disclosure relates to the field of cell therapy, and more specifically, NKG2D chimeric antigen receptors.

BACKGROUND

[0003] Human cancers are by their nature comprised of normal cells that have undergone a genetic or epigenetic conversion to become abnormal cancer cells. In doing so, cancer cells begin to express proteins and other antigens that are distinct from those expressed by normal cells. These aberrant tumor antigens can be used by the body's innate immune system to specifically target and kill cancer cells. However, cancer cells employ various mechanisms to prevent immune cells, such as T and B lymphocytes, from successfully targeting cancer cells.

[0004] Current T cell therapies rely on enriched or modified human T cells to target and kill cancer cells in a patient. To increase the ability of T cells to target and kill a particular cancer cell, methods have been developed to engineer T cells to express constructs which direct T cells to a particular target cancer cell. Chimeric antigen receptors (CARs) and engineered T cell receptors (TCRs), which comprise binding domains capable of interacting with a particular tumor antigen, allow T cells to target and kill cancer cells that express the particular tumor antigen. However, some tumor types, and in particular solid tumors, are resistant to T cell immunotherapy, and a need for development of next-generation enhancement strategies to target tumor intrinsic resistance mechanisms to T cell immunotherapy. NKG2D ligands are expressed on most types of tumors, and they demonstrate relative selectivity of ligand expression on tumor cells compared with healthy cells and represent a target to augment traditional T cell therapies.

SUMMARY

[0005] Disclosed is a chimeric antigen receptor (CAR), comprising a NKG2D ecto domain; a transmembrane domain; a 4-1BB costimulatory domain; and a signaling domain comprising a CD3-zeta signaling domain. In embodiments, the 4-1BB costimulatory domain comprises the amino acid sequence according to SEQ ID NO: 33. In embodiments, the CD3zeta signaling

domain comprises the amino acid sequence according to SEQ ID NO: 27. In embodiments, the CAR further comprises a CD8-alpha hinge domain. In embodiments. the CD8-alpha hinge domain comprises the amino acid sequence according to SEQ ID NO: 15. In embodiments, the NKG2D ecto domain comprises the amino acid sequence according to SEQ ID NO: 3. In embodiments, the transmembrane domain comprises a CD28 transmembrane domain. In embodiments, the CD28 transmembrane domain comprises the amino acid sequence according to SEQ ID NO: 21. In embodiments, the signaling domain further comprises a CD3-epsilon signaling domain. In embodiments, the CD3-epsilon signaling domain comprises the amino acid sequence according to SEQ ID NO: 31.

[0006] Disclosed is a nucleic acid encoding a disclosed CAR and a vector comprising the same. In embodiments, the recombinant vector or nucleic acid further comprises a nucleic acid encoding an engineered T cell receptor (TCR) specific for a tumor antigen. In embodiments, the recombinant vector or nucleic acid further comprises a nucleic acid encoding a second CAR that is specific for a tumor antigen. In embodiments, the tumor antigen comprises HPV–16 E6, HPV–16 E7, alpha folate receptor, 5T4, αvβ6 integrin, BCMA, B7–H3, B7–H6, CAIX, CD19, CD20, CD22, CD28, CD30, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD137 (4–1BB), CD138, CD171, CEA, CSPG4, CLL-1, CS1. EGFR, EGFR family including ErbB2 (HERII), EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, Flt3, FAP, fetal AchR, FRa, GD2, GD3, Glypican–3 (GPC3), HLA–Al + MAGEI, HLA–A2 + MAGE1, HLAA3 + MAGE1, HLA–Al + NY–ES0–1, HLA–A2 + NY–ES0–1, HLA–A3 + NY–ES0–1, IL–llRα, IL–13Rα2, Lambda, Lewis–Y, Kappa, Mesothelin, Mucl, Muc16, NCAM, NKG2D Ligands, NYE-S0–1, PRAME, PSCA, PSMA, RORI, SSX, Survivin, TACI, TAG72, TEMs, or VEGFRII.

[0007] Disclosed is a host cell transformed with a disclosed nucleic acid or recombinant vector. In embodiments, a host cell is transformed with disclosed nucleic acid or recombinant vector and a nucleic acid or recombinant vector encoding an engineered T cell receptor (TCR) that is specific for a tumor antigen or a second CAR that is specific for a tumor antigen. In embodiments, the tumor antigen comprises HPV–16 E6, HPV–16 E7, alpha folate receptor, 5T4, αvβ6 integrin, BCMA, B7–H3, B7–H6, CAIX, CD19, CD20, CD22, CD28, CD30, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD137 (4–1BB), CD138, CD171, CEA, CSPG4, CLL-1, CS1, EGFR, EGFR family including ErbB2 (HERII), EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, FAP, fetal AchR, FRa, Flt3, GD2, GD3, Glypican–3 (GPC3), HLA–Al + MAGEI, HLA–A2 + MAGE1, HLAA3 + MAGE1, HLA–Al + NY–ES0–1, HLA–A2 + NY–ES0–1, HLA–A3 + NY–ES0–1, IL–llRα, IL–13Rα2, Lambda, Lewis–Y, Kappa, Mesothelin, Mucl, Muc16, NCAM, NKG2D Ligands, NYE-S0–1, PRAME, PSCA, PSMA, RORI, SSX, Survivin, TACI, TAG72, TEMs, or VEGFRII. In embodiments, the host cell comprises an induced

pluripotent stem cell (iPSC), a T cell, or a NK cell. Disclosed is a pharmaceutical composition comprising a disclosed T cell and/or an NK cell. Disclosed is a method of treating disease in a patient in need of thereof, comprising administering a disclosed T cell and/or an NK cell, or the pharmaceutical composition to the patient. In embodiments, the host cell is allogeneic to the patient.

DETAILED DESCRIPTION OF THE INVENTION

[0008] Terms

[0009] In order for the present disclosure to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms are set forth throughout the Specification.

[0010] As used in this Specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

[0011] Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive and covers both "or" and "and".

[0012] The term "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include A and B; A or B; A (alone); and B (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0013] The term "*e.g.*," as used herein, is used merely by way of example, without limitation intended, and should not be construed as referring only those items explicitly enumerated in the specification.

[0014] The terms "or more", "at least", "more than", and the like, *e.g.*, "at least one" are understood to include but not be limited to at least 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, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 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, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149 or 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000 or more than the stated value. Also included is any greater number or fraction in between.

[0015] Conversely, the term "no more than" includes each value less than the stated value. For example, "no more than 100 nucleotides" includes 100, 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 79, 78, 77, 76, 75, 74, 73, 72, 71, 70, 69, 68, 67, 66, 65, 64, 63, 62, 61, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, and 0 nucleotides. Also included is any lesser number or fraction in between.

[0016] The terms "plurality", "at least two", "two or more", "at least second", and the like, are understood to include but not limited to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 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, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149 or 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000 or more. Also included is any greater number or fraction in between.

[0017] Throughout the specification the word "comprising," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps. It is understood that wherever aspects are described herein with the language "comprising," otherwise analogous aspects described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0018] Unless specifically stated or evident from context the term "about" refers to a value or composition that is within an acceptable error range for the particular value or composition as determined by one of ordinary skill in the art, which will depend in part on how the value or composition is measured or determined, *i.e.*, the limitations of the measurement system. For example, "about" or "comprising essentially of" can mean within one or more than one standard deviation per the practice in the art. "About" or "comprising essentially of" can mean a range of up to 10% (*i.e.*, ±10%). Thus, "about" can be understood to be within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, or 0.001% greater or less than the stated value. For example, about 5 mg can include any amount between 4.5 mg and 5.5 mg. Furthermore, particularly with respect to biological systems or processes, the terms can mean up to an order of magnitude or up to 5-fold of a value. When particular values or compositions are provided in the

instant disclosure, unless otherwise stated, the meaning of "about" or "comprising essentially of" should be assumed to be within an acceptable error range for that particular value or composition.

[0019] As described herein, any concentration range, percentage range, ratio range or integer range is to be understood to be inclusive of the value of any integer within the recited range and, when appropriate, fractions thereof (such as one-tenth and one-hundredth of an integer), unless otherwise indicated.

[0020] Units, prefixes, and symbols used herein are provided using their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range.

[0021] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, Juo, "The Concise Dictionary of Biomedicine and Molecular Biology", 2nd ed., (2001), CRC Press; "The Dictionary of Cell & Molecular Biology", 5th ed., (2013), Academic Press; and "The Oxford Dictionary Of Biochemistry And Molecular Biology", Cammack *et al.* eds., 2nd ed, (2006), Oxford University Press, provide those of skill in the art with a general dictionary for many of the terms used in this disclosure.

[0022] "Administering" refers to the physical introduction of an agent to a subject, such as a modified T cell disclosed herein, using any of the various methods and delivery systems known to those skilled in the art. Exemplary routes of administration for the formulations disclosed herein include intravenous, intramuscular, subcutaneous, intraperitoneal, spinal or other parenteral routes of administration, for example by injection or infusion. The phrase "parenteral administration" means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intralymphatic, intralesional, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion, as well as *in vivo* electroporation. In some embodiments, the formulation is administered via a non-parenteral route, *e.g.,* orally. Other non-parenteral routes include a topical, epidermal or mucosal route of administration, for example, intranasally, vaginally, rectally, sublingually or topically. Administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods.

[0023] The terms, "activated" and "activation" refer to the state of a T cell that has been sufficiently stimulated to induce detectable cellular proliferation. In one embodiment, activation may also be associated with induced cytokine production, and detectable effector functions. The term "activated T cells" refers to, among other things, T cells that are proliferating. Signals generated through the TCR alone may be insufficient for full activation of the T cell and one or more secondary or costimulatory signals may also be required. Thus, T cell activation comprises

a primary stimulation signal through the TCR/CD3 complex and one or more secondary costimulatory signals. Costimulation may be evidenced by proliferation and/or cytokine production by T cells that have received a primary activation signal, such as stimulation through the TCR/CD3 complex.

[0024] The term "antibody" (Ab) includes, without limitation, a glycoprotein immunoglobulin which binds specifically to an antigen. In general, and antibody can comprise at least two heavy (H) chains and two light (L) chains interconnected by disulfide bonds, or an antigen-binding molecule thereof. Each H chain comprises a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region comprises three constant domains, CH1, CH2 and CH3. Each light chain comprises a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprises one constant domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL comprises three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the Abs may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (C1q) of the classical complement system. In general, human antibodies are approximately 150 kD tetrameric agents composed of two identical heavy (H) chain polypeptides (about 50 kD each) and two identical light (L) chain polypeptides (about 25 kD each) that associate with each other into what is commonly referred to as a "Y-shaped" structure. The heavy and light chains are linked or connected to one another by a single disulfide bond; two other disulfide bonds connect the heavy chain hinge regions to one another, so that the dimers are connected to one another and the tetramer is formed. Naturally-produced antibodies are also glycosylated, *e.g.*, on the C_H2 domain.

[0025] The term "human antibody" is intended to comprise antibodies having variable and constant domain sequences generated, assembled, or derived from human immunoglobulin sequences, or sequences indistinguishable therefrom. In some embodiments, antibodies (or antibody components) may be considered to be "human" even though their amino acid sequences comprise residues or elements not encoded by human germline immunoglobulin sequences (*e.g.*, variations introduced by *in vitro* random or site-specific mutagenesis or introduced by *in vivo* somatic mutation). The term "humanized" is intended to comprise antibodies having a variable domain with a sequence derived from a variable domain of a non-human species (*e.g.*, a mouse), modified to be more similar to a human germline encoded sequence. In some embodiments, a

"humanized" antibody comprises one or more framework domains having substantially the amino acid sequence of a human framework domain, and one or more complementary determining regions having substantially the amino acid sequence as that of a non-human antibody. In some embodiments, a humanized antibody comprises at least a portion of an immunoglobulin constant region (Fc), generally that of a human immunoglobulin constant domain. In some embodiments, a humanized antibodies may comprise a C_H1, hinge, C_H2, C_H3, and, optionally, a C_H4 region of a human heavy chain constant domain.

[0026] Antibodies can include, for example, monoclonal antibodies, recombinantly produced antibodies, monospecific antibodies, multispecific antibodies (including bispecific antibodies), human antibodies, engineered antibodies, humanized antibodies, chimeric antibodies, immunoglobulins, synthetic antibodies, tetrameric antibodies comprising two heavy chain and two light chain molecules, an antibody light chain monomer, an antibody heavy chain monomer, an antibody light chain dimer, an antibody heavy chain dimer, an antibody light chain- antibody heavy chain pair, intrabodies, antibody fusions (sometimes referred to herein as "antibody conjugates"), heteroconjugate antibodies, single domain antibodies, monovalent antibodies, single chain antibodies or single-chain Fvs (scFv), camelized antibodies, affybodies, Fab fragments, $F(ab')_2$ fragments, disulfide-linked Fvs (sdFv), anti-idiotypic (anti-Id) antibodies (including, *e.g.*, anti-anti-Id antibodies), minibodies, domain antibodies, synthetic antibodies (sometimes referred to herein as "antibody mimetics"), and antigen binding fragments of any of the above. In certain embodiments, antibodies described herein refer to polyclonal antibody populations. Antibodies may also comprise, for example, Fab′ fragments, Fd′ fragments, Fd fragments, isolated CDRs, single chain Fvs, polypeptide-Fc fusions, single domain antibodies (e.g., shark single domain antibodies such as IgNAR or fragments thereof, and human heavy-chain antibodies (UniAbs)), camelid antibodies, single chain or Tandem diabodies (TandAb®), Anticalins®, Nanobodies® minibodies, BiTE®s, ankyrin repeat proteins or DARPINs®, Avimers®, DARTs, TCR-like antibodies, Adnectins®, Affilins®, Trans-bodies®, Affibodies®, TrimerX®, MicroProteins, Fynomers®, Centyrins®, and KALBITOR®s.

[0027] An immunoglobulin may derive from any of the commonly known isotypes, including but not limited to IgA, secretory IgA, IgG, IgE and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgG1, IgG2, IgG3 and IgG4. "Isotype" refers to the Ab class or subclass (*e.g.*, IgM or IgG1) that is encoded by the heavy chain constant region genes. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring Abs; monoclonal and polyclonal Abs; chimeric and humanized Abs; human or nonhuman Abs; wholly synthetic Abs; and single chain Abs. A nonhuman Ab may be humanized by recombinant methods to reduce its immunogenicity in man. Where not expressly

stated, and unless the context indicates otherwise, the term "antibody" also includes an antigen binding fragment or an antigen-binding portion of any of the aforementioned immunoglobulins, and includes a monovalent and a divalent fragment or portion, and a single chain Ab.

[0028] An "antigen binding molecule," "antigen binding portion," "antigen binding fragment," or "antibody fragment" or "antigen binding domain" refers to any molecule that comprises the antigen binding parts of the molecule. In an example an antigen binding molecule is an antibody, or portion thereof, such as an scFv. In an example an antigen biding molecule is a portion of a TCR that binds antigen, and may be the antigen binding portion of the TCR alpha chain and/or the antigen binding portion of a TCR alpha chain. In an example, an antigen biding molecule may be a portion of NKG2D that binds an NKG2D ligand. An antigen binding molecule can include the antigenic complementarity determining regions (CDRs). Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')2, and Fv fragments, dAb, linear antibodies, scFv antibodies, and multispecific antibodies formed from antigen binding molecules. Peptibodies (i.e., Fc fusion molecules comprising peptide binding domains) are another example of suitable antigen binding molecules. In some embodiments, the antigen binding molecule binds to an antigen on a tumor cell. In some embodiments, the antigen binding molecule binds to an antigen on a cell involved in a hyperproliferative disease or to a viral or bacterial antigen. In embodiments, an antigen binding molecule is a chimeric antigen receptor (CAR) or an engineered T cell receptor (TCR). In certain embodiments, the antigen binding molecule or domain is an antibody fragment that specifically binds to the antigen, including one or more of the complementarity determining regions (CDRs) thereof. In further embodiments, the antigen binding molecule is a single chain variable fragment (scFv). In some embodiments, the antigen binding molecule or domain comprises or consists of avimers.

[0029] In some instances, a CDR is substantially identical to one found in a reference antibody (*e.g.,* an antibody of the present disclosure) and/or the sequence of a CDR provided in the present disclosure. In some embodiments, a CDR is substantially identical to a reference CDR in that it is either identical in sequence or contains between 1, 2, 3, 4, or 5 (*e.g.,* 1-5) amino acid substitutions as compared with the reference CDR. In some embodiments a CDR is substantially identical to a reference CDR in that it shows at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the reference CDR (*e.g.,* 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%). In some embodiments a CDR is substantially identical to a reference CDR in that it shows at least 96%, 96%, 97%, 98%, 99%, or 100% sequence identity with the reference CDR. In some embodiments a CDR is substantially identical to a reference CDR in that one amino acid within the CDR is deleted, added, or substituted as compared with the reference CDR while the CDR has an amino acid sequence that is otherwise

identical with that of the reference CDR. In some embodiments a CDR is substantially identical to a reference CDR in that 2, 3, 4, or 5 (*e.g.,* 2-5) amino acids within the CDR are deleted, added, or substituted as compared with the reference CDR while the CDR has an amino acid sequence that is otherwise identical to the reference CDR. In various embodiments, an antigen binding fragment binds a same antigen as a reference antibody. In various embodiments, an antigen binding fragment cross-competes with the reference antibody, for example, binding to substantially the same or identical epitope as the reference antibody

[0030] An antigen binding fragment may be produced by any means. For example, in some embodiments, an antigen binding fragment may be enzymatically or chemically produced by fragmentation of an intact antibody. In some embodiments, an antigen binding fragment may be recombinantly produced (such as by expression of an engineered nucleic acid sequence). In some embodiments, an antigen binding fragment may be wholly or partially synthetically produced. In some embodiments, an antigen binding fragment may have a length of at least about 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 amino acids or more; in some embodiments at least about 200 amino acids (e.g., 50-100, 50-150, 50-200, or 100-200 amino acids).

[0031] The term "variable region" or "variable domain" is used interchangeably. The variable region typically refers to a portion of an antibody, generally, a portion of a light or heavy chain, typically about the amino-terminal 110 to 120 amino acids in the mature heavy chain and about 90 to 115 amino acids in the mature light chain, which differ extensively in sequence among antibodies and are used in the binding and specificity of a particular antibody for its particular antigen. The variability in sequence is concentrated in those regions called complementarity determining regions (CDRs) while the more highly conserved regions in the variable domain are called framework regions (FR). Without wishing to be bound by any particular mechanism or theory, it is believed that the CDRs of the light and heavy chains are primarily responsible for the interaction and specificity of the antibody with antigen. In certain embodiments, the variable region is a human variable region. In certain embodiments, the variable region comprises rodent or murine CDRs and human framework regions (FRs). In embodiments, the variable region is a primate (*e.g.*, non-human primate) variable region. In certain embodiments, the variable region comprises rodent or murine CDRs and primate (*e.g.*, non-human primate) framework regions (FRs).

[0032] The terms "VL" and "VL domain" are used interchangeably to refer to the light chain variable region of an antibody or an antigen-binding molecule thereof.

[0033] The terms "VH" and "VH domain" are used interchangeably to refer to the heavy chain variable region of an antibody or an antigen-binding molecule thereof.

[0034] A number of definitions of the CDRs are commonly in use: Kabat numbering, Chothia numbering, AbM numbering, or contact numbering. The AbM definition is a compromise between the two used by Oxford Molecular's AbM antibody modelling software. The contact definition is based on an analysis of the available complex crystal structures.

[0035] Table 1. CDR Numbering

Loop	Kabat	AbM	Chothia	Contact
L1	L24--L34	L24--L34	L24--L34	L30--L36
L2	L50--L56	L50--L56	L50--L56	L46--L55
L3	L89--L97	L89--L97	L89--L97	L89--L96
H1	H31--H35B (Kabat Numbering)	H26--H35B	H26--H32..34	H30--H35B
H1	H31--H35 (Chothia Numbering)	H26--H35	H26--H32	H30--H35
H2	H50--H65	H50--H58	H52--H56	H47--H58
H3	H95--H102	H95--H102	H95--H102	H93--H101

[0036] The term "Kabat numbering" and like terms are recognized in the art and refer to a system of numbering amino acid residues in the heavy and light chain variable regions of an antibody, or an antigen-binding molecule thereof. In certain aspects, the CDRs of an antibody can be determined according to the Kabat numbering system (*see*, *e.g.*, Kabat EA & Wu TT (1971) Ann NY Acad Sci 190: 382-391 and Kabat EA *et al.*, (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). Using the Kabat numbering system, CDRs within an antibody heavy chain molecule are typically present at amino acid positions 31 to 35, which optionally can include one or two additional amino acids, following 35 (referred to in the Kabat numbering scheme as 35A and 35B) (CDR1), amino acid positions 50 to 65 (CDR2), and amino acid positions 95 to 102 (CDR3). Using the Kabat numbering system, CDRs within an antibody light chain molecule are typically present at amino acid positions 24 to 34 (CDR1), amino acid positions 50 to 56 (CDR2), and amino acid positions 89 to 97 (CDR3). In a specific embodiment, the CDRs of the antibodies described herein have been determined according to the Kabat numbering scheme.

[0037] In certain aspects, the CDRs of an antibody can be determined according to the Chothia numbering scheme, which refers to the location of immunoglobulin structural loops (*see*, *e.g.*, Chothia C & Lesk AM, (1987), J Mol Biol 196: 901-917; Al-Lazikani B *et al.*, (1997) J Mol Biol 273: 927-948; Chothia C *et al.*, (1992) J Mol Biol 227: 799-817; Tramontano A *et al.*, (1990) J

Mol Biol 215(1): 175-82; and U.S. Patent No. 7,709,226). Typically, when using the Kabat numbering convention, the Chothia CDR-H1 loop is present at heavy chain amino acids 26 to 32, 33, or 34, the Chothia CDR-H2 loop is present at heavy chain amino acids 52 to 56, and the Chothia CDR-H3 loop is present at heavy chain amino acids 95 to 102, while the Chothia CDR-L1 loop is present at light chain amino acids 24 to 34, the Chothia CDR-L2 loop is present at light chain amino acids 50 to 56, and the Chothia CDR-L3 loop is present at light chain amino acids 89 to 97. The end of the Chothia CDR-HI loop when numbered using the Kabat numbering convention varies between H32 and H34 depending on the length of the loop (this is because the Kabat numbering scheme places the insertions at H35A and H35B; if neither 35A nor 35B is present, the loop ends at 32; if only 35A is present, the loop ends at 33; if both 35A and 35B are present, the loop ends at 34). In a specific embodiment, the CDRs of the antibodies described herein have been determined according to the Chothia numbering scheme.

[0038] The terms "constant region" and "constant domain" are interchangeable and have a meaning common in the art. The constant region is an antibody portion, *e.g.*, a carboxyl terminal portion of a light and/or heavy chain which is not directly involved in binding of an antibody to antigen but which can exhibit various effector functions, such as interaction with the Fc receptor. The constant region of an immunoglobulin molecule generally has a more conserved amino acid sequence relative to an immunoglobulin variable domain.

[0039] The term "heavy chain" when used in reference to an antibody can refer to any distinct type, *e.g.*, alpha (α), delta (δ), epsilon (ε), gamma (γ) and mu (μ), based on the amino acid sequence of the constant domain, which give rise to IgA, IgD, IgE, IgG and IgM classes of antibodies, respectively, including subclasses of IgG, *e.g.*, IgG_1, IgG_2, IgG_3 and IgG_4.

[0040] The term "light chain" when used in reference to an antibody can refer to any distinct type, *e.g.*, kappa (κ) or lambda (λ) based on the amino acid sequence of the constant domains. Light chain amino acid sequences are well known in the art. In specific embodiments, the light chain is a human light chain.

[0041] An "antigen" refers to a compound, composition, or substance that may stimulate the production of antibodies or a T cell response in a human or animal, including compositions (such as one that includes a tumor–specific protein) that are injected or absorbed into a human or animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous antigens, such as the disclosed antigens. A "target antigen" or "target antigen of interest" is an antigen that is not substantially found on the surface of other normal (desired) cells and to which a binding domain of a TCR or CAR contemplated herein, is designed to bind. A person of skill in the art would readily understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. An antigen can be endogenously

expressed, *i.e.* expressed by genomic DNA, or can be recombinantly expressed. An antigen can be specific to a certain tissue, such as a cancer cell, or it can be broadly expressed. In addition, fragments of larger molecules can act as antigens. A "target" is any molecule bound by a binding motif, CAR, TCR or antigen binding agent, *e.g.*, an antibody.

[0042] "Antigen-specific targeting region" (ASTR) refers to the region of the CAR or TCR which targets specific antigens. The targeting regions on the CAR or TCR are extracellular. In some embodiments, the antigen-specific targeting regions comprise an antibody or a functional equivalent thereof or a fragment thereof or a derivative thereof and each of the targeting regions target a different antigen. The targeting regions may comprise full length heavy chain, Fab fragments, single chain Fv (scFv) fragments, divalent single chain antibodies or diabodies, each of which are specific to the target antigen. There are, however, numerous alternatives, such as linked cytokines (which leads to recognition of cells bearing the cytokine receptor), affibodies, ligand binding domains from naturally occurring receptors, such as NKG2D, soluble protein/peptide ligand for a receptor (for example on a tumor cell), peptides, and vaccines to prompt an immune response, which may each be used in various embodiments of this disclosure. In fact, almost any molecule that binds a given antigen with high affinity can be used as an antigen-specific targeting region, as will be appreciated by those of skill in the art.

[0043] "Antigen presenting cell" or "APC" refers to cells that process and present antigens to T cells. Exemplary APCs comprise dendritic cells, macrophages, B cells, certain activated epithelial cells, and other cell types capable of TCR stimulation and appropriate T cell costimulation.

[0044] An "anti-tumor effect" refers to a biological effect that can present as a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in tumor cell proliferation, a decrease in the number of metastases, an increase in overall or progression-free survival, an increase in life expectancy, or amelioration of various physiological symptoms associated with the tumor. An anti-tumor effect can also refer to the prevention of the occurrence of a tumor.

[0045] Two events or entities are "associated" with one another if the presence, level, and/or form of one is correlated with that of the other. For example, an entity (*e.g.*, polypeptide, genetic signature, metabolite, microbe, etc.) is considered to be associated with a disease, disorder, or condition, if its presence, level, and/or form correlates with incidence of and/or susceptibility to the disease, disorder, or condition (*e.g.*, across a relevant population). For example, two or more entities are physically "associated" with one another if they interact, directly or indirectly, so that they are and/or remain in physical proximity with one another (*e.g.*, bind). In additional examples, two or more entities that are physically associated with one another are covalently linked or connected to one another, or non-covalently associated, for example by means of hydrogen bonds, van der Waals interaction, hydrophobic interactions, magnetism, and combinations thereof.

[0046] The term "autologous" refers to any material derived from the same individual to which it is later to be re-introduced. For example, the engineered autologous cell therapy (eACT™) method described herein involves collection of lymphocytes from a patient, which are then engineered to express, *e.g.*, a CAR construct, and then administered back to the same patient.

[0047] "Binding affinity" generally refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (*e.g.*, an antibody) and its binding partner (*e.g.*, an antigen). Unless indicated otherwise "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (*e.g.*, antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_D). Affinity can be measured and/or expressed in a number of ways known in the art, including, but not limited to, equilibrium dissociation constant (K_D), and equilibrium association constant (K_A). The K_D is calculated from the quotient of k_{off}/k_{on}, whereas K_A is calculated from the quotient of k_{on}/k_{off}. k_{on} refers to the association rate constant of, *e.g.*, an antibody to an antigen, and k_{off} refers to the dissociation of, *e.g.*, an antibody to an antigen. The k_{on} and k_{off} can be determined by techniques known to one of ordinary skill in the art, such as BIACORE® or KinExA.

[0048] The term "KD" (M) refers to the dissociation equilibrium constant of a particular antibody-antigen interaction, or the dissociation equilibrium constant of an antibody or antibody-binding fragment binding to an antigen. There is an inverse relationship between K_D and binding affinity, therefore the smaller the K_D value, the higher, i.e. stronger, the affinity. Thus, the terms "higher affinity" or "stronger affinity" relate to a higher ability to form an interaction and therefore a smaller K_D value, and conversely the terms "lower affinity" or "weaker affinity" relate to a lower ability to form an interaction and therefore a larger K_D value. In some circumstances, a higher binding affinity (or K_D) of a particular molecule (e.g. antibody) to its interactive partner molecule (e.g. antigen X) compared to the binding affinity of the molecule (e.g. antibody) to another interactive partner molecule (e.g. antigen Y) may be expressed as a binding ratio determined by dividing the larger K_D value (lower, or weaker, affinity) by the smaller K_D (higher, or stronger, affinity), for example expressed as 5-fold or 10-fold greater binding affinity, as the case may be.

[0049] The term "k_d" (sec -1 or 1/s) refers to the dissociation rate constant of a particular binding pair, such as an antibody-antigen interaction, or the dissociation rate constant of a binding pair, such as an antibody or antibody-binding fragment. Said value is also referred to as the $k_{0i}r$ value.

[0050] The term "k_a" (M-1 x sec-1 or 1/M) refers to the association rate constant of a particular binding pair, such as antibody-antigen interaction, or the association rate constant of a particular binding pair, such as an antibody or antibody-binding fragment.

[0051] The term "K_A" (M-1 or 1/M) refers to the association equilibrium constant of a particular binding pair, such as antibody-antigen interaction, or the association equilibrium constant of a binding pair, such as an antibody or antibody binding fragment. The association equilibrium constant is obtained by dividing the k_a by the k_d.

[0052] The term "binding" generally refers to a non-covalent association between or among two or more entities. Direct binding involves physical contact between entities or moieties. "Indirect" binding involves physical interaction by way of physical contact with one or more intermediate entities. Binding between two or more entities may be assessed in any of a variety of contexts, *e.g.*, where interacting entities or moieties are studied in isolation or in the context of more complex systems (*e.g.*, while covalently or otherwise associated with a carrier entity and/or in a biological system such as a cell).

[0053] The terms "immunospecifically binds," "immunospecifically recognizes," "specifically binds," and "specifically recognizes" are analogous terms in the context of antibodies and refer to molecules that bind to an antigen (*e.g.*, epitope or immune complex) as such binding is understood by one skilled in the art. For example, a molecule that specifically binds to an antigen may bind to other peptides or polypeptides, generally with lower affinity as determined by, *e.g.*, immunoassays, BIACORE®, KinExA 3000 instrument (Sapidyne Instruments, Boise, ID), or other assays known in the art. In a specific embodiment, molecules that specifically bind to an antigen bind to the antigen with a K_A that is at least 2 logs, 2.5 logs, 3 logs, 4 logs or greater than the K_A when the molecules bind to another antigen. Binding may comprise preferential association of a binding domain, antibody, or antigen binding system with a target of the binding domain, antibody, or antigen binding system as compared to association of the binding domain, antibody, or antigen binding system with an entity that is not the target (*i.e.* non-target). In some embodiments, a binding domain, antibody, or antigen binding system selectively binds a target if binding between the binding domain, antibody, or antigen binding system and the target is greater than 2-fold, greater than 5-fold, greater than 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, or greater than 100-fold as compared with binding of the binding domain, antibody, or antigen binding system and a non-target. In some embodiments, a binding domain, antibody, or antigen binding system selectively binds a target if the binding affinity is less than about 10^{-5} M, less than about 10^{-6} M, less than about 10^{-7} M, less than about 10^{-8} M, or less than about 10^{-9} M.

[0054] In another embodiment, molecules that specifically bind to an antigen bind with a dissociation constant (K_d) of about 1×10^{-7} M. In some embodiments, the antigen binding molecule specifically binds an antigen with "high affinity" when the K_d is about 1×10^{-9} M to about 5×10^{-9} M. In some embodiments, the antigen binding molecule specifically binds an

antigen with "very high affinity" when the K_d is 1 x 10^{-10} M to about 5 x 10^{-10} M. In one embodiment, the antigen binding molecule has a K_d of 10^{-9} M. In one embodiment, the off-rate is less than about 1 x 10^{-5}.

[0055] In certain embodiments, provided herein is an antibody or an antigen binding molecule thereof that binds to the target human antigen with a 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70% or higher affinity than to another species of the target antigen as measured by, *e.g.*, a radioimmunoassay, surface plasmon resonance, or kinetic exclusion assay. In a specific embodiment, an antibody or an antigen binding molecule thereof described herein, which binds to a target human antigen, will bind to another species of the target antigen with less than 10%, 15%, or 20% of the binding of the antibody or an antigen binding molecule thereof to the human antigen as measured by, *e.g.*, a radioimmunoassay, surface plasmon resonance, or kinetic exclusion assay.

[0056] "Cancer" refers to a broad group of various diseases characterized by the uncontrolled growth of abnormal cells in the body. Unregulated cell division and growth results in the formation of malignant tumors that invade neighboring tissues and may also metastasize to distant parts of the body through the lymphatic system or bloodstream. A "cancer" or "cancer tissue" can include a tumor. In some embodiments, the methods of the present disclosure can be used to reduce the tumor size of a tumor derived from, for example, prostate cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, multiple myeloma, Hodgkin's Disease, non-Hodgkin's lymphoma (NHL), primary mediastinal large B cell lymphoma (PMBC), diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), transformed follicular lymphoma, splenic marginal zone lymphoma (SMZL), cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, chronic or acute leukemia, acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia (ALL) (including non T cell ALL), chronic lymphocytic leukemia (CLL), solid tumors of childhood, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T cell lymphoma, environmentally induced cancers including those induced by asbestos, other B cell malignancies, multiple myeloma, and combinations of said cancers. The particular cancer can be responsive to chemo- or radiation

therapy or the cancer can be refractory. A refractory cancer refers to a cancer that is not amendable to surgical intervention and the cancer is either initially unresponsive to chemo- or radiation therapy or the cancer becomes unresponsive over time.

[0057] "Chemokines" are a type of cytokine that mediates cell chemotaxis, or directional movement. Examples of chemokines include, but are not limited to, IL-8, IL-16, eotaxin, eotaxin-3, macrophage-derived chemokine (MDC or CCL22), monocyte chemotactic protein 1 (MCP-1 or CCL2), MCP-4, macrophage inflammatory protein 1α (MIP-1α, MIP-1a), MIP-1β (MIP-1b), gamma-induced protein 10 (IP-10), and thymus and activation regulated chemokine (TARC or CCL17).

[0058] "Chimeric antigen receptor" or "CAR" refers to a molecule engineered to comprise a binding domain and a means of activating immune cells (for example T cells such as naive T cells, central memory T cells, effector memory T cells, NK cells or combination thereof) upon antigen binding. CARs are also known as artificial T cell receptors, chimeric T cell receptors or chimeric immunoreceptors. In some embodiments, a CAR comprises a binding domain, an extracellular domain, a transmembrane domain, one or more co-stimulatory domains, and an intracellular signaling domain. A T cell that has been genetically engineered to express a chimeric antigen receptor may be referred to as a CAR T cell. Similarly, an NK cell that has been genetically engineered to express a chimeric antigen receptor may be referred to as a CAR NK cell.

[0059] By "decrease" or "lower," or "lessen," or "reduce," or "abate" refers generally to the ability of a composition contemplated herein to produce, elicit, or cause a lesser physiological response (i.e., a downstream effect) compared to the response caused by either the vehicle alone (i.e., an active moiety) or a control molecule/composition. A "decrease" or "reduced" amount is typically a "statistically significant" amount, and may include an decrease that is 1.1, 1.2, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 15, 20, 30 or more times (e.g., 500, 1000 times) (including all integers and decimal points in between and above 1, e.g., 1.5, 1.6, 1.7. 1.8, etc.) the response (reference response) produced by vehicle, a control composition.

[0060] "Extracellular domain" (or "ECD") refers to a portion of a polypeptide that, when the polypeptide is present in a cell membrane, is understood to reside outside of the cell membrane, in the extracellular space. Ecto domain may be used herein interchangeably with extracellular domain.

[0061] The term "extracellular ligand-binding domain," as used herein, refers to an oligo- or polypeptide that is capable of binding a ligand, e.g., a cell surface molecule. For example, the extracellular ligand-binding domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state (e.g., cancer). Examples of cell

surface markers that may act as ligands include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells.

[0062] The binding domain of the CAR may be followed by a "spacer," or, "hinge," 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). The hinge region in a CAR is generally between the transmembrane (TM) and the binding domain. In certain embodiments, a hinge region is an immunoglobulin hinge region and may be a wild type immunoglobulin hinge region or an altered wild type immunoglobulin hinge region, such as an Igg4 hinge. Other exemplary hinge regions used in the CARs described herein include the hinge region derived from the extracellular regions of type 1 membrane proteins such as CD8alpha, CD4, CD28 and CD7, which may be wild-type hinge regions from these molecules or may be altered.

[0063] The "transmembrane" region or domain is the portion of the CAR that anchors the extracellular binding portion to the plasma membrane of the immune effector cell and facilitates binding of the binding domain to the target antigen. The transmembrane domain may be a CD3zeta transmembrane domain, however other transmembrane domains that may be employed include those obtained from CD8alpha, CD4, CD28, CD45, CD9, CD16, CD22, CD33, CD64, CD80, CD86, CD134, CD137, NKG2D, 2B4 and CD154. In certain embodiments, the transmembrane domain is synthetic in which case it would comprise predominantly hydrophobic residues such as leucine and valine.

[0064] The "intracellular signaling domain" or “signaling domain” refers to the part of the chimeric antigen receptor protein that participates in transducing the message of effective CAR binding to a target antigen 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 term "effector function" refers to a specialized function of the 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 terms "intracellular signaling domain" or “signaling domain,” used interchangeably herein, refer 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 intracellular 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 an intracellular 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 intracellular signaling domain is meant to include any truncated portion of the intracellular signaling domain sufficient

to transducing effector function signal. The intracellular signaling domain is also known as the, "signal transduction domain," and is typically derived from portions of the human CD3 or FcRy chains.

[0065] It is known that signals generated through the T cell receptor alone are insufficient for full activation of the T cell and that a secondary, or costimulatory signal is also required. Thus, T cell activation can be said to be mediated by two distinct classes of cytoplasmic signaling sequences: those that initiate antigen dependent primary activation through the T cell receptor (primary cytoplasmic signaling sequences) and those that act in an antigen independent manner to provide a secondary or costimulatory signal (secondary cytoplasmic signaling sequences). Cytoplasmic signaling sequences that act in a costimulatory manner may contain signaling domains which are known as immunoreceptor tyrosine-based activation domain or ITAMs.

[0066] Examples of ITAM containing primary cytoplasmic signaling sequences that are of particular use in the disclosure include those derived from DAP10, DAP12, TCRzeta, FcRgamma, FcRbeta, CD3zeta, CD3gamma, CD3delta, CD3epsilon, CD5, CD22, CD79a, CD79b and CD66d.

[0067] As used herein, the term, "costimulatory signaling domain," or "costimulatory domain", refers to the portion of the CAR comprising the intracellular domain of a costimulatory molecule. Costimulatory 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. Examples of such co-stimulatory molecules include CD27, CD28, 4-1 BB (CD137), OX40 (CD134), CD30, CD40, PD-1, ICOS (CD278), LFA-1, CD2, CD7, LIGHT, NKD2C, 2B4, CD137, DAP12, B7-H2 and a ligand that specifically binds CD83. Accordingly, while the present disclosure provides exemplary costimulatory domains derived from CD28, CD3-epsilon, 4-1BB, other costimulatory domains are contemplated for use with the CARs described herein. The inclusion of one or more co stimulatory signaling domains may enhance the efficacy and expansion of T cells and NK cells expressing CAR receptors. The intracellular signaling and costimulatory signaling domains may be linked in any order in tandem to the carboxyl terminus of the transmembrane domain.

[0068] Although CARs engineered to contain a signaling domain from CD3 or FcRgamma have been shown to deliver a potent signal for T cell activation and effector function, they are not sufficient to elicit signals that promote T cell survival and expansion in the absence of a concomitant costimulatory signal. Other CARs containing a binding domain, a hinge, a transmembrane and the signaling domain derived from CD3zeta or FcRgamma together with one or more costimulatory signaling domains (e.g., intracellular costimulatory domains derived from 4-1BB, CD28, CD134 and CD278) may more effectively direct antitumor activity as well as increased cytokine secretion, lytic activity, survival and proliferation in CAR expressing T cells

in vitro, and in animal models and cancer patients (Milone et al., Molecular Therapy, 2009; 17: 1453-1464; Zhong et al., Molecular Therapy, 2010; 18: 413-420; Carpenito et al., PNAS, 2009; 106:3360-3365).

[0069] A "costimulatory signal" refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to a T cell response, such as, but not limited to, proliferation and/or upregulation or down regulation of key molecules.

[0070] A "costimulatory ligand" includes a molecule on an antigen presenting cell that specifically binds a cognate co-stimulatory molecule on a T cell. Binding of the costimulatory ligand provides a signal that mediates a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A costimulatory ligand induces a signal that is in addition to the primary signal provided by a stimulatory molecule, for instance, by binding of a T cell receptor (TCR)/CD3 complex with a major histocompatibility complex (MHC) molecule loaded with peptide. A co-stimulatory ligand can include, but is not limited to, 3/TR6, 4-1BB ligand, agonist or antibody that binds Toll ligand receptor, B7-1 (CD80), B7-2 (CD86), CD30 ligand, CD40, CD7, CD70, CD83, herpes virus entry mediator (HVEM), human leukocyte antigen G (HLA-G), ILT4, immunoglobulin-like transcript (ILT) 3, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), ligand that specifically binds with B7-H3, lymphotoxin beta receptor, MHC class I chain-related protein A (MICA), MHC class I chain-related protein B (MICB), OX40 ligand, PD-L2, or programmed death (PD) L1. A co-stimulatory ligand includes, without limitation, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as, but not limited to, 4-1BB, B7-H3, CD2, CD27, CD28, CD30, CD40, CD7, ICOS, ligand that specifically binds with CD83, lymphocyte function-associated antigen-1 (LFA-1), natural killer cell receptor C (NKG2C), OX40, PD-1, or tumor necrosis factor superfamily member 14 (TNFSF14 or LIGHT).

[0071] A "costimulatory molecule" is a cognate binding partner on a T cell that specifically binds with a costimulatory ligand, thereby mediating a costimulatory response by the T cell, such as, but not limited to, proliferation. Costimulatory molecules include, but are not limited to, A "costimulatory molecule" is a cognate binding partner on a T cell that specifically binds with a costimulatory ligand, thereby mediating a costimulatory response by the T cell, such as, but not limited to, proliferation. Costimulatory molecules include, but are not limited to, 4-1BB/CD137, B7-H3, BAFFR, BLAME (SLAMF8), BTLA, CD 33, CD 45, CD100 (SEMA4D), CD103, CD134, CD137, CD154, CD16, CD160 (BY55), CD18, CD19, CD19a, CD2, CD22, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3 (alpha; beta; delta; epsilon; gamma; zeta), CD30, CD37, CD4, CD4, CD40, CD49a, CD49D, CD49f, CD5, CD64, CD69, CD7, CD80, CD83 ligand, CD84, CD86, CD8alpha, CD8beta, CD9, CD96 (Tactile), CDl-la, CDl-lb, CDl-lc, CDl-ld, CDS,

CEACAM1, CRT AM, DAP-10, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM (LIGHTR), IA4, ICAM-1, ICAM-1, ICOS, Ig alpha (CD79a), IL2R beta, IL2R gamma, IL7R alpha, integrin, ITGA4, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGBl, KIRDS2, LAT, LFA-1, LFA-1, LIGHT, LIGHT (tumor necrosis factor superfamily member 14; TNFSF14), LTBR, Ly9 (CD229), lymphocyte function-associated antigen-1 (LFA-1 (CDl la/CD18), MHC class I molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX40, PAG/Cbp, PD-1, PSGL1, SELPLG (CD162), signaling lymphocytic activation molecule, SLAM (SLAMF1; CD150; IPO-3), SLAMF4 (CD244; 2B4), SLAMF6 (NTB-A; Lyl08), SLAMF7, SLP-76, TNF, TNFr, TNFR2, Toll ligand receptor, TRANCE/RANKL, VLA1, or VLA-6, or fragments, truncations, or combinations thereof.

[0072] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.,* lysine, arginine, histidine), acidic side chains (*e.g.,* aspartic acid, glutamic acid), uncharged polar side chains (*e.g.,* glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (*e.g.,* alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (*e.g.,* threonine, valine, isoleucine) and aromatic side chains (*e.g.,* tyrosine, phenylalanine, tryptophan, histidine). In certain embodiments, one or more amino acid residues within a CDR(s) or within a framework region(s) of an antibody or antigen-binding molecule thereof can be replaced with an amino acid residue with a similar side chain. In general, two sequences are generally considered to be "substantially similar" if they contain a conservative amino acid substitution in corresponding positions. For example, certain amino acids are generally classified as "hydrophobic" or "hydrophilic" amino acids, and/or as having "polar" or "non-polar" side chains. Substitution of one amino acid for another of the same type may be considered a conservative substitution. Exemplary amino acid categorizations are summarized in Tables 2 and 3 below:

Table 2

Amino Acid	3-Letter	1-Letter	Property	Property	Hydropathy Index
Alanine	Ala	A	nonpolar	neutral	1.8
Arginine	Arg	R	polar	positive	-4.5
Asparagine	Asn	N	polar	neutral	-3.5
Aspartic acid	Asp	D	polar	negative	-3.5
Cysteine	Cys	C	nonpolar	neutral	2.5
Glutamic acid	Glu	E	polar	negative	-3.5
Glutamine	Gln	Q	polar	neutral	-3.5
Glycine	Gly	G	nonpolar	neutral	-0.4
Histidine	His	H	polar	positive	-3.2
Isoleucine	Ile	I	nonpolar	neutral	4.5
Leucine	Leu	L	nonpolar	neutral	3.8
Lysine	Lys	K	polar	positive	-3.9
Methionine	Met	M	nonpolar	neutral	1.9
Phenylalanine	Phe	F	nonpolar	neutral	2.8
Proline	Pro	P	nonpolar	neutral	-1.6
Serine	Ser	S	polar	neutral	-0.8
Threonine	Thr	T	polar	neutral	-0.7
Tryptophan	Trp	W	nonpolar	neutral	-0.9
Tyrosine	Tyr	Y	polar	neutral	-1.3
Valine	Val	V	nonpolar	neutral	4.2

Table 3

Ambiguous Amino Acids	3-Letter	1-Letter
Asparagine or aspartic acid	Asx	B
Glutamine or glutamic acid	Glx	Z
Leucine or Isoleucine	Xle	J
Unspecified or unknown amino acid	Xaa	X

[0073] "Combination therapy" refers to those situations in which a subject is simultaneously exposed to two or more therapeutic regimens (*e.g.*, two or more therapeutic moieties). In some embodiments, the two or more regimens may be administered simultaneously; in some embodiments, such regimens may be administered sequentially (*e.g.*, all "doses" of a first regimen

are administered prior to administration of any doses of a second regimen); in some embodiments, such agents are administered in overlapping dosing regimens. In some embodiments, "administration" of combination therapy may involve administration of one or more agent(s) or modality(ies) to a subject receiving the other agent(s) or modality(ies) in the combination. For clarity, combination therapy does not require that individual agents be administered together in a single composition (or even necessarily at the same time), although in some embodiments, two or more agents, or active moieties thereof, may be administered together in a combination composition, or even in a combination compound (*e.g.*, as part of a single chemical complex or covalent entity).

[0074] "Corresponding to" may be used to designate the position/identity of a structural element in a molecule or composition through comparison with an appropriate reference molecule or composition. For example, in some embodiments, a monomeric residue in a polymer (*e.g.*, an amino acid residue in a polypeptide or a nucleic acid residue in a polynucleotide) may be identified as "corresponding to" a residue in an appropriate reference polymer. For example, for purposes of simplicity, residues in a polypeptide may be designated using a canonical numbering system based on a reference related polypeptide, so that an amino acid "corresponding to" a residue at position 100, for example, need not actually be the 100th amino acid in an amino acid chain provided it corresponds to the residue found at position 100 in the reference polypeptide. Various sequence alignment strategies are available, comprising software programs such as, for example, BLAST, CS-BLAST, CUDASW++, DIAMOND, FASTA, GGSEARCH/GLSEARCH, Genoogle, HMMER, HHpred/HHsearch, IDF, Infernal, KLAST, USEARCH, parasail, PSI-BLAST, PSI-Search, ScalaBLAST, Sequilab, SAM, SSEARCH, SWAPHI, SWAPHI-LS, SWIMM, or SWIPE that may be utilized, for example, to identify "corresponding" residues in polypeptides and/or nucleic acids in accordance with the present disclosure.

[0075] An antigen binding molecule, such as an antibody, an antigen binding fragment thereof, CAR or TCR, "cross-competes" with a reference binding molecule, such as an antibody or an antigen binding fragment thereof, if the interaction between an antigen and the first antigen binding molecule blocks, limits, inhibits, or otherwise reduces the ability of the reference binding molecule to interact with the antigen. Cross competition can be complete, *e.g.*, binding of the antigen binding molecule to the antigen completely blocks the ability of the reference binding molecule to bind the antigen, or it can be partial, *e.g.*, binding of the antigen binding molecule to the antigen reduces the ability of the reference antigen binding molecule to bind the antigen. In certain embodiments, an antigen binding molecule that cross-competes with a reference antigen binding molecule binds the same or an overlapping epitope as the reference antigen binding molecule. In other embodiments, the antigen binding molecule that cross-competes with a

reference antigen binding molecule binds a different epitope than the reference antigen binding molecule. Numerous types of competitive binding assays can be used to determine if one antigen binding molecule competes with another, for example: solid phase direct or indirect radioimmunoassay (RIA); solid phase direct or indirect enzyme immunoassay (EIA); sandwich competition assay (Stahli et al., 1983, Methods in Enzymology 9:242-253); solid phase direct biotin-avidin EIA (Kirkland et al., 1986, J. Immunol. 137:3614-3619); solid phase direct labeled assay, solid phase direct labeled sandwich assay (Harlow and Lane, 1988, Antibodies, A Laboratory Manual, Cold Spring Harbor Press); solid phase direct label RIA using 1-125 label (Morel et al., 1988, Molec. Immunol. 25:7-15); solid phase direct biotin-avidin EIA (Cheung, et al., 1990, Virology 176:546-552); and direct labeled RIA (Moldenhauer et al., 1990, Scand. J. Immunol. 32:77-82).

[0076] A "cytokine," refers to a non-antibody protein that is released by one cell in response to contact with a specific antigen, wherein the cytokine interacts with a second cell to mediate a response in the second cell. A cytokine can be endogenously expressed by a cell or administered to a subject. Cytokines may be released by immune cells, including macrophages, B cells, T cells, and mast cells to propagate an immune response. Cytokines can induce various responses in the recipient cell. Cytokines can include homeostatic cytokines, chemokines, pro-inflammatory cytokines, effectors, and acute-phase proteins. For example, homeostatic cytokines, including interleukin (IL) 7 and IL-15, promote immune cell survival and proliferation, and pro-inflammatory cytokines can promote an inflammatory response. Examples of homeostatic cytokines include, but are not limited to, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12p40, IL-12p70, IL-15, and interferon (IFN) gamma. Examples of pro-inflammatory cytokines include, but are not limited to, IL-1a, IL-1b, IL-6, IL-13, IL-17a, tumor necrosis factor (TNF)-alpha, TNF-beta, fibroblast growth factor (FGF) 2, granulocyte macrophage colony-stimulating factor (GM-CSF), soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular adhesion molecule 1 (sVCAM-1), vascular endothelial growth factor (VEGF), VEGF-C, VEGF-D, and placental growth factor (PLGF). Examples of effectors include, but are not limited to, granzyme A, granzyme B, soluble Fas ligand (sFasL), and perforin. Examples of acute phase-proteins include, but are not limited to, C-reactive protein (CRP) and serum amyloid A (SAA).

[0077] The term "domain" refers to a portion of an entity. In some embodiments, a "domain" is associated with a structural and/or functional feature of the entity, *e.g.*, so that, when the domain is physically separated from the rest of its parent entity, it substantially or entirely retains the structural and/or functional feature. In some embodiments, a domain may comprise a portion of an entity that, when separated from that (parent) entity and linked or connected with a different (recipient) entity, substantially retains and/or imparts on the recipient entity one or more structural

and/or functional features, *e.g.*, that characterized it in the parent entity. In some embodiments, a domain is a portion of a molecule (*e.g.*, a small molecule, carbohydrate, lipid, nucleic acid, or polypeptide). In some embodiments, a domain is a section of a polypeptide; in some such embodiments, a domain is characterized by a structural element (*e.g.*, an amino acid sequence or sequence domain, α-helix character, β-sheet character, coiled-coil character, random coil character, etc.), and/or by a functional feature (*e.g.*, binding activity, enzymatic activity, folding activity, signaling activity, etc.).

[0078] The term "dosage form" may be used to refer to a physically discrete unit of an active agent (*e.g.*, an antigen binding system or antibody) for administration to a subject. Generally, each such unit contains a predetermined quantity of active agent. In some embodiments, such quantity is a unit dosage amount (or a whole fraction thereof) appropriate for administration in accordance with a dosing regimen that has been determined to correlate with a desired or beneficial outcome when administered to a relevant population. The total amount of a therapeutic composition or agent administered to a subject is determined by one or more medical practitioners and may involve administration of more than one dosage forms.

[0079] The term "dosing regimen" may be used to refer to a set of one or more unit doses that are administered individually to a subject. In some embodiments, a given therapeutic agent has a recommended dosing regimen, which may involve one or more doses. In some embodiments, a dosing regimen comprises a plurality of doses each of which is separated in time from other doses. In some embodiments, a dosing regimen comprises a plurality of doses and consecutive doses are separated from one another by time periods of equal length; in some embodiments, a dosing regimen comprises a plurality of doses and consecutive doses are separated from one another by time periods of at least two different lengths. In some embodiments, all doses within a dosing regimen are of the same unit dose amount. In some embodiments, different doses within a dosing regimen are of different amounts. In some embodiments, a dosing regimen comprises a first dose in a first dose amount, followed by one or more additional doses in a second dose amount different from the first dose amount. In some embodiments, a dosing regimen is periodically adjusted to achieve a desired or beneficial outcome.

[0080] "Effector cell" refers to a cell of the immune system that expresses one or more Fc receptors and mediates one or more effector functions. In some embodiments, effector cells may comprise, without limitation, one or more of monocytes, macrophages, neutrophils, dendritic cells, eosinophils, mast cells, platelets, large granular lymphocytes, Langerhans' cells, natural killer (NK) cells, T-lymphocytes, and B-lymphocytes. Effector cells may be of any organism comprising, without limitation, humans, mice, rats, rabbits, and monkeys.

[0081] "Effector function" refers to a biological result of interaction of an antibody Fc region with an Fc receptor or ligand. Effector functions comprise, without limitation, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-mediated cytotoxicity (CMC). An effector function may be antigen binding dependent, antigen binding independent, or both. ADCC refers to lysis of antibody-bound target cells by immune effector cells. Without wishing to be bound by any theory, ADCC is generally understood to involve Fc receptor (FcR)-bearing effector cells recognizing and subsequently killing antibody-coated target cells (*e.g.*, cells that express on their surface antigens to which an antibody is bound). Effector cells that mediate ADCC may comprise immune cells, comprising yet not limited to, one or more of natural killer (NK) cells, macrophages, neutrophils, eosinophils.

[0082] The term "engineered Autologous Cell Therapy," which can be abbreviated as "eACT™," also known as adoptive cell transfer, is a process by which a patient's own T cells are collected and subsequently genetically altered to recognize and target one or more antigens expressed on the cell surface of one or more specific tumor cells or malignancies. T cells or NK cells can be engineered to express, for example, chimeric antigen receptors (CAR) and/or T cell receptor (TCR). In some examples, CAR positive (+) T or NK cells are engineered to express an extracellular single chain variable fragment (scFv) with specificity for a particular tumor antigen linked to an intracellular signaling part comprising at least one costimulatory domain and at least one activating domain. In some examples, CAR positive (+) T or NK cells are engineered to express an extracellular domain of NKG2D, with specificity for NKG2D antigens, linked to an intracellular signaling part comprising at least one costimulatory domain and at least one activating domain. The costimulatory domain can be derived from a naturally-occurring costimulatory domain, or a variant thereof, *e.g.*, a variant having a truncated hinge domain ("THD"), and the activating domain can be derived from, *e.g.*, CD3-zeta and/or CD3-epsilon. In certain embodiments, the CAR is designed to have two, three, four, or more costimulatory domains.

[0083] In some embodiments, the CAR is engineered such that the costimulatory domain is expressed as a separate polypeptide chain. Example CAR T cell therapies and constructs are described in U.S. Patent Publication Nos. 2013/0287748, 2014/0227237, 2014/0099309, and 2014/0050708, which are incorporated by reference in their entirety. "Adoptive cell therapy" or "ACT" involves transfer of immune cells with anti-tumor activity into a subject, *e.g.*, a cancer patient. In some embodiments, ACT is a treatment approach that involves the use of lymphocytes (*e.g.*, engineered lymphocytes) with anti-tumor activity.

[0084] An "epitope" refers to a localized region of an antigen to which an antibody can specifically bind. An epitope can be, for example, contiguous amino acids of a polypeptide (linear

or contiguous epitope) or an epitope can, for example, come together from two or more non-contiguous regions of a polypeptide or polypeptides (conformational, non-linear, discontinuous, or non-contiguous epitope). In certain embodiments, the epitope to which an antibody binds can be determined by, *e.g.*, NMR spectroscopy, X-ray diffraction crystallography studies, ELISA assays, hydrogen/deuterium exchange coupled with mass spectrometry (*e.g.*, liquid chromatography electrospray mass spectrometry), array-based oligo-peptide scanning assays, and/or mutagenesis mapping (*e.g.*, site-directed mutagenesis mapping). For X-ray crystallography, crystallization may be accomplished using any of the known methods in the art (*e.g.*, Giegé R *et al.,* (1994) Acta Crystallogr D Biol Crystallogr 50(Pt 4): 339-350; McPherson A (1990) Eur J Biochem 189: 1-23; Chayen NE (1997) Structure 5: 1269-1274; McPherson A (1976) J Biol Chem 251: 6300-6303). Antibody:antigen crystals may be studied using well known X-ray diffraction techniques and may be refined using computer software such as X-PLOR (Yale University, 1992, distributed by Molecular Simulations, Inc.; see e.g. Meth Enzymol (1985) volumes 114 & 115, eds Wyckoff HW *et al.,*; U.S. 2004/0014194), and BUSTER (Bricogne G (1993) Acta Crystallogr D Biol Crystallogr 49(Pt 1): 37-60; Bricogne G (1997) Meth Enzymol 276A: 361-423, ed Carter CW; Roversi P *et al.,* (2000) Acta Crystallogr D Biol Crystallogr 56(Pt 10): 1316-1323). Mutagenesis mapping studies may be accomplished using any method known to one of skill in the art. *See*, *e.g.*, Champe M *et al.,* (1995) J Biol Chem 270: 1388-1394 and Cunningham BC & Wells JA (1989) Science 244: 1081-1085 for a description of mutagenesis techniques, including alanine scanning mutagenesis techniques.

[0085] "Endogenous" with reference to a gene, protein, and/or nucleic acid refers to the natural presence of that gene, protein, and/or nucleic acid in a cell, such as an immune cell.

[0086] "Exogenous" refers to an introduced agent, such as a nucleic acid, gene, or protein, into a cell, for example from an outside source. A nucleic acid introduced into a cell is exogenous even if it encodes a protein which is naturally found in the cell. Such exogenous introduction of a nucleic acid encoding a protein can be used to increase the expression of the protein over the level that would naturally be found in the cell under similar conditions, e.g. without introduction of the exogenous nucleic acid.

[0087] The term "excipient" refers to an agent that may be comprised in a composition, for example to provide or contribute to a desired consistency or stabilizing effect. In some embodiments, a suitable excipient may comprise, for example, starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, or the like.

[0088] A "fragment" or "portion" of a material or entity as described herein has a structure that comprises a discrete portion of the whole, *e.g.*, of a physical entity or abstract entity. In some

embodiments, a fragment lacks one or more moieties found in the whole. In some embodiments, a fragment consists of or comprises a characteristic structural element, domain or moiety found in the whole. In some embodiments, a polymer fragment comprises or consists of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or more monomeric units (*e.g.*, residues) as found in the whole polymer. In some embodiments, a polymer fragment comprises or consists of at least about 5%, 10%, 15%, 20%, 25%, 30%, 25%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more of the monomeric units (*e.g.*, residues) found in the whole polymer (e.g., 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%). The whole material or entity may in some embodiments be referred to as the "parent" of the fragment.

[0089] The term "fusion polypeptide" or "fusion protein" generally refers to a polypeptide comprising at least two segments. Generally, a polypeptide containing at least two such segments is considered to be a fusion polypeptide if the two segments are moieties that (1) are not comprised in nature in the same peptide, and/or (2) have not previously been linked or connected to one another in a single polypeptide, and/or (3) have been linked or connected to one another through action of the hand of man. In embodiments, a CAR is a fusion protein. In embodiments, a TCR is a fusion protein.

[0090] The term "gene product" or "expression product" generally refers to an RNA transcribed from the gene (pre-and/or post-processing) or a polypeptide (pre- and/or post-modification) encoded by an RNA transcribed from the gene.

[0091] The term "genetically engineered" or "engineered" refers to a method of modifying the genome of a cell, including, but not limited to, deleting a coding or non-coding region or a portion thereof or inserting a coding region or a portion thereof. In some embodiments, the cell that is modified is a lymphocyte, *e.g.*, a T cell or NK cell, which can either be obtained from a patient or a donor. In some embodiments, the cell that is modified is an induced pluripotent stem cell (iPSC) which can be differentiated to a lymphocyte, such as a T cell or NK cell. The cell can be modified to express an exogenous construct, such as, *e.g.*, a chimeric antigen receptor (CAR) or a T cell receptor (TCR), which is incorporated into the cell's genome. Other gene edits can also be done., for example to reduce rejection and/or enhance cell fitness. Engineering generally comprises manipulation by the hand of man. For example, a polynucleotide is considered to be "engineered" when two or more sequences, that are not linked or connected together in that order in nature, are manipulated by the hand of man to be directly linked or connected to one another in the engineered polynucleotide. In the context of manipulation of cells by techniques of molecular biology, a cell or organism is considered to be "engineered" if it has been manipulated so that its genetic

information is altered (*e.g.*, new genetic material not previously present has been introduced, for example by transformation, somatic hybridization, transfection, transduction, or other mechanism, or previously present genetic material is altered or removed, for example by substitution or deletion mutation, or by other protocols). In some embodiments, a binding agent is a modified lymphocyte, *e.g.*, a T cell or NK cell, may be obtained from a patient or a donor. An engineered cell may be modified to express an exogenous construct, such as, *e.g.*, a chimeric antigen receptor (CAR) or a T cell receptor (TCR), which is incorporated into the cell's genome. Progeny of an engineered polynucleotide or binding agent are generally referred to as "engineered" even though the actual manipulation was performed on a prior entity. In some embodiments, "engineered" refers to an entity that has been designed and produced. The term "designed" refers to an agent (i) whose structure is or was selected by the hand of man; (ii) that is produced by a process requiring the hand of man; and/or (iii) that is distinct from natural substances and other known agents.

[0092] A "T cell receptor" or "TCR" refers to antigen-recognition molecules present on the surface of T cells. During normal T cell development, each of the four TCR genes, α, β, γ, and δ, may rearrange leading to highly diverse TCR proteins. Examples of TCR based T cell therapies are disclosed in International Patent Application Nos. PCT/US2013/059608 and PCT/US2015/033129, which are hereby incorporated herein by reference in their entirety.

[0093] The term "heterologous" means from any source other than naturally occurring sequences. For example, a heterologous sequence included as a part of a costimulatory protein is amino acids that do not naturally occur as, i.e., do not align with, the wild type human costimulatory protein. For example, a heterologous nucleotide sequence refers to a nucleotide sequence other than that of the wild type human costimulatory protein-encoding sequence.

[0094] Term "identity" refers to the overall relatedness between polymeric molecules, *e.g.*, between nucleic acid molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Methods for the calculation of a percent identity as between two provided polypeptide sequences are known. Calculation of the percent identity of two nucleic acid or polypeptide sequences, for example, may be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps may be introduced in one or both of a first and a second sequences for optimal alignment and non-identical sequences may be disregarded for comparison purposes). The nucleotides or amino acids at corresponding positions are then compared. When a position in the first sequence is occupied by the same residue (*e.g.*, nucleotide or amino acid) as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, optionally taking into account the number of gaps, and the length of each gap, which may need to be introduced for optimal alignment of the two sequences. Comparison

or alignment of sequences and determination of percent identity between two sequences may be accomplished using a mathematical algorithm, such as BLAST (basic local alignment search tool). In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical (e.g., 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%).

[0095] To calculate percent identity, the sequences being compared are typically aligned in a way that gives the largest match between the sequences. One example of a computer program that can be used to determine percent identity is the GCG program package, which includes GAP (Devereux et al., 1984, Nucl. Acid Res. 12:387; Genetics Computer Group, University of Wisconsin, Madison, Wis.). The computer algorithm GAP is used to align the two polypeptides or polynucleotides for which the percent sequence identity is to be determined. The sequences are aligned for optimal matching of their respective amino acid or nucleotide (the "matched span," as determined by the algorithm). In certain embodiments, a standard comparison matrix (*see*, Dayhoff et al., 1978, Atlas of Protein Sequence and Structure 5:345-352 for the PAM 250 comparison matrix; Henikoff et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:10915-10919 for the BLOSUM 62 comparison matrix) is also used by the algorithm. Other algorithms are also available for comparison of amino acid or nucleic acid sequences, comprising those available in commercial computer programs such as BLASTN for nucleotide sequences and BLASTP, gapped BLAST, and PSI-BLAST for amino acid sequences. Exemplary such programs are described in Altschul, et al., Basic local alignment search tool, J. Mol. Biol., 215(3): 403-410, 1990; Altschul, et al., Methods in Enzymology; Altschul, et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," Nucleic Acids Res. 25:3389-3402, 1997; Baxevanis, et al., Bioinformatics : A Practical Guide to the Analysis of Genes and Proteins, Wiley, 1998; and Misener, et al., (eds.), Bioinformatics Methods and Protocols (Methods in Molecular Biology, Vol. 132), Humana Press, 1999. In addition to identifying similar sequences, the programs mentioned above generally provide an indication of the degree of similarity. In some embodiments, two sequences are considered to be substantially similar if at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or more of their corresponding residues are similar and/or identical over a relevant stretch of residues (e.g., 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%). In some embodiments, the relevant stretch is a complete sequence. In some embodiments, the relevant stretch is at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at

least 85, at least 90, at least 95, at least 100, at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, at least 300, at least 325, at least 350, at least 375, at least 400, at least 425, at least 450, at least 475, at least 500 or more residues. Sequences with substantial sequence similarity may be homologs of one another.

[0096] The term "substantial identity" or "substantially identical," when referring to a nucleic acid or fragment thereof, indicates that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 95%, and more preferably at least about 96%, 97%, 98% or 99% of the nucleotide bases, as measured by any well-known algorithm of sequence identity, such as FASTA, BLAST or Gap, as discussed below. A nucleic acid molecule having substantial identity to a reference nucleic acid molecule may, in certain instances, encode a polypeptide having the same or substantially similar amino acid sequence as the polypeptide encoded by the reference nucleic acid molecule.

[0097] As applied to polypeptides, the term "substantial similarity" or "substantially similar" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 95% sequence identity, even more preferably at least 98% or 99% sequence identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

[0098] The terms “improve,” “increase,” “inhibit,” and “reduce” indicate values that are relative to a baseline or other reference measurement. In some embodiments, an appropriate reference measurement may comprise a measurement in certain system (*e.g.*, in a single individual) under otherwise comparable conditions absent presence of (*e.g.*, prior to and/or after) an agent or treatment, or in presence of an appropriate comparable reference agent. In some embodiments, an appropriate reference measurement may comprise a measurement in comparable system known or expected to respond in a comparable way, in presence of the relevant agent or treatment.

[0099] An “immune response” refers to the action of a cell of the immune system (for example, T lymphocytes, B lymphocytes, natural killer (NK) cells, macrophages, eosinophils, mast cells, dendritic cells and neutrophils) and soluble macromolecules produced by any of these cells or the liver (including Abs, cytokines, and complement) that results in selective targeting, binding to, damage to, destruction of, and/or elimination from a vertebrate's body of invading pathogens, cells or tissues infected with pathogens, cancerous or other abnormal cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

[0100] The term “immunotherapy” refers to the treatment of a subject afflicted with, or at risk of contracting or suffering a recurrence of, a disease by a method comprising inducing, enhancing, suppressing or otherwise modifying an immune response. Examples of immunotherapy include,

but are not limited to, NK cells and T cell therapies. T cell therapy can include adoptive T cell therapy, tumor-infiltrating lymphocyte (TIL) immunotherapy, autologous cell therapy, engineered autologous cell therapy (eACT™), and allogeneic T cell transplantation. However, one of skill in the art would recognize that the conditioning methods disclosed herein would enhance the effectiveness of any transplanted T cell therapy. Examples of T cell therapies are described in U.S. Patent Publication Nos. 2014/0154228 and 2002/0006409, U.S. Patent No. 5,728,388, and International Publication No. WO 2008/081035. Examples of TCR based T cell therapies are disclosed in International Patent Application Nos. PCT/US2013/059608 and PCT/US2015/033129, which are hereby incorporated herein by reference in their entirety.

[0101] The T cells or NK cells of the immunotherapy can come from any source known in the art. For example, T cells and NK cells can be differentiated *in vitro* from a hematopoietic stem cell population (for example iPSCs) or can be obtained from a subject. T cells and NK cells can be obtained from, *e.g.*, peripheral blood mononuclear cells (PBMCs), bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In addition, the T cells can be derived from one or more T cell lines available in the art. T cells can also be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as FICOLL™ separation and/or apheresis. Additional methods of isolating T cells for a T cell therapy are disclosed in U.S. Patent Publication No. 2013/0287748, which is herein incorporated by references in its entirety.

[0102] The term "*in vitro*" refers to events occurring in an artificial environment, *e.g.*, in a test tube, reaction vessel, cell culture, etc., rather than within a multi-cellular organism. The term "*in vitro* cell" refers to any cell which is cultured *ex vivo*. In particular, an *in vitro* cell can include a T cell or an NK cell. The term "*in vivo*" refers to events that occur within a multi-cellular organism, such as a human or a non-human animal.

[0103] The term "isolated" refers to a substance that (1) has been separated from at least some components with which it was associated at an earlier time or with which the substance would otherwise be associated, and/or (2) is present in a composition that comprises a limited or defined amount or concentration of one or more known or unknown contaminants. An isolated substance, in some embodiments, may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% (e.g., 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%) of other non-substance components with which the substance was associated at an earlier time, *e.g.*, other components or contaminants with which the substance was previously or otherwise would be associated. In certain instances, a substance is isolated if it is present in a composition that comprises a limited

or reduced amount or concentration of molecules of a same or similar type. For instance, in certain instances, a nucleic acid, DNA, or RNA substance is isolated if it is present in a composition that comprises a limited or reduced amount or concentration of non-substance nucleic acid, DNA, or RNA molecules. For instance, in certain instances, a polypeptide substance is isolated if it is present in a composition that comprises a limited or reduced amount or concentration of non-substance polypeptide molecules. In certain embodiments, an amount may be, *e.g.*, an amount measured relative to the amount of a desired substance present in a composition. In certain embodiments, a limited amount may be an amount that is no more than 100% of the amount of substance in a composition, *e.g.*, no more than 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of the amount of substance in a composition (e.g., 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%). In certain instances, a composition is pure or substantially pure with respect to a selected substance. In some embodiments, an isolated substance is about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure (e.g., 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%). A substance is “pure” if it is substantially free of other components or of contaminants. In some embodiments, a substance may still be considered “isolated” or even “pure,” after having been combined with certain other components such as, for example, one or more carriers or excipients (*e.g.*, buffer, solvent, water, etc.); in such embodiments, percent isolation or purity of the substance is calculated without comprising such carriers or excipients.

[0104] "Linker" (L) or "linker domain" or "linker region" refers to an oligo- or polypeptide region from about 1 to 100 amino acids in length, for example linking together any of the domains/regions of a CAR, TCR, and/or scFv, or ever one of more of those polypeptides together. Linkers may be composed of flexible residues like glycine and serine so that the adjacent protein domains are free to move relative to one another. Longer linkers may be used when it is desirable to ensure that two adjacent domains do not sterically interfere with one another. Linkers may be cleavable or non-cleavable. Examples of cleavable linkers include 2A linkers (for example T2A), 2A-like linkers or functional equivalents thereof and combinations thereof. In some embodiments, the linkers include the picornaviral 2A-like linker, CHYSEL (SEQ ID NO: 1) sequences of porcine teschovirus (P2A), virus (T2A) or combinations, variants and functional equivalents thereof. In other embodiments, the linker sequences may comprise Asp-Val/Ile-Glu-X-Asn-Pro-Gly$^{(2A)}$-Pro$^{(2B)}$ domain (SEQ ID NO: 2), which results in cleavage between the 2A glycine and the 2B proline. Other linkers will be apparent to those of skill in the art and may be used in connection with this disclosure. A linker may be a portion of a multi-element agent that connects different elements to one another. For example, a polypeptide comprises two or more functional or

structural domains may comprise a stretch of amino acids between such domains that links them to one another. In some embodiments, a polypeptide comprising a linker element has an overall structure of the general form S1-L-S2, wherein S1 and S2 may be the same or different and represent two domains associated with one another by the linker. A linker may connect or link together any of the domains/regions of a CAR or TCR. In some embodiments, a polypeptide linker is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids in length (*e.g.*, 1 to 10, 1 to 20, 1 to 30, 1 to 40, 1 to 50, 1 to 60, 1 to 70, 1 to 80, 1 to 90, 1 to 100, 10 to 20, 10 to 30, 10 to 40, 10 to 50, 10 to 60, 10 to 70, 10 to 80, 10 to 90, or 10 to 100 amino acids in length). In some embodiments, a linker is characterized in that it tends not to adopt a rigid three-dimensional structure, and instead provides flexibility to the polypeptide. In another example it may be used to connect to or more polypeptides to be expressed, such as a CAR and/or TCR.

[0105] Other linkers include non-cleavable linkers. A number of linkers are employed to realize the subject invention including "flexible linkers." The latter are rich in glycine. Klein et al., Protein Engineering, Design & Selection Vol. 27, No. 10, pp. 325–330, 2014; Priyanka et al., Protein Sci., 2013 Feb; 22(2): 153–167.

[0106] In some embodiments, the linker is a synthetic linker. A synthetic linker can have a length of from about 10 amino acids to about 200 amino acids, e.g., from 10 to 25 amino acids, from 25 to 50 amino acids, from 50 to 75 amino acids, from 75 to 100 amino acids, from 100 to 125 amino acids, from 125 to 150 amino acids, from 150 to 175 amino acids, or from 175 to 200 amino acids. A synthetic linker can have a length of from 10 to 30 amino acids, e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids. A synthetic linker can have a length of from 30 to 50 amino acids, e.g., from 30 to 35 amino acids, from 35 to 40 amino acids, from 40 to 45 amino acids, or from 45 to 50 amino acids.

[0107] In some embodiments, the linker is a flexible linker. In some embodiments, the linker is rich in glycine (Gly or G) residues. In some embodiments, the linker is rich in serine (Ser or S) residues. In some embodiments, the linker is rich in glycine and serine residues.

[0108] The term "lymphocyte" includes natural killer (NK) cells, T cells, or B cells. NK cells are a type of cytotoxic (cell toxic) lymphocyte that represent a component of the inherent immune system. NK cells reject tumors and cells infected by viruses. It works through the process of apoptosis or programmed cell death. They were termed "natural killers" because they do not require activation in order to kill cells. T cells play a role in cell-mediated-immunity (no antibody involvement). Its T cell receptors (TCR) differentiate themselves from other lymphocyte types. The thymus, a specialized organ of the immune system, is primarily responsible for the T cell's maturation. There are six types of T cells, namely: Helper T cells (*e.g.*, CD4+ cells), Cytotoxic T

cells (also known as TC, cytotoxic T lymphocyte, CTL, T-killer cell, cytolytic T cell, CD8+ T cells or killer T cell), Memory T cells ((i) stem memory T_{SCM} cells, like naive cells, are CD45RO−, CCR7+, CD45RA+, CD62L+ (L-selectin), CD27+, CD28+ and IL-7Rα+, but they also express large amounts of CD95, IL-2Rβ, CXCR3, and LFA-1, and show numerous functional attributes distinctive of memory cells); (ii) central memory T_{CM} cells express L-selectin and the CCR7, they secrete IL-2, but not IFNγ or IL-4, and (iii) effector memory T_{EM} cells, however, do not express L-selectin or CCR7 but produce effector cytokines like IFNγ and IL-4), Regulatory T cells (Tregs, suppressor T cells, or CD4+CD25+ regulatory T cells), Natural Killer T cells (NKT) and Gamma Delta T cells. B-cells, on the other hand, play a role in humoral immunity (with antibody involvement). It makes antibodies and antigens and performs the role of antigen-presenting cells (APCs) and turns into memory B-cells after activation by antigen interaction. In mammals, immature B-cells are formed in the bone marrow, where its name is derived from.

[0109] The term "neutralizing" refers to an antigen binding molecule, scFv, antibody, or a fragment thereof, that binds to a ligand and prevents or reduces the biological effect of that ligand. In some embodiments, the antigen binding molecule, scFv, antibody, or a fragment thereof, directly blocking a binding site on the ligand or otherwise alters the ligand's ability to bind through indirect means (such as structural or energetic alterations in the ligand). In some embodiments, the antigen binding molecule, scFv, antibody, or a fragment thereof prevents the protein to which it is bound from performing a biological function.

[0110] "Nucleic acid" refers to any polymeric chain of nucleotides. A nucleic acid may be DNA, RNA, or a combination thereof. In some embodiments, a nucleic acid comprises one or more natural nucleic acid residues. In some embodiments, a nucleic acid comprises of one or more nucleic acid analogs. In some embodiments, nucleic acids are prepared by one or more of isolation from a natural source, enzymatic synthesis by polymerization based on a complementary template (*in vivo* or *in vitro*), reproduction in a recombinant cell or system, and chemical synthesis. In some embodiments, a nucleic acid is at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 1 10, 120, 130, 140, 150, 160, 170, 180, 190, 20, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 or more residues long (e.g., 20 to 100, 20 to 500, 20 to 1000, 20 to 2000, or 20 to 5000 or more residues). In some embodiments, a nucleic acid is partly or wholly single stranded; in some embodiments, a nucleic acid is partly or wholly double stranded. In some embodiments a nucleic acid has a nucleotide sequence comprising at least one element that encodes, or is the complement of a sequence that encodes, a polypeptide.

[0111] "Operably linked" refers to a juxtaposition where the components described are in a relationship permitting them to function in their intended manner. For example, a control element

"*operably linked*" to a functional element is associated in such a way that expression and/or activity of the functional element is achieved under conditions compatible with the control element. In embodiments, a promotor is operably linked to nucleic acids.

[0112] A "patient" includes any human who is afflicted with a cancer (*e.g.*, multiple myeloma). The terms "subject" and "patient" are used interchangeably herein.

[0113] The terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide contains at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

[0114] The term "pharmaceutically acceptable" refers to a molecule or composition that, when administered to a recipient, is not deleterious to the recipient thereof, or that any deleterious effect is outweighed by a benefit to the recipient thereof. With respect to a carrier, diluent, or excipient used to formulate a composition as disclosed herein, a pharmaceutically acceptable carrier, diluent, or excipient must be compatible with the other ingredients of the composition and not deleterious to the recipient thereof, or any deleterious effect must be outweighed by a benefit to the recipient. The term "pharmaceutically acceptable carrier" means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent encapsulating material, involved in carrying or transporting an agent from one portion of the body to another (*e.g.*, from one organ to another). Each carrier present in a pharmaceutical composition must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the patient, or any deleterious effect must be outweighed by a benefit to the recipient. Some examples of materials which may serve as pharmaceutically acceptable carriers comprise: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin,

sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; pH buffered solutions; polyesters, polycarbonates and/or polyanhydrides; and other non-toxic compatible substances employed in pharmaceutical formulations.

[0115] The term "pharmaceutical composition" refers to a composition in which an active agent is formulated together with one or more pharmaceutically acceptable carriers. In some embodiments, the active agent is present in a unit dose amount appropriate for administration in a therapeutic regimen that shows a statistically significant probability of achieving a predetermined therapeutic effect when administered to a relevant subject or population. In some embodiments, a pharmaceutical composition may be formulated for administration in solid or liquid form, comprising, without limitation, a form adapted for the following: oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, *e.g.*, those targeted for buccal, sublingual, and systemic absorption, boluses, powders, granules, pastes for application to the tongue; parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation; topical application, for example, as a cream, ointment, or a controlled-release patch or spray applied to the skin, lungs, or oral cavity; intravaginally or intrarectally, for example, as a pessary, cream, or foam; sublingually; ocularly; transdermally; or nasally, pulmonary, and to other mucosal surfaces.

[0116] The term "proliferation" refers to an increase in cell division, either symmetric or asymmetric division of cells. In some embodiments, "proliferation" refers to the symmetric or asymmetric division of T cells. "Increased proliferation" occurs when there is an increase in the number of cells in a treated sample compared to cells in a non–treated sample.

[0117] The term "reference" describes a standard or control relative to which a comparison is performed. For example, in some embodiments, an agent, animal, individual, population, sample, sequence, or value of interest is compared with a reference or control that is an agent, animal, individual, population, sample, sequence, or value. In some embodiments, a reference or control is tested, measured, and/or determined substantially simultaneously with the testing, measuring, or determination of interest. In some embodiments, a reference or control is a historical reference or control, optionally embodied in a tangible medium. Generally, a reference or control is determined or characterized under comparable conditions or circumstances to those under assessment. When sufficient similarities are present to justify reliance on and/or comparison to a selected reference or control.

[0118] "Regulatory T cells" ("Treg", "Treg cells", or "Tregs") refer to a lineage of CD4+ T lymphocytes that participate in controlling certain immune activities, *e.g.*, autoimmunity, allergy, and response to infection. Regulatory T cells may regulate the activities of T cell populations, and may also influence certain innate immune system cell types. Tregs may be identified by the expression of the biomarkers CD4, CD25 and Foxp3, and low expression of CD127. Naturally occurring Treg cells normally constitute about 5-10% of the peripheral CD4+ T lymphocytes. However, Treg cells within a tumor microenvironment (i.e. tumor-infiltrating Treg cells), Treg cells may make up as much as 20-30% of the total CD4+ T lymphocyte population.

[0119] The term "sample" generally refers to an aliquot of material obtained or derived from a source of interest. In some embodiments, a source of interest is a biological or environmental source. In some embodiments, a source of interest may comprise a cell or an organism, such as a cell population, tissue, or animal (*e.g.*, a human). In some embodiments, a source of interest comprises biological tissue or fluid. In some embodiments, a biological tissue or fluid may comprise amniotic fluid, aqueous humor, ascites, bile, bone marrow, blood, breast milk, cerebrospinal fluid, cerumen, chyle, chime, ejaculate, endolymph, exudate, feces, gastric acid, gastric juice, lymph, mucus, pericardial fluid, perilymph, peritoneal fluid, pleural fluid, pus, rheum, saliva, sebum, semen, serum, smegma, sputum, synovial fluid, sweat, tears, urine, vaginal secretions, vitreous humour, vomit, and/or combinations or component(s) thereof. In some embodiments, a biological fluid may comprise an intracellular fluid, an extracellular fluid, an intravascular fluid (blood plasma), an interstitial fluid, a lymphatic fluid, and/or a transcellular fluid. In some embodiments, a biological fluid may comprise a plant exudate. In some embodiments, a biological tissue or sample may be obtained, for example, by aspirate, biopsy (*e.g.*, fine needle or tissue biopsy), swab (*e.g.*, oral, nasal, skin, or vaginal swab), scraping, surgery, washing or lavage (*e.g.*, brocheoalvealar, ductal, nasal, ocular, oral, uterine, vaginal, or other washing or lavage). In some embodiments, a biological sample comprises cells obtained from an individual. In some embodiments, a sample is a "primary sample" obtained directly from a source of interest by any appropriate means. In some embodiments, as will be clear from context, the term "sample" refers to a preparation that is obtained by processing (*e.g.*, by removing one or more components of and/or by adding one or more agents to) a primary sample. Such a "processed sample" may comprise, for example nucleic acids or proteins extracted from a sample or obtained by subjecting a primary sample to one or more techniques such as amplification or reverse transcription of nucleic acid, isolation and/or purification of certain components, *etc.*

[0120] "Single chain variable fragment", "single-chain antibody variable fragments" or "scFv" antibodies refer to forms of antibodies comprising the variable regions of only the heavy and light chains, connected by a linker peptide.

[0121] The term "stage of cancer" refers to a qualitative or quantitative assessment of the level of advancement of a cancer. In some embodiments, criteria used to determine the stage of a cancer may comprise, without limitation, one or more of where the cancer is located in a body, tumor size, whether the cancer has spread to lymph nodes, whether the cancer has spread to one or more different parts of the body, etc. In some embodiments, cancer may be staged using the so-called TNM System, according to which T refers to the size and extent of the main tumor, usually called the primary tumor; N refers to the number of nearby lymph nodes that have cancer; and M refers to whether the cancer has metastasized. In some embodiments, a cancer may be referred to as Stage 0 (abnormal cells are present without having spread to nearby tissue, also called carcinoma *in situ*, or CIS; CIS is not cancer, though could become cancer), Stage I-III (cancer is present; the higher the number, the larger the tumor and the more it has spread into nearby tissues), or Stage IV (the cancer has spread to distant parts of the body). In some embodiments, a cancer may be assigned to a stage selected from the group consisting of: *in situ*; localized (cancer is limited to the place where it started, with no sign that it has spread); regional (cancer has spread to nearby lymph nodes, tissues, or organs): distant (cancer has spread to distant parts of the body); and unknown (there is not enough information to determine the stage).

[0122] "Stimulation," refers to a primary response induced by binding of a stimulatory molecule with its cognate ligand, wherein the binding mediates a signal transduction event. A "stimulatory molecule" is a molecule on a T cell, *e.g.*, the T cell receptor (TCR)/CD3 complex, that specifically binds with a cognate stimulatory ligand present on an antigen present cell. A "stimulatory ligand" is a ligand that when present on an antigen presenting cell (*e.g.*, an APC, a dendritic cell, a B-cell, and the like) can specifically bind with a stimulatory molecule on a T cell, thereby mediating a primary response by the T cell, including, but not limited to, activation, initiation of an immune response, proliferation, and the like. Stimulatory ligands include, but are not limited to, an anti-CD3 antibody (such as OKT3), an MHC Class I molecule loaded with a peptide, a superagonist anti-CD2 antibody, and a superagonist anti-CD28 antibody.

[0123] The phrase "therapeutic agent" may refer to any agent that elicits a desired pharmacological effect when administered to an organism. In some embodiments, an agent is considered to be a therapeutic agent if it demonstrates a statistically significant effect across an appropriate population. In some embodiments, the appropriate population may be a population of model organisms or human subjects. In some embodiments, an appropriate population may be defined by various criteria, such as a certain age group, gender, genetic background, preexisting clinical conditions, in accordance with presence or absence of a biomarker, etc. In some embodiments, a therapeutic agent is a substance that may be used to alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of, and/or reduce incidence of one or more

symptoms or features of a disease, disorder, and/or condition. In some embodiments, a therapeutic agent is an agent that has been or is required to be approved by a government agency before it may be marketed for administration to humans. In some embodiments, a therapeutic agent is an agent for which a medical prescription is required for administration to humans.

[0124] A "therapeutically effective amount," "effective dose," "effective amount," or "therapeutically effective dosage" of a therapeutic agent, *e.g.,* engineered CAR T cells or NK cells, is any amount that, when used alone or in combination with another therapeutic agent, protects a subject against the onset of a disease or promotes disease regression evidenced by a decrease in severity of disease symptoms, an increase in frequency and duration of disease symptom-free periods, or a prevention of impairment or disability due to the disease affliction. The ability of a therapeutic agent to promote disease regression can be evaluated using a variety of methods known to the skilled practitioner, such as in human subjects during clinical trials, in animal model systems predictive of efficacy in humans, or by assaying the activity of the agent in *in vitro* assays.

[0125] The terms "transduction" and "transduced" refer to the process whereby foreign DNA is introduced into a cell via viral vector (*see* Jones et al., "Genetics: principles and analysis," Boston: Jones & Bartlett Publ. (1998)). In some embodiments, the vector is a retroviral vector, a DNA vector, a RNA vector, an adenoviral vector, a baculoviral vector, an Epstein Barr viral vector, a papovaviral vector, a vaccinia viral vector, a herpes simplex viral vector, an adenovirus associated vector, a lentiviral vector, or any combination thereof.

[0126] "Transformation" refers to any process by which exogenous DNA is introduced into a host cell. Transformation may occur under natural or artificial conditions using various methods. Transformation may be achieved using any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. In some embodiments, some transformation methodology is selected based on the host cell being transformed and/or the nucleic acid to be inserted. Methods of transformation may comprise, yet are not limited to, viral infection, electroporation, and lipofection. In some embodiments, a "transformed" cell is stably transformed in that the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. In some embodiments, a transformed cell may express introduced nucleic acid.

[0127] "Treatment" or "treating" of a subject refers to any type of intervention or process performed on, or the administration of an active agent to, the subject with the objective of reversing, alleviating, ameliorating, inhibiting, slowing down or preventing the onset, progression, development, severity or recurrence of a symptom, complication or condition, or biochemical indicia associated with a disease. In one embodiment, "treatment" or "treating" includes a partial

remission. In another embodiment, "treatment" or "treating" includes a complete remission. In some embodiments, treatment may be of a subject who does not exhibit signs of the relevant disease, disorder and/or condition and/or of a subject who exhibits only early signs of the disease, disorder, and/or condition. In some embodiments, such treatment may be of a subject who exhibits one or more established signs of the relevant disease, disorder and/or condition. In some embodiments, treatment may be of a subject who has been diagnosed as suffering from the relevant disease, disorder, and/or condition. In some embodiments, treatment may be of a subject known to have one or more susceptibility factors that are statistically correlated with increased risk of development of the relevant disease, disorder, and/or condition.

[0128] The term "vector" refers to a recipient nucleic acid molecule modified to comprise or incorporate a provided nucleic acid sequence. One type of vector is a "plasmid," which refers to a circular double stranded DNA molecule into which additional DNA may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) may be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors comprise sequences that direct expression of inserted genes to which they are operatively linked. Such vectors may be referred to herein as "expression vectors." Standard techniques may be used for engineering of vectors, e.g., as found in Sambrook et al., Molecular Cloning: A Laboratory Manual (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)), which is incorporated herein by reference.

[0129] The term "sequence" refers to a nucleotide sequence of any length, which can be DNA or RNA; can be linear, circular or branched and can be either single-stranded or double stranded. The term "donor sequence" refers to a nucleotide sequence that is inserted into a genome. A donor sequence can be of any length, for example between 2 and 10,000 nucleotides in length (or any integer value therebetween or thereabove), preferably between about 100 and 1,000 nucleotides in length (or any integer therebetween), more preferably between about 200 and 500 nucleotides in length.

[0130] A "gene," for the purposes of the present disclosure, includes a DNA region encoding a gene product (see infra), as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal

ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

[0131] A "transmembrane domain" is a domain of a polypeptide that includes at least one contiguous amino acid sequence that traverses a lipid bilayer when present in the corresponding endogenous polypeptide when expressed in a mammalian cell. For example, a transmembrane domain can include one, two, three, four, five, six, seven, eight, nine, or ten contiguous amino acid sequences that each traverse a lipid bilayer when present in the corresponding endogenous polypeptide when expressed in a mammalian cell. A transmembrane domain can, e.g., include at least one (e.g., two, three, four, five, six, seven, eight, nine, or ten) contiguous amino acid sequence (that traverses a lipid bilayer when present in the corresponding endogenous polypeptide when expressed in a mammalian cell) that has α-helical secondary structure in the lipid bilayer. In some embodiments, a transmembrane domain can include two or more contiguous amino acid sequences (that each traverse a lipid bilayer when present in the corresponding endogenous polypeptide when expressed in a mammalian cell) that form a β-barrel secondary structure in the lipid bilayer. Non-limiting examples of transmembrane domains are described herein. Additional examples of transmembrane domains are known in the art.

[0132] The phrase "extracellular side of the plasma membrane" when used to describe the location of a polypeptide means that the polypeptide includes at least one transmembrane domain that traverses the plasma membrane and at least one domain (e.g., at least one antigen-binding domain) that is located in the extracellular space.

[0133] The disclosure may employ, unless indicated specifically to the contrary, methods of chemistry, biochemistry, organic chemistry, molecular biology, microbiology, recombinant DNA techniques, genetics, immunology, and cell biology that are within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, e.g., Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (3rd Edition, 2001); Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (1982); Ausubel *et al.*, *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, 1985); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); *Transcription and Translation* (B. Hames & S. Higgins, Eds., 1984); Perbal, *A Practical Guide to Molecular Cloning* (1984); Harlow and Lane, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998) *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*.

[0134] The present disclosure provides antigen receptors (CARs) comprising a portion of the extracellular domain of NKG2D that is capable of binding one or more NKG2D ligands, referred to herein as NKG2D CARs. Among other things, the present disclosure provides methods and compositions useful for treatment of cancer and/or for initiating or modulating immune responses. In some embodiments, the NKG2D CAR is expressed with a TCR specific for one or more tumor antigens and/or one or more additional CARs specific for one or more tumor antigens.

[0135] Various embodiments of the present disclosure provide a vector encoding a NKG2D CAR provided herein, *e.g.*, a vector encoding a NKG2D CAR. Various embodiments of the present disclosure provide a vector encoding a TCR or one or more additional CARs (e.g. a CAR that binds a different target than the NKG2D CAR), *e.g.*, a vector encoding a NKG2D CAR and TCR or one or more additional CARs. In some embodiments the NKG2D CAR is encoded in a separate vector from the vector encoding the TCR or one or more additional CARs. In some embodiments the NKG2D CAR is encoded in the same vector encoding the TCR or one or more additional CARs.

[0136] Various embodiments of the present disclosure provide a cell encoding or expressing a NKG2D CAR, *e.g.*, induce pluripotent cells (iPSC) a T cell or NK cell engineered to encode or express NKG2D CAR. Various embodiments of the present disclosure provide a cell encoding or expressing a NKG2D CAR and a TCR or one or more additional CARs, *e.g.*, a T cell or NK cell engineered to encode or express a NKG2D CAR and a TCR or one or more additional CARs. The present disclosure provides immune cells genetically modified with an integrated gene, *e.g.*, a nucleotide sequence of interest (*e.g.*, a constitutive expression construct and/or an inducible expression construct that comprises such nucleotide sequence. In embodiments, the immune cells are further engineered to express a TCR or one or more additional CARs. In some embodiments, the present disclosure provides methods of treating a subject having a tumor, comprising administering to the subject a NKG2D CAR therapy described herein. In some embodiments, methods further comprise administration of one or more additional therapies (*e.g.*, a second binding agent (*e.g.*, CAR T cell, CAR-NK cell, TCR-T cell, TIL cell, allogeneic NK cell, and autologous NK cell), an antibody-drug conjugate, an antibody, a bispecific antibody, a T cell-engaging bispecific antibody, an engineered antibody, and/or a polypeptide described herein).

[0137] Natural killer cells preferentially express several calcium-dependent (C-type) lectins, which have been implicated in the regulation of NK cell function. NKG2D (NCBI Gene ID: 22914 as updated March 7, 2021, which is incorporated herein by reference) is a transmembrane protein belonging to the NKG2 family of C-type lectin-like receptors. The NKG2 gene family is located

within the NK complex, a region that contains several C-type lectin genes preferentially expressed in NK cells. NKG2D is a recognition receptor for the detection and elimination of transformed and infected cells as its ligands are induced during cellular stress, either as a result of infection or genomic stress such as in cancer. NKG2D binds to a diverse family of ligands that include MHC class I chain-related A and B proteins and UL-16 binding proteins. The surface expression of these ligands is important for the recognition of stressed cells by the immune system, and thus this protein and its ligands are therapeutic targets for the treatment of immune diseases and cancers.

[0138] NKG2D ligands are induced-self proteins which are absent or present only at low levels on surface of normal cells but are overexpressed by infected, transformed, senescent and stressed cells. Their expression is regulated at different stages (transcription, mRNA and protein stabilization, cleavage from the cell surface) by various stress pathways. The NKG2D ligands are homologous to MHC class I molecules and are divided into two families: MIC and RAET1/ULBP. Human MIC genes are located within the MHC locus and are composed of seven members (MICA-G), of which only MICA and MICB produce functional transcripts. Among ten known human RAET1/ULBP genes, six encode functional proteins: RAET1E/ULBP4, RAET1G/ULBP5, RAET1H/ULBP2, RAET1/ULBP1, RAET1L/ULBP6, RAET1N/ULBP3.

[0139] Chimeric antigen receptors (CARs) are engineered receptors that may direct or redirect T cells or NK cells (*e.g.*, patient or donor T or NK cells) to a selected target. A CAR may be engineered to recognize a target (such as an antigen and in the case of a disclosed NKG2D CAR a NKG2D ligand) and, when bound to that target, activate the immune cell to attack and destroy the cell bearing that target. When these targets exist on tumor cells, an immune cell that expresses the CAR may target and kill the tumor cell. CARs generally comprise an extracellular binding domain that mediates antigen binding (*e.g.*, a NKG2D ecto domain), a transmembrane domain that spans, or is understood to span, the cell membrane when the CAR is present at a cell surface or cell membrane, and an intracellular (or cytoplasmic) signaling domain.

[0140] According to at least one non-limiting view, there have been at least three "generations" of CAR compositions. In a first generation of CARs, a binding domain (*e.g.*, a single chain fragment variable, binding domain) is linked or connected to a signaling domain (*e.g.*, CD3ζ) via a transmembrane domain, optionally comprising a hinge domain and one or more spacers. In a second generation of CARs, a costimulatory domain (CM1, such as CD28, 4-1BB, or OX-40) is introduced with the signaling domain (*e.g.*, CD3ζ). In a third generation of CARs, a second costimulatory domain (CM2) is comprised.

[0141] TCRs are heterodimers composed of an α-chain and a β-chain. TCR signaling requires recruitment of signaling proteins that generate an immune synapse. In addition, TCR localization at the plasma membrane depends on CD3 complex, which is expressed in T cells. Engineered

single chain TCRs may be generated, *e.g.*, using transmembrane and signaling domains of CAR constructs, methods and constructs for which are known (*e.g.*, sTCR and TCR-CAR molecules, *e.g.*, fusion of a TCRβ chain with CD28 TM and CD28 and CD3ζ signaling modules).

[0142] A NKG2D CAR of the present disclosure may comprise an extracellular NKG2D domain that binds NKG2D ligands. In some embodiments, an antigen binding system further comprises a costimulatory domain, and/or an extracellular domain (*e.g.*, a "hinge" or "spacer" region), and/or a transmembrane domain, and/or an intracellular (signaling) domain, a CD3-zeta and/or CD3-epsilon activation domain.

[0143] In certain embodiments, a NKG2D CAR comprises a NKG2D ecto domain (extracellular domain) polypeptide refers to a polypeptide which has at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to SEQ ID 3. LFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYS KEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFK GYIENCSTPNTYICMQRTV (SEQ ID NO: 3). In embodiments, a NKG2D ecto domain is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to TTATTCAACCAAGAAGTCCAGATTCCCTTGACCGAAAGTTACTGCGGCCCATGTCCG AAAAACTGGATATGTTATAAAAATAACTGTTACCAGTTCTTCGATGAATCTAAAAA CTGGTATGAGAGCCAGGCATCTTGTATGTCTCAAAATGCCAGCCTGCTCAAAGTATA CAGCAAGGAGGACCAGGATTTACTTAAACTGGTGAAGTCATATCACTGGATGGGAT TGGTACACATTCCCACAAATGGATCTTGGCAGTGGGAAGACGGCTCCATTCTCTCAC CCAACCTACTAACAATAATTGAAATGCAGAAGGGAGACTGCGCACTCTATGCATCG AGCTTTAAAGGTTATATAGAAAACTGTTCAACTCCAAATACATACATCTGCATGCAA AGGACTGTA (SEQ ID NO: 4). In embodiments, a NKG2D ecto domain is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to TTATTCAACCAAGAAGTCCAAATTCCCTTGACCGAAAGTTACTGTGGCCCATGTCCT AAAAACTGGATATGTTACAAAAATAACTGTTACCAATTCTTCGATGAAAGTAAAAA CTGGTATGAGAGCCAGGCTTCTTGTATGTCTCAAAATGCCAGCCTTCTGAAAGTATA CAGCAAGGAGGACCAGGATTTACTTAAACTGGTGAAGTCATATCATTGGATGGGAC TAGTACACATTCCAACAAATGGATCTTGGCAGTGGGAAGACGGCTCCATTCTCTCAC CCAACCTACTAACAATAATTGAAATGCAGAAGGGAGACTGTGCACTCTATGCATCG

AGCTTTAAAGGCTATATAGAAAACTGTTCAACTCCAAATACATACATCTGCATGCA AAGGACTGTG (SEQ ID NO: 5). In embodiments, a NKG2D ecto domain is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to TTATTCAACCAAGAAGTCCAAATTCCCTTGACCGAAAGTTACTGTGGCCCATGTCCT AAGAACTGGATATGTTACAAAAATAACTGTTACCAATTCTTCGATGAATCTAAGAA TTGGTATGAGAGCCAGGCTTCTTGTATGTCTCAAAATGCCAGCCTTCTTAAAGTATA CAGCAAAGAGGACCAGGATTTACTTAAACTGGTGAAGTCATATCATTGGATGGGAC TAGTACACATTCCAACAAATGGATCTTGGCAGTGGGAAGACGGCTCCATTCTCTCAC CCAACCTACTAACAATAATTGAAATGCAGAAGGGAGACTGTGCACTCTATGCATCG AGCTTTAAAGGCTATATAGAAAACTGTTCAACTCCAAATACATATATTTGCATGCAA AG GACTGTG (SEQ ID NO: 55).

[0144] In some embodiments, a NKG2D CAR of the present disclosure may comprise an antigen binding system that comprises one or more, or all, of a leader peptide (P), NKG2D ecto domain (B), a hinge (E), a transmembrane domain (T), a costimulatory domain (C), a second costimulatory domain (C′), and an activation domain (A). In some instances, a NKG2D CAR is configured according to the following: B E T A. In certain instances, the activation domain comprises one or more activation domains. In certain aspects, the activation domain comprises CD3ζ, CD3ε, or both CD3ζ and CD3ε. In some instances, a NKG2D CAR is configured according to the following: P B E T A. In some instances, a NKG2D CAR is configured according to the following: B E T C A. In some instances a NKG2D CAR is configured according to the following: P B E T C A. In some instances, a NKG2D CAR is configured according to the following: B E T C C′ A. In some instances, a NKG2D CAR is configured according to the following: P B E T C C′ A.

[0145] In certain embodiments, the CARs contemplated herein may comprise linker residues between the various domains, domains, added for appropriate spacing conformation of the molecule. CARs contemplated herein, may comprise one, two, three, four, or five or more linkers. In some embodiments, 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.

[0146] 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 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)). Other linkers contemplated herein include Whitlow linkers (see Whitlow, *Protein Eng.* 6(8): 989–95 (1993)). The ordinarily skilled artisan will recognize that design of a CAR in some embodiments may include linkers that are all or partially flexible, such that the linker may include a flexible linker as well as one or more portions that confer less flexible structure to provide for a desired CAR structure. In one embodiment, any of the constructs described herein may comprise a "GS" linker. In another embodiment, any of the constructs described herein comprise a "GSG" linker. In an example a glycine-serine linker comprises or consists of the amino acid sequence GS (SEQ ID NO: 6), which may be encoded by the nucleic acid sequence according to ggatcc (SEQ ID NO: 7) or gggtcc (SEQ ID NO: 8). In an example a glycine-serine linker comprises or consists of the amino acid sequence GGGSGGGS (SEQ ID NO: 9), which may be encoded by the nucleic acid sequence according to ggcggtggaagcggaggaggttcc (SEQ ID NO: 10). In another embodiment, the CARs described herein comprise the amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) of SEQ ID NO: 11 (GSTSGSGKPGSGEGSTKG (SEQ ID NO: 11). In an embodiment, a linker is encoded by a nucleic acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid sequence according to gggagcactagcggctctggcaaacctggatctggcgagggatctaccaagggc (SEQ ID NO: 12), gggagcacaagcggctctggcaaacctggatctggcgagggatctaccaagggc (SEQ ID NO: 13), or gggagcacaagcggctctggcaaacctggatccggcgagggatctaccaagggc (SEQ ID NO: 14).

[0147] The binding domain of the CAR may generally be followed by one or more "hinge domains," which plays a role in positioning the antigen binding domain away from the effector cell surface to enable proper cell/cell contact, antigen binding and activation. A CAR generally comprises one or more hinge domains between the binding domain and the transmembrane domain. The hinge domain may be derived either from a natural, synthetic, semi–synthetic, or recombinant source. The hinge domain may include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region.

[0148] In some embodiments, the CARs contemplated herein re may comprise a hinge that is, is from, or is derived from (*e.g.*, comprises all or a fragment of) an immunoglobulin-like hinge domain. In some embodiments, a hinge domain is from or derived from an immunoglobulin. In some embodiments, a hinge domain is selected from the hinge of IgG1, IgG2, IgG3, IgG4, IgA, IgD, IgE, or IgM, or a fragment thereof. A hinge may be derived from a natural source or from a

synthetic source. Hinge domains suitable for use in the CARs described herein include the hinge region derived from the extracellular regions of type 1 membrane proteins such as CD8α, CD4, CD28 and CD7, which may be wild–type hinge regions from these molecules or may be altered. A hinge may be derived from a natural source or from a synthetic source. In some embodiments, an Antigen binding system of the present disclosure may comprise a hinge that is, is from, or is derived from (*e.g.*, comprises all or a fragment of) CD2, CD3 delta, CD3 epsilon, CD3 gamma, CD4, CD7, CD8α, CD8β, CD11a (ITGAL), CD11b (ITGAM), CD11c (ITGAX), CD11d (ITGAD), CD18 (ITGB2), CD19 (B4), CD27 (TNFRSF7), CD28, CD28T, CD29 (ITGB1), CD30 (TNFRSF8), CD40 (TNFRSF5), CD48 (SLAMF2), CD49a (ITGA1), CD49d (ITGA4), CD49f (ITGA6), CD66a (CEACAM1), CD66b (CEACAM8), CD66c (CEACAM6), CD66d (CEACAM3), CD66e (CEACAM5), CD69 (CLEC2), CD79A (B–cell antigen receptor complex-associated alpha chain), CD79B (B–cell antigen receptor complex-associated beta chain), CD84 (SLAMF5), CD96 (Tactile), CD100 (SEMA4D), CD103 (ITGAE), CD134 (OX40), CD137 (4–1BB), CD150 (SLAMF1), CD158A (KIR2DL1), CD158B1 (KIR2DL2), CD158B2 (KIR2DL3), CD158C (KIR3DP1), CD158D (KIRDL4), CD158F1 (KIR2DL5A), CD158F2 (KIR2DL5B), CD158K (KIR3DL2), CD160 (BY55), CD162 (SELPLG), CD226 (DNAM1), CD229 (SLAMF3), CD244 (SLAMF4), CD247 (CD3–zeta), CD258 (LIGHT), CD268 (BAFFR), CD270 (TNFSF14), CD272 (BTLA), CD276 (B7–H3), CD279 (PD–1), CD314 (NKG2D), CD319 (SLAMF7), CD335 (NK–p46), CD336 (NK–p44), CD337 (NK–p30), CD352 (SLAMF6), CD353 (SLAMF8), CD355 (CRTAM), CD357 (TNFRSF18), inducible T cell co–stimulator (ICOS), LFA–1 (CD11a/CD18), NKG2C, DAP–10, ICAM–1, NKp80 (KLRF1), IL–2R beta, IL–2R gamma, IL–7R alpha, LFA1–1, SLAMF9, LAT, GADS (GrpL), SLP-76 (LCP2), PAG1/CBP, a CD83 ligand, Fc gamma receptor, MHC class 1 molecule, MHC class 2 molecule, a TNF receptor protein, an immunoglobulin protein, a cytokine receptor, an integrin, activating NK cell receptors, or Toll ligand receptor, or which is a fragment or combination thereof.

[0149] Polynucleotide and polypeptide sequences of these hinge domains are known. In some embodiments, the polynucleotide encoding a hinge domain comprises a nucleotide sequence at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% (e.g., 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%) identical to a nucleotide sequence known. In some embodiments, the polypeptide sequence of a hinge domain comprises a polypeptide sequence at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at

least about 99%, or about 100% (e.g., 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%) identical to a known polypeptide sequence.

[0150] In embodiments, the hinge domain comprises a CD8α hinge region. In embodiments the CARs described herein comprise a hinge domain from CD8α having the amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 15 (TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO: 15)). In embodiments, hinge domain from CD8α is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: ACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCGCGTCGCAACC CCTGTCCCTGCGCCCAGAGGCGTGCCGGCCAGCGGCGGGGGGCGCAGTGCACACGA GGGGGCTGGACTTCGCCTGTGAT (SEQ ID NO: 16). In embodiments, a hinge domain from CD8α is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: ACAACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCGCGTCGCAACC CCTGTCCCTGAGGCCTGAAGCGTGCCGGCCAGCGGCGGGGGGCGCAGTGCACACGA GGGGGCTGGACTTCGCTTGTGAC (SEQ ID NO: 17). In embodiments, hinge domain from CD8α is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: ACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCGCGTCGCAACC CCTGTCCCTGCGCCCCGAGGCGTGCCGGCCAGCGGCGGGGGGCGCAGTGCACACGA GGGGGCTGGACTTCGCCTGTGAT (SEQ ID NO: 18). In embodiments, hinge domain from CD8α is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: ACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCGCGTCGCAACC CCTGTCCCTGAGGCCTGAAGCGTGCCGGCCAGCGGCGGGCGGCGCAGTGCACACGA GAGGGCTGGACTTCGCCTGTGAT (SEQ ID NO: 56).

[0151] In embodiments, the hinge domain comprises a truncated CD28 hinge region (CD28T) hinge region, such as disclosed in International Patent Application No: PCT/US2017/025351, filed March 31, 2017, which is incorporated herein by reference in its entirety. In embodiments

the CARs described herein comprise a CD28T hinge domain having the amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 19 (LDNEKSNGTIIHVKGKHLCPSPLFPGPSKP (SEQ ID NO: 19)). In embodiments, a CD28T hinge domain is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: CTAGACAATGAGAAGAGCAATGGAACCATTATCCATGTGAAAGGGAAACACCTTTG TCCAAGTCCCCTATTTCCCGGACCTTCTAAGCCC (SEQ ID NO: 20).

[0152] In general, a "transmembrane domain" (e.g., of an antigen binding system) refers to a domain having an attribute of being present in the membrane when present in a molecule at a cell surface or cell membrane (*e.g.*, spanning a portion or all of a cellular membrane). A costimulatory domain for an antigen binding system of the present disclosure may further comprise a transmembrane domain and/or an intracellular signaling domain. It is not required that every amino acid in a transmembrane domain be present in the membrane. For example, in some embodiments, a transmembrane domain is characterized in that a designated stretch or portion of a protein is substantially located in the membrane. Amino acid or nucleic acid sequences may be analyzed using a variety of algorithms to predict protein subcellular localization (*e.g.*, transmembrane localization). The programs psort (PSORT.org) and Prosite (prosite.expasy.org) are exemplary of such programs.

[0153] The type of transmembrane domain comprised in an antigen binding system described herein is not limited to any type. In some embodiments, a transmembrane domain is selected that is naturally associated with a binding domain and/or intracellular domain. In some instances, a transmembrane domain comprises a modification of one or more amino acids (*e.g.*, deletion, insertion, and/or substitution), *e.g.*, to avoid binding of such domains to a transmembrane domain of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

[0154] A transmembrane domain may be derived either from a natural or from a synthetic source. Where the source is natural, a domain may be derived from any membrane-bound or transmembrane protein. Exemplary transmembrane domains may be derived from (*e.g.*, may comprise at least a transmembrane domain of) an alpha, beta or zeta chain of a T-cell receptor, 2B4, CD28, CD3 epsilon, CD3 delta, CD3 gamma, CD45, CD4, CD5, CD7, CD8, CD8 alpha, CD8beta, CD9, CD11a, CD11b, CD11c, CD11d, CD16, CD22, CD27, CD33, CD37, CD64, CD80, CD86, CD134, CD137, TNFSFR25, CD154, 4-1BB/CD137, activating NK cell receptors,

an Immunoglobulin protein, B7-H3, BAFFR, BLAME (SLAMF8), BTLA, CD100 (SEMA4D), CD103, CD160 (BY55), CD18, CD19, CD19a, CD2, CD247, CD276 (B7-H3), CD29, CD30, CD40, CD49a, CD49D, CD49f, CD69, CD84, CD96 (Tactile), CDS, CEACAM1, CRT AM, cytokine receptor, DAP-10, DAP-12, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM (LIGHTR), IA4, ICAM-1, ICAM-1, Ig alpha (CD79a), IL-2R beta, IL-2R gamma, IL-7R alpha, inducible T cell costimulator (ICOS), integrins, ITGA4, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, LAT, LFA-1, LFA-1, a ligand that binds with CD83, LIGHT, LIGHT, LTBR, Ly9 (CD229), lymphocyte function-associated antigen-1 (LFA-1; CD1-1a/CD18), MHC class 1 molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX-40, PAG/Cbp, programmed death-1 (PD-1), PSGL1, SELPLG (CD162), Signaling Lymphocytic Activation Molecules (SLAM proteins), SLAM (SLAMF1; CD150; IPO-3), SLAMF4 (CD244; 2B4), SLAMF6 (NTB-A; Ly108), SLAMF7, SLP-76, TNF receptor proteins, TNFR2, TNFSF14, a Toll ligand receptor, TRANCE/RANKL, VLA1, or VLA-6, or a fragment, truncation, or a combination thereof. In some embodiments, a transmembrane domain may be synthetic (and can, *e.g.*, comprise predominantly hydrophobic residues such as leucine and valine). In some embodiments, a triplet of phenylalanine, tryptophan and valine are comprised at each end of a synthetic transmembrane domain. In some embodiments, a transmembrane domain is directly linked or connected to a cytoplasmic domain. In some embodiments, a short oligo- or polypeptide linker (*e.g.*, between 2 and 10 amino acids in length) may form a linkage between a transmembrane domain and an intracellular domain. In some embodiments, a linker is a glycine-serine doublet.

[0155] Polynucleotide and polypeptide sequences of transmembrane domains provided herein are known. In some embodiments, the polynucleotide encoding a transmembrane domain comprises a nucleotide sequence at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% (e.g., 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%) identical to a nucleotide sequence known. In some embodiments, the polypeptide sequence of a transmembrane domain comprises a polypeptide sequence at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% (e.g., 85-90%, 85-95%, 85-100%, 90-95%, 90-100%, or 95-100%) identical to a polypeptide sequence known. Optionally, short spacers may form linkages between any or some of the extracellular, transmembrane, and intracellular domains of the CAR.

[0156] In embodiments a NKG2D CAR described herein comprise a TM domain from CD28 having the amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 21 (FWVLVVVGGVLACYSLLVTVAFIIFWV (SEQ ID NO: 21)). In embodiments, TM domain from CD28 is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: TTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAACA GTGGCCTTTATTATTTTCTGGGTG (SEQ ID NO: 22). In embodiments, TM domain from CD28 is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: TTTTGGGTATTGGTAGTAGTGGGCGGAGTCCTGGCTTGCTATAGTCTGCTAGTAACA GTGGCTTTTATTATATTTTGGGTG (SEQ ID NO: 23). In embodiments, TM domain from CD28 is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: TTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAACA GTGGCCTTTATTATTTTCTGGGTG (SEQ ID NO: 24).

[0157] In embodiments the CARs described herein comprise a TM domain from CD8α having the amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 25(IYIWAPLAGTCGVLLLSLVITLYC (SEQ ID NO: 25)). In embodiments, TM domain from CD8α is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: ATCTACATCTGGGCGCCCTTGGCCGGGACTTGTGGGGTCCTTCTCCTGTCACTGGTT ATCACCCTTTATTGC (SEQ ID NO: 26). In embodiments, TM domain from CD8α is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: ATCTACATCTGGGCGCCCTTGGCCGGGACTTGTGGGGTCCTTCTCCTGTCACTGGTT ATCACCCTTTACTGC (SEQ ID NO: 57).

[0158] Intracellular signaling domains that may transduce a signal upon binding of an antigen to an immune cell are known, any of which may be comprised in an antigen binding system of the present disclosure. For example, cytoplasmic sequences of a T cell receptor (TCR) are known to initiate signal transduction following TCR binding to an antigen (see, *e.g.*, Brownlie et al., Nature Rev. Immunol. 13:257-269 (2013)).

[0159] In some embodiments, CARs contemplated herein comprise an intracellular signaling domain. An "intracellular signaling domain," refers to the part of a CAR that participates in transducing the message of effective CAR binding to a target antigen 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. In some embodiments, a signaling domain and/or activation domain comprises an immunoreceptor tyrosine-based activation domain (ITAM). Examples of ITAM containing cytoplasmic signaling sequences comprise those derived from TCR zeta, FcR gamma, FcR beta, CD3 zeta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d (see, *e.g.*, Love et al., Cold Spring Harb. Perspect. Biol. 2:a002485 (2010); Smith-Garvin et al., Annu. Rev. Immunol. 27:591-619 (2009)). In certain embodiments, suitable signaling domains comprise, without limitation, 4-1BB/CD137, activating NK cell receptors, an Immunoglobulin protein, B7-H3, BAFFR, BLAME (SLAMF8), BTLA, CD100 (SEMA4D), CD103, CD160 (BY55), CD18, CD19, CD19a, CD2, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3 delta, CD3 epsilon, CD3 gamma, CD30, CD4, CD40, CD49a, CD49D, CD49f, CD69, CD7, CD84, CD8alpha, CD8beta, CD96 (Tactile), CD11a, CD11b, CD11c, CD11d, CDS, CEACAM1, CRT AM, cytokine receptor, DAP-10, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM (LIGHTR), IA4, ICAM-1, ICAM-1, Ig alpha (CD79a), IL-2R beta, IL-2R gamma, IL-7R alpha, inducible T cell costimulator (ICOS), integrins, ITGA4, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, LAT, LFA-1, LFA-1, ligand that binds with CD83, LIGHT, LIGHT, LTBR, Ly9 (CD229), Ly108), lymphocyte function-associated antigen-1 (LFA-1; CD1-1a/CD18), MHC class 1 molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX-40, PAG/Cbp, programmed death-1 (PD-1), PSGL1, SELPLG (CD162), Signaling Lymphocytic Activation Molecules (SLAM proteins), SLAM (SLAMF1; CD150; IPO-3), SLAMF4 (CD244; 2B4), SLAMF6 (NTB-A, SLAMF7, SLP-76, TNF receptor proteins, TNFR2, TNFSF14, a Toll ligand receptor, TRANCE/RANKL, VLA1, or VLA-6, or a fragment, truncation, or a combination thereof.

[0160] The term "effector function" refers to a specialized function of the 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 "intracellular 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 intracellular signaling domain may be employed, in many cases it is not necessary to use the entire domain. To the extent that a truncated portion of an intracellular 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 intracellular signaling domain is meant to include any truncated portion of the intracellular signaling domain sufficient to transducing effector function signal.

[0161] It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary or costimulatory signal may also be required. Thus, T cell activation may be said to be mediated by two distinct classes of intracellular signaling domains: primary signaling domains that initiate antigen–dependent primary activation through the TCR (e.g., a TCR/CD3 complex) and costimulatory signaling domains that act in an antigen independent manner to provide a secondary or costimulatory signal. In some embodiments, a CAR contemplated herein comprises an intracellular signaling domain that comprises one or more "costimulatory signaling domain" and a "primary signaling domain."

[0162] Illustrative examples of ITAM containing primary signaling domains that are useful in the present disclosure include those derived from TCRζ, FcRγ, FcRβ, DAP12, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d. In some embodiments, a CAR comprises a CD3ζ primary signaling domain and one or more costimulatory signaling domains. The intracellular primary signaling and costimulatory signaling domains may be linked in any order in tandem to the carboxyl terminus of the transmembrane domain. In one embodiment, the CARs have a CD3ζ domain having the amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 27. LRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 27). In embodiments, a CD3ζ domain is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: CTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGGCAGA ACCAACTCTATAACGAGCTCAATCTAGGAAGGAGAGAAGAGTACGATGTTCTAGAC AAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCACGAAGGAAGAACCCTC AGGAAGGCCTGTACAACGAACTACAAAAAGATAAAATGGCGGAGGCCTACAGTGA

GATTGGCATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAG
GGCCTCAGTACAGCCACCAAGGACACCTATGACGCCCTTCACATGCAAGCTCTGCC
CCCTCGC (SEQ ID NO: 28). In embodiments, a CD3ζ domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to:
CTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGA
ACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGAC
AAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTC
AGGAAGGCCTGTACAACGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGA
GATTGGCATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGACGGCCTTTACCAG
GGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCC
CCCTCGC (SEQ ID NO: 29).

[0163] In embodiments, a CD3ζ domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to:
CTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGA
ACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGAC
AAGAGGCGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTC
AGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGA
GATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAG
GGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCC
CCCTCGC (SEQ ID NO: 30). In embodiments, a CD3ζ domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to:
CTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAAGGGCAGA
ACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGAC
AAGAGGCGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTC
AGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGA
GATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAG
GGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAAGCTCTGCC
CCCTCGCTGA (SEQ ID NO: 58). In embodiments, a CD3ζ domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at

least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: CTGAGAGTTAAGTTCAGCAGGAGCGCCGACGCCCCTGCCTACCAGCAAGGACAGAA TCAACTGTACAACGAGCTGAACCTGGGCAGACGGGAGGAATACGATGTGCTGGACA AGAGGAGAGGCAGAGACCCCGAGATGGGCGGCAAACCTAGAAGAAAGAACCCCCA GGAGGGCCTGTATAACGAGCTCCAGAAGGACAAGATGGCCGAGGCCTACAGCGAG ATCGGCATGAAGGGCGAAAGAAGAAGAGGCAAGGGCCACGACGGCCTCTACCAGG GCTTAAGCACAGCTACAAAGGACACCTACGACGCCCTGCACATGCAGGCCCTGCCC CCTAGATGA (SEQ ID NO: 59). In embodiments, a CD3ζ domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: CTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAAGGGCAGA ACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGAC AAGAGGCGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTC AGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGA GATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAG GGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAAGCTCTGCC CCCTCGCTGA (SEQ ID NO: 60).

[0164] In some embodiments, a CAR comprises a CD3ζ signaling domain, a CD3ε signaling domain and one or more costimulatory signaling domains. The intracellular primary signaling and costimulatory signaling domains may be linked in any order in tandem to the carboxyl terminus of the transmembrane domain. In embodiments, the CARs have a CD3ε domain having the amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 31. KNRKAKAKPVTRGAGAGGRQRGQNKERPPPVPNPDYEPIRKGQRDLYSGL (SEQ ID NO: 31). In embodiments, the CARs have a CD3ε domain having the amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: __. KNRKAKAKPVTRGAGAGGRQRGQNKERPPPVPNPDYEPIRKGQRDLYSGLNQRRI (SEQ ID NO: 61).

[0165] In embodiments, a CD3ε domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity;

e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: AAGAACCGAAAAGCAAAAGCCAAGCCTGTTACAAGAGGAGCAGGGGCAGGAGGCC GACAGAGAGGGCAAAACAAAGAAAGGCCCCCGCCCGTCCCAAACCCGGATTATGA GCCAATTAGGAAGGGTCAGAGAGACCTGTATTCTGGGCTC (SEQ ID NO: 32). In embodiments, a CD3ε domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: AAGAACCGCAAAGCAAAGGCAAAACCCGTCACACGAGGAGCGGGCGCAGGGGGAC GACAACGCGGTCAGAATAAGGAACGCCCGCCTCCAGTACCAAATCCAGATTATGAA CCAATTCGGAAGGGACAACGCGATCTCTACTCCGGTCTCAATCAGAGGCGAATT (SEQ ID NO: 62).

[0166] CARs contemplated herein comprise one or more costimulatory signaling domains to enhance the efficacy and expansion of T cells expressing CAR receptors. As used herein, the term, "costimulatory signaling domain," or "costimulatory domain", refers to an intracellular signaling domain of a costimulatory molecule. In some embodiments, costimulatory molecules may include DAP-10, DAP-12, CD27, CD28, CD137(4–IBB), OX40 (CD134), CD30, CD40, PD–I, ICOS (CD278), CTLA4, LFA–1, CD2, CD7, LIGHT, TRIM, LCK3, SLAM, DAPIO, LAG3, HVEM, B7-H3, NKD2C, GITR, CD5, ICAM-l, CDl la, Lck, TNFR-I, TNFR-II, FasR, NKG2C, and B7-H3, and CD83.

[0167] In embodiments, the CARs comprise a 4–1BB costimulatory domain having the amino acid sequence of having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 33. KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCE (SEQ ID NO: 33). In embodiments, the CARs comprise a 4–1BB costimulatory domain having the amino acid sequence of having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: __. KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO: 63). In embodiments, a 4–IBB costimulatory domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: AAACGAGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTATGAGACCAGT

ACAAACAACTCAGGAGGAGGATGGCTGTAGCTGCCGATTCCCGGAAGAAGAAGAA GGTGGCTGTGAA (SEQ ID NO: 34). In embodiments, a 4–IBB costimulatory domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: AAACGGGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTATGAGACCAGT ACAAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAA GGAGGATGTGAA (SEQ ID NO: 35). In embodiments, a 4–IBB costimulatory domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: AAACGGGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTATGAGACCAGT ACAAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAA GGAGGATGTGAA (SEQ ID NO: 36). In embodiments, a 4–IBB costimulatory domain is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: AAGAGAGGCCGGAAGAAGCTGCTGTACATCTTCAAGCAGCCCTTCATGAGACCTGT GCAGACCACACAGGAGGAAGACGGCTGCAGCTGTAGATTCCCCGAGGAAGAGGAG GGCGGCTGTGAGCTG (SEQ ID NO: 64).

[0168] In embodiments, the CARs comprise a CD28 costimulatory domain having the amino acid sequence of having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 37. RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS (SEQ ID NO: 37). In embodiments, a CD28 costimulatory domain is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCGCCG CCCCGGGCCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAG CCTATCGCTCC (SEQ ID NO: 38).

[0169] The engineered NKG2D CARs described herein may also comprise an N-terminal signal peptide or tag at the N-terminus of the NKG2D ecto domain. In one embodiment, a heterologous signal peptide may be used. The antigen binding domain may be fused to a leader or a signal peptide that directs the nascent protein into the endoplasmic reticulum and subsequent

translocation to the cell surface. It is understood that, once a polypeptide containing a signal peptide is expressed at the cell surface, the signal peptide is generally proteolytically removed during processing of the polypeptide in the endoplasmic reticulum and translocation to the cell surface. Thus, a polypeptide such as the CAR constructs described herein, are generally expressed at the cell surface as a mature protein lacking the signal peptide, whereas the precursor form of the polypeptide includes the signal peptide. Any suitable signal sequence known in the art may be used. Similarly, any known tag sequence known in the art may also be used.

[0170] In embodiments, a signal sequence is a CD8α signal sequence. In embodiments, the NKG2D CARs described herein comprise a CD8α signal sequence having the amino acid sequence of having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to SEQ ID NO: 39; MALPVTALLLPLALLLHAARP (SEQ ID NO: 39). In embodiments, a CD8α signal sequence is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: ATGGCTCTTCCTGTGACTGCACTACTGCTGCCCCTGGCCTTACTTCTTCATGCTGCGC GTCCT (SEQ ID NO: 40). In embodiments, a CD8α signal sequence is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: ATGGCTCTTCCTGTGACAGCTCTTCTGCTGCCCCTGGCCCTGCTTCTGCATGCTGCTA GACCT (SEQ ID NO: 65).

[0171] In one embodiment a signal sequence is a CSF2RA signal sequence. In embodiments, the NKG2D CARs described herein comprise a CSF2RA signal sequence having the amino acid sequence of having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to MLLLVTSLLLCELPHPAFLLIP (SEQ ID NO: 41), MEWTWVFLFLLSVTAGVHS (SEQ ID NO: 42), or MALPVTALLLPLALLLHAARP (SEQ ID NO: 43). In embodiments, a CSF2RA signal sequence is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to: ATGGCTCTTCCTGTGACAGCTCTTCTGCTGCCCCTGGCCCTGCTTCTGCATGCTGCTA GACCT (SEQ ID NO: 44).

[0172] Components of a CAR may be exchanged or "swapped" using routine techniques of biotechnology for equivalent components. To provide just a few non-limiting and partial examples, a CAR of the present disclosure may comprise a binding domain as provided herein in combination with a hinge provided herein and a costimulatory domain provided herein. In certain examples, a CAR of the present disclosure may comprise a leader sequence as provided herein together with a binding domain as provided herein in combination with a hinge provided herein and s costimulatory domain provided herein.

[0173] In one embodiment described herein, a NKG2D CAR construct has an amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 45. MALPVTALLLPLALLLHAARPLFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKN WYESQASCMSQNASLLKVYSKEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSP NLLTIIEMQKGDCALYASSFKGYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSL RPEACRPAAGGAVHTRGLDFACDFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKL LYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERR RGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 45). In embodiments a NKG2D CAR binding CAR is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) the nucleic acid having the sequence according to: ATGGCTCTTCCTGTGACTGCACTACTGCTGCCCCTGGCCTTACTTCTTCATGCTGCGC GTCCTTTATTCAACCAAGAAGTCCAGATTCCCTTGACCGAAAGTTACTGCGGCCCAT GTCCGAAAAACTGGATATGTTATAAAAATAACTGTTACCAGTTCTTCGATGAATCTA AAAACTGGTATGAGAGCCAGGCATCTTGTATGTCTCAAAATGCCAGCCTGCTCAAA GTATACAGCAAGGAGGACCAGGATTTACTTAAACTGGTGAAGTCATATCACTGGAT GGGATTGGTACACATTCCCACAAATGGATCTTGGCAGTGGGAAGACGGCTCCATTC TCTCACCCAACCTACTAACAATAATTGAAATGCAGAAGGGAGACTGCGCACTCTAT GCATCGAGCTTTAAAGGTTATATAGAAAACTGTTCAACTCCAAATACATACATCTGC ATGCAAAGGACTGTAACAACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCA CCATCGCGTCGCAACCCCTGTCCCTGAGGCCTGAAGCGTGCCGGCCAGCGGCGGGG GGCGCAGTGCACACGAGGGGGCTGGACTTCGCTTGTGACTTTTGGGTATTGGTAGT AGTGGGCGGAGTCCTGGCTTGCTATAGTCTGCTAGTAACAGTGGCTTTTATTATATT TTGGGTGAAACGAGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTATGA

GACCAGTACAAACAACTCAGGAGGAGGATGGCTGTAGCTGCCGATTCCCGGAAGA
AGAAGAAGGTGGCTGTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCC
GCGTACCAGCAGGGGCAGAACCAACTCTATAACGAGCTCAATCTAGGAAGGAGAG
AAGAGTACGATGTTCTAGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAA
GCCACGAAGGAAGAACCCTCAGGAAGGCCTGTACAACGAACTACAAAAAGATAAA
ATGGCGGAGGCCTACAGTGAGATTGGCATGAAAGGCGAGCGCCGGAGGGGCAAGG
GGCACGATGGCCTTTACCAGGGCCTCAGTACAGCCACCAAGGACACCTATGACGCC
CTTCACATGCAAGCTCTGCCCCCTCGC (SEQ ID NO: 46).

[0174] In embodiments, a NKG2D CAR construct has an amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 47.
LFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYS
KEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFK
GYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF
ACDFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGC
SCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPE
MGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDT
YDALHMQALPPR (SEQ ID NO: 47). In embodiments, a NKG2D CAR binding CAR is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) the nucleic acid having the sequence according to:
TTATTCAACCAAGAAGTCCAGATTCCCTTGACCGAAAGTTACTGCGGCCCATGTCCG
AAAAACTGGATATGTTATAAAAATAACTGTTACCAGTTCTTCGATGAATCTAAAAA
CTGGTATGAGAGCCAGGCATCTTGTATGTCTCAAAATGCCAGCCTGCTCAAAGTATA
CAGCAAGGAGGACCAGGATTTACTTAAACTGGTGAAGTCATATCACTGGATGGGAT
TGGTACACATTCCCACAAATGGATCTTGGCAGTGGGAAGACGGCTCCATTCTCTCAC
CCAACCTACTAACAATAATTGAAATGCAGAAGGGAGACTGCGCACTCTATGCATCG
AGCTTTAAAGGTTATATAGAAAACTGTTCAACTCCAAATACATACATCTGCATGCAA
AGGACTGTAACAACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCGC
GTCGCAACCCCTGTCCCTGAGGCCTGAAGCGTGCCGGCCAGCGGCGGGGGGCGCAG
TGCACACGAGGGGGCTGGACTTCGCTTGTGACTTTTGGGTATTGGTAGTAGTGGGCG
GAGTCCTGGCTTGCTATAGTCTGCTAGTAACAGTGGCTTTTATTATATTTTGGGTGA
AACGAGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTATGAGACCAGTA
CAAACAACTCAGGAGGAGGATGGCTGTAGCTGCCGATTCCCGGAAGAAGAAGAAG
GTGGCTGTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAG

CAGGGGCAGAACCAACTCTATAACGAGCTCAATCTAGGAAGGAGAGAAGAGTACG
ATGTTCTAGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCACGAAG
GAAGAACCCTCAGGAAGGCCTGTACAACGAACTACAAAAAGATAAAATGGCGGAG
GCCTACAGTGAGATTGGCATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATG
GCCTTTACCAGGGCCTCAGTACAGCCACCAAGGACACCTATGACGCCCTTCACATG
CAAGCTCTGCCCCCTCGC (SEQ ID NO: 48).

[0175] In embodiments, a NKG2D CAR construct has an amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 49.
MALPVTALLLPLALLLHAARPLFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKN
WYESQASCMSQNASLLKVYSKEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSP
NLLTIIEMQKGDCALYASSFKGYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSL
RPEACRPAAGGAVHTRGLDFACDFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKL
LYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEKNRKAKAKPVTRGAGAGGRQRGQN
KERPPPVPNPDYEPIRKGQRDLYSGLLRVKFSRSADAPAYQQGQNQLYNELNLGRREEY
DVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG
LYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 49). In embodiments, a NKG2D CAR binding CAR is encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to:
ATGGCTCTTCCTGTGACAGCTCTTCTGCTGCCCCTGGCCCTGCTTCTGCATGCTGCTA
GACCTTTATTCAACCAAGAAGTCCAAATTCCCTTGACCGAAAGTTACTGTGGCCCAT
GTCCTAAAAACTGGATATGTTACAAAAATAACTGTTACCAATTCTTCGATGAAAGTA
AAAACTGGTATGAGAGCCAGGCTTCTTGTATGTCTCAAAATGCCAGCCTTCTGAAA
GTATACAGCAAGGAGGACCAGGATTTACTTAAACTGGTGAAGTCATATCATTGGAT
GGGACTAGTACACATTCCAACAAATGGATCTTGGCAGTGGGAAGACGGCTCCATTC
TCTCACCCAACCTACTAACAATAATTGAAATGCAGAAGGGAGACTGTGCACTCTAT
GCATCGAGCTTTAAAGGCTATATAGAAAACTGTTCAACTCCAAATACATACATCTGC
ATGCAAAGGACTGTGACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCAC
CATCGCGTCGCAACCCCTGTCCCTGCGCCCAGAGGCGTGCCGGCCAGCGGCGGGGG
GCGCAGTGCACACGAGGGGGCTGGACTTCGCCTGTGATTTTTGGGTGCTGGTGGTG
GTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGTAACAGTGGCCTTTATTATTTTCT
GGGTGAAACGGGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTATGAGA
CCAGTACAAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGA
AGAAGGAGGATGTGAAAAGAACCGAAAAGCAAAAGCCAAGCCTGTTACAAGAGGA

GCAGGGGCAGGAGGCCGACAGAGAGGGCAAAACAAAGAAAGGCCCCCGCCCGTCC
CAAACCCGGATTATGAGCCAATTAGGAAGGGTCAGAGAGACCTGTATTCTGGGCTC
CTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGA
ACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGAC
AAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTC
AGGAAGGCCTGTACAACGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGA
GATTGGCATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGACGGCCTTTACCAG
GGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCC
CCCTCGC (SEQ ID NO: 50).

[0176] In embodiments, a NKG2D CAR construct has an amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; *e.g.*, 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) SEQ ID NO: 51.
LFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYS
KEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFK
GYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF
ACDFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGC
SCRFPEEEEGGCEKNRKAKAKPVTRGAGAGGRQRGQNKERPPPVPNPDYEPIRKGQRD
LYSGLLRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQ
ALPPR (SEQ ID NO: 51). In embodiments, a NKG2D CAR binding CAR is encoded by a nucleic acid having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to the nucleic acid having the sequence according to:
TTATTCAACCAAGAAGTCCAAATTCCCTTGACCGAAAGTTACTGTGGCCCATGTCCT
AAAAACTGGATATGTTACAAAAATAACTGTTACCAATTCTTCGATGAAAGTAAAAA
CTGGTATGAGAGCCAGGCTTCTTGTATGTCTCAAAATGCCAGCCTTCTGAAAGTATA
CAGCAAGGAGGACCAGGATTTACTTAAACTGGTGAAGTCATATCATTGGATGGGAC
TAGTACACATTCCAACAAATGGATCTTGGCAGTGGGAAGACGGCTCCATTCTCTCAC
CCAACCTACTAACAATAATTGAAATGCAGAAGGGAGACTGTGCACTCTATGCATCG
AGCTTTAAAGGCTATATAGAAAACTGTTCAACTCCAAATACATACATCTGCATGCA
AAGGACTGTGACCACGACGCCAGCGCCGCGACCACCAACACCGGCGCCCACCATCG
CGTCGCAACCCCTGTCCCTGCGCCCAGAGGCGTGCCGGCCAGCGGCGGGGGGCGCA
GTGCACACGAGGGGGCTGGACTTCGCCTGTGATTTTTGGGTGCTGGTGGTGGTTGGT
GGAGTCCTGGCTTGCTATAGCTTGCTAGTAACAGTGGCCTTTATTATTTTCTGGGTG
AAACGGGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTATGAGACCAGT

ACAAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAA
GGAGGATGTGAAAAGAACCGAAAAGCAAAAGCCAAGCCTGTTACAAGAGGAGCAG
GGGCAGGAGGCCGACAGAGAGGGCAAAACAAAGAAAGGCCCCCGCCCGTCCCAAA
CCCGGATTATGAGCCAATTAGGAAGGGTCAGAGAGACCTGTATTCTGGGCTCCTGA
GAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAGAACCA
GCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGA
GACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGA
AGGCCTGTACAACGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATT
GGCATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGACGGCCTTTACCAGGGTC
TCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCTGCCCCCT
CGC (SEQ ID NO: 52).

[0177] The present disclosure contemplates the use of the NKG2D CARs described herein with engineered T cell receptors (TCRs) used in T cell immunotherapy. Libraries of TCRs may be screened for their selectivity to target antigens. In this manner, natural TCRs, which have a high avidity and reactivity toward target antigens may be selected, cloned, and subsequently introduced into a population of T cells used for adoptive immunotherapy. A T cell or NK cell with an engineered TCR that also expresses the NKG2D CAR described herein would not only be able to target specific antigens due to TCR specificity but also cells expressing NKG2D ligands. Thus, combining TCRs with the NKG2D CAR described herein may provide a way to maintain or enhance the therapeutic effect of adoptive T cell or NK cell immunotherapy. In embodiments, a NKG2D CAR described herein is co–expressed with a TCR.

[0178] In one embodiment described herein, T cells or NK-cells are modified by introducing a polynucleotide encoding subunit of a TCR that may form TCRs that confer specificity to T cells or NK cells for tumor cells expressing a target antigen and/or a NKG2D ligand. In some embodiments, the subunits have one or more amino acid substitutions, deletions, insertions, or modifications compared to the naturally occurring subunit, so long as the subunits retain the ability to form TCRs conferring upon transfected T cells and NK cells the ability to home to target cells, and participate in immunologically–relevant cytokine signaling. The engineered TCRs may also bind target cells displaying the relevant tumor–associated peptide with high avidity, and optionally mediate efficient killing of target cells presenting the relevant peptide in vivo.

[0179] The nucleic acids encoding engineered TCRs may be isolated from their natural context in a (naturally–occurring) chromosome of a T cell, and may be incorporated into suitable vectors as described elsewhere herein. Both the nucleic acids and the vectors comprising them may be transferred into a cell, which cell may be a T cell. The modified T cells are then able to express one or more chains of a TCR (and in some aspects two chains) encoded by the transduced nucleic

acid or nucleic acids. In some embodiments, the engineered TCR is an exogenous TCR because it is introduced into T cells that do not normally express the introduced TCR. The essential aspect of the engineered TCRs is that it has high avidity for a tumor antigen presented by a major histocompatibility complex (MHC) or similar immunological component. In contrast to engineered TCRs, CARs are engineered to bind target antigens in an MHC independent manner.

[0180] The protein encoded by the nucleic acids described herein may be expressed with additional polypeptides attached to the amino–terminal or carboxyl–terminal portion of the α–chain or the β–chain of a TCR so long as the attached additional polypeptide does not interfere with the ability of the α–chain or the β–chain to form a functional T cell receptor and the MHC dependent antigen recognition.

[0181] Antigens that are recognized by the engineered TCRs contemplated herein include, but are not limited to cancer antigens, including antigens on both hematological cancers and solid tumors and viral induced cancers. TCR therapy for the treatment of HPV induced cervical carcinoma is an area of interest that holds promise. The oncolytic proteins HPV–16 E6 and HPV–16 E7 may thus be potential target antigens for use with TCR (see for example International Patent Application No. PCT/US2015/033129). Other illustrative antigens include, but are not limited HPV oncoproteins, including HPV–16 E6 and HPV–16 E7, alpha folate receptor, 5T4, αvβ6 integrin, BCMA, B7–H3, B7–H6, CAIX, CD19, CD20, CD22, CD28, CD30, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD137 (4–1BB), CD138, CD171, CEA, CSPG4, CLL-1, EGFR, EGFR family including ErbB2 (HERII), EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, FAP, fetal AchR, FRa, GD2, GD3, Glypican–3 (GPC3), HLA–Al+MAGEI, HLA–A2 + MAGE1, HLAA3 + MAGE1, MAGA-A3, HLA–Al + NY–ES0–1, HLA–A2 + NY–ES0–1, HLA–A3 + NY–ES0–1, IL–11Rα, IL–13Rα2, Lambda, Lewis–Y, Kappa, Mesothelin, Mucl, Muc16, NCAM, NKG2D Ligands, NY-ES0–1, PRAME, PSCA, PSMA, RORI, SSX, Survivin, TAG72, TACI, TEMs, and VEGFRII.

[0182] Combining any TCR construct as described herein with the NKG2D CARs of the present disclosure may restore, maintain or enhance the therapeutic effect of TCR therapy. Thus, in one embodiment described herein, the NKG2D CARs are co–expressed in a T cell or NK cell with a TCR directed against HPV. In another embodiment, the NKG2D CARs are co–expressed in a T cell or NK cell with a TCR directed against the HPV–16 E6 protein. In another embodiment described herein, the NKG2D CARs are co–expressed in a T cell or NK cells with a TCR directed against the HPV–16 E7 protein.

[0183] T cells or NK cells may also be genetically engineered with vectors designed to express a second CAR (in addition to a NKG2D CAR) that redirect cytotoxicity toward tumor cells. In some embodiments, CARs combine antibody based specificity for a target antigen (e.g., tumor antigen)

with an activating intracellular domain to generate a chimeric protein that exhibits a specific anti–tumor cellular immune activity. The present disclosure contemplates the use of the NKG2D CARs described herein with one or more additional CARs. Like with the use of TCRs, co–expression of the NKG2D CARs with one or more additional CARs may promote expansion enhance, protect, and in some cases restore, CAR therapies. In embodiments, a NKG2D CAR is co–expressed with one or more additional CARs.

[0184] The one or more additional CARs contemplated herein comprise an extracellular domain that binds to a specific target antigen (also referred to as a binding domain or antigen–specific binding domain), a transmembrane domain and an intracellular signaling domain. The main characteristic of one or more additional CARs are their ability to redirect immune effector cell specificity, thereby triggering proliferation, cytokine production, phagocytosis or production of molecules that may mediate cell death of the target antigen expressing cell in a major histocompatibility (MHC) independent manner, exploiting the cell specific targeting abilities of monoclonal antibodies, soluble ligands or cell specific co–receptors.

[0185] In some embodiments, the one or more additional CAR comprises an extracellular binding domain including but not limited to an antibody or antigen binding fragment thereof, a tethered ligand, or the extracellular domain of a co–receptor, that specifically binds a target antigen. By way of non–limiting examples, target antigens may include: HPV oncoproteins, including HPV–16 E6 and HPV–16 E7, alpha folate receptor, 5T4, αvβ6 integrin, BCMA, B7–H3, B7–H6, CAIX, CD19, CD20, CD22, CD28, CD30, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD137 (4–1BB), CD138, CD171, CEA, CSPG4, CLL-1, EGFR, EGFR family including ErbB2 (HERII), EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, FAP, fetal AchR, FRa, GD2, GD3, Glypican–3 (GPC3), HLA–Al + MAGEI, HLA–A2 + MAGE1, HLAA3 + MAGE1, HLA–Al + NY–ES0–1, HLA–A2 + NY–ES0–1, HLA–A3 + NY–ES0–1, IL–llRα, IL–13Rα2, Lambda, Lewis–Y, Kappa, Mesothelin, Mucl, Muc16, NCAM, NKG2D Ligands, NYE-S0–1, PRAME, PSCA, PSMA, RORI, SSX, Survivin, TACI, TAG72, TEMs, and VEGFRII; In embodiments described herein, the CAR binds to a tumor antigen comprising BCMA, CLL-1, CD19, CD20, CD22, CD28, CD137 (4–1BB), Glypican–3 (GPC3), PSCA, PSMA, or TACI.

[0186] In some embodiments, the one or more additional CARs contemplated herein comprise an extracellular binding domain that specifically binds to a target polypeptide, e.g., target antigen, expressed on tumor cell. As used herein, the terms, "binding domain," "extracellular domain," "extracellular binding domain," "antigen–specific binding domain," “antigen 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. A binding domain may comprise any protein, polypeptide, oligopeptide, or peptide that possesses the ability to specifically

recognize and bind to a biological molecule (e.g., a cell surface receptor or tumor protein, lipid, polysaccharide, or other cell surface target molecule, or component thereof). A binding domain includes any naturally occurring, synthetic, semi–synthetic, or recombinantly produced binding partner for a biological molecule of interest.

[0187] In some embodiments, the extracellular binding domain of the one or more additional 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 a target antigen, such as a peptide, lipid, polysaccharide, or nucleic acid containing an antigenic determinant, such as those recognized by an immune cell. Antibodies include antigen binding fragments thereof. The term also includes genetically engineered forms such as chimeric antibodies (for example, humanized murine antibodies), hetero–conjugate 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.

[0188] In some embodiments, the target antigen is an epitope of an HPV oncoproteins, including HPV–16 E6 and HPV–16 E7, alpha folate receptor, 5T4, αvβ6 integrin, BCMA, B7–H3, B7–H6, CAIX, CD19, CD20, CD22, , CD28, CD30, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD137 (4–1BB), CD138, CD171, CEA, CSPG4, CLL-1, EGFR, EGFR family including ErbB2 (HERII), EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, F AP, fetal AchR, FRa, GD2, GD3, Glypican–3 (GPC3), HLA–Al + MAGEI, HLA–A2 + MAGE1, HLAA3 + MAGE1, HLA–Al + NY–ES0–1, HLA–A2 + NY–ES0–1, HLA–A3 + NY–ES0–1, IL–llRα, IL–13Rα2, Lambda, Lewis–Y, Kappa, Mesothelin, Mucl, Muc16, NCAM, NKG2D Ligands, NYE-S0–1, PRAME, PSCA, PSMA, RORI, SSX, Survivin, TAG72, TEMs, TACI, and VEGFRII polypeptide. In one embodiment described herein, the CAR binds to a tumor antigen epitope comprising BCMA, CLL-1, CD19, CD20, CD22, CD28, CD137 (4–1BB), Glypican–3 (GPC3), PSCA, PSMA, or TACI.

[0189] In certain embodiments, the one or more additional CARs contemplated herein may comprise linker residues between the various domains, e.g., between VH and VL domains, added for appropriate spacing conformation of the molecule. CARs contemplated herein, may comprise one, two, three, four, or five or more linkers. In some embodiments, 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.

[0190] Illustrative examples of linkers include glycine polymers (G)n; glycine–serine polymers (G1–5S1–5)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 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)). Other linkers contemplated herein include Whitlow linkers (see Whitlow, Protein Eng. 6(8): 989–95 (1993)). The ordinarily skilled artisan will recognize that design of a CAR in some embodiments may include linkers that are all or partially flexible, such that the linker may include a flexible linker as well as one or more portions that confer less flexible structure to provide for a desired CAR structure. In one embodiment, any of the constructs described herein may comprise a "GS" linker. In another embodiment, any of the constructs described herein comprise a "GSG" linker. In another embodiment, the CARs described herein comprise the amino acid sequence having at least 75% sequence identity to (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) of SEQ ID NO: 40. GSTSGSGKPGSGEGSTKG (SEQ ID NO: 40).

[0191] In other embodiments, a CAR comprises a scFv that further comprises a variable region linking sequence. A "variable region linking sequence," is an amino acid sequence that connects a heavy chain variable region to a light chain variable region and provides a spacer function compatible with interaction of the two sub–binding domains so that the resulting polypeptide retains a specific binding affinity to the same target molecule as an antibody that comprises the same light and heavy chain variable regions. In one embodiment, the variable region linking sequence 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.

[0192] In other embodiments, the binding domain of the CAR is followed by 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). 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 may include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region.

[0193] The binding domain of the CAR may generally be followed by one or more "hinge domains," which plays a role in positioning the antigen binding domain away from the effector cell surface to enable proper cell/cell contact, antigen binding and activation. A CAR generally comprises one or more hinge domains between the binding domain and the transmembrane

domain. The hinge domain may be derived either from a natural, synthetic, semi–synthetic, or recombinant source. The hinge domain may include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region.

[0194] Where expression of two or more polypeptides is desired, the polynucleotide sequences encoding them may be separated by an IRES sequence. In another embodiment, two or more polypeptides may be expressed as a fusion protein that comprises one or more self–cleaving polypeptide sequences, such as a T2A polypeptide. In other embodiments, they are expressed from different promotors and can be in two or more vectors. In some embodiments, a NKG2D CAR is encoded in the same vector as a TCR and/or one or more non-NKG2D CARs and is operably linked to the same promotor where the sequences are separated by an IRES sequence. In some embodiments, a NKG2D CAR is encoded in the same vector as a TCR and/or one or more non-NKG2D CARs and is operably linked to the same promotor where the sequences are separated by a cleavable linker. In some embodiments, a NKG2D CAR is encoded in the same vector as a TCR and/or one or more non-NKG2D CARs and the NKG2D CAR is operably linked to a different promotor than the TCR and/or one or more non-NKG2D CARs. In some embodiments, a NKG2D CAR is encoded in a different vector than a TCR and/or one or more non-NKG2D CARs.

[0195] "Polypeptide," "polypeptide fragment," "peptide" and "protein" are, 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 protein sequence or a fragment of a full length protein, and may include 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 various embodiments, the polypeptides contemplated herein 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.

[0196] Polypeptides include "polypeptide variants." Polypeptide variants may differ from a naturally occurring polypeptide in one or more substitutions, deletions, additions and/or insertions. 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 engineered NKG2D CARs by introducing one or more substitutions, deletions, additions and/or insertions. Preferably, polypeptides of the disclosure include polypeptides having at least about 50%, 60%, 65%, 70%, 75%, 85%, 90%, 95%, 98%, or 99% amino acid identity thereto. Polypeptides of the disclosure include variants having at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any of the

reference sequences described herein (see, e.g., Sequence Listing), typically where the variant maintains at least one biological activity of the reference sequence. Polypeptides include "polypeptide fragments." Polypeptide fragments refer to a polypeptide, which may be monomeric or multi–meric that has an amino–terminal deletion, a carboxyl–terminal deletion, and/or an internal deletion or substitution of a naturally–occurring or recombinantly–produced polypeptide. In certain embodiments, a polypeptide fragment may comprise an amino acid chain at least 5 to about 500 amino acids long. It will be appreciated that in certain embodiments, fragments are at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 150, 200, 250, 300, 350, 400, or 450 amino acids long.

[0197] The polypeptide may also be fused in–frame or conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (e.g., poly–His), or to enhance binding of the polypeptide to a solid support. As noted above, polypeptides of the present disclosure 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 may 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.).

[0198] In certain embodiments, a variant will contain conservative substitutions. 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 of the present disclosure and still obtain a functional molecule that encodes a variant or derivative polypeptide with desirable characteristics.

[0199] Polypeptide variants further include glycosylated forms, aggregative conjugates with other molecules, and covalent conjugates with unrelated chemical moieties (e.g., pegylated molecules). Covalent variants may 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.

[0200] Polypeptides of the present disclosure include fusion polypeptides. In some embodiments, fusion polypeptides and polynucleotides encoding fusion polypeptides are provided. 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 may 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 may be in any order or a specified order. Fusion polypeptides or fusion proteins may 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 common techniques. Ligated DNA sequences comprising the fusion polypeptide are operably linked to suitable transcriptional or translational control elements as discussed elsewhere herein.

[0201] 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.

[0202] Fusion polypeptides may further comprise a polypeptide cleavage signal between each of the polypeptide domains described herein. In addition, polypeptide site may 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). Suitable protease cleavages sites and self–cleaving peptides are known to the skilled person (see, e.g., in Ryan *et al.*, 1997. *J Gener. Viral.* 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 Pl (P35) proteases, byovirus Nia proteases, byovirus RNA–2–encoded proteases, aphthovirus L proteases, enterovirus 2A proteases, rhinovirus 2A proteases, picoma 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. Due to its high cleavage stringency, TEV (tobacco etch virus) protease cleavage sites may be used. In other embodiments,

self–cleaving peptides may include those polypeptide sequences obtained from potyvirus and cardiovirus 2A peptides, FMDV (foot–and–mouth disease virus), equine rhinitis A virus, Thosea asigna virus and porcine teschovirus. In other embodiments, the self–cleaving polypeptide site comprises a 2A or 2A–like site, sequence or domain (Donnelly *et al.*, 2001. *J Gen. Viral.* 82:1027–1041).

[0203] Generally, it is understood that any appropriate viral vector or vectors may be used for transduction of the engineered constructs described herein. In one embodiment described herein, a cell (e.g., T cell, NK cell or iPSC) is transduced with a retroviral vector, e.g., a lentiviral vector. As used herein, the term "retrovirus" refers to an RNA virus that reverse transcribes its genomic RNA into a linear double–stranded DNA copy and subsequently covalently integrates its genomic DNA into a host genome. Illustrative retroviruses suitable for use in some embodiments, include, but are not limited to: Moloney murine leukemia virus (M–MuLV), Moloney murine sarcoma virus (MoMSV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), feline leukemia virus (FLV), spumavirus, Friend murine leukemia virus, Murine Stem Cell Virus (MSCV) and Rous Sarcoma Virus (RSV) and lentivirus.

[0204] As used herein, the term "lentivirus" refers to a group (or genus) of complex retroviruses. Illustrative lentiviruses include, but are not limited to: HIV (human immunodeficiency virus; including HIV type 1, and HIV type 2); visna–maedi virus (VMV) virus; the caprine arthritis encephalitis virus (CAEV); equine infectious anemia virus (EIAV); feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV).

[0205] 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. Useful vectors include, for example, plasmids (e.g., DNA plasmids or RNA plasmids), transposons, cosmids, bacterial artificial chromosomes, and viral vectors. Useful viral vectors include, e.g., replication defective retroviruses and lentiviruses.

[0206] As will be evident to one of skill in the art, the term "viral vector" is widely used to refer either to a nucleic acid molecule (e.g., a transfer plasmid) that includes virus–derived nucleic acid elements that typically facilitate transfer of the nucleic acid molecule or integration into the genome of a cell or to a viral particle that mediates nucleic acid transfer. Viral particles will typically include various viral components and sometimes also host cell components in addition to nucleic acid(s).

[0207] The term viral vector may refer either to a virus or viral particle capable of transferring a nucleic acid into a cell or to the transferred nucleic acid itself. Viral vectors and transfer plasmids contain structural and/or functional genetic elements that are primarily derived from a virus. The term "retroviral vector" refers to a viral vector or plasmid containing structural and functional genetic elements, or portions thereof, that are primarily derived from a retrovirus. The term "lentiviral vector" refers to a viral vector or plasmid containing structural and functional genetic elements, or portions thereof, including LTRs that are primarily derived from a lentivirus. The term "hybrid vector" refers to a vector, LTR or other nucleic acid containing both retroviral, e.g., lentiviral, sequences and non–retroviral viral sequences. In one embodiment, a hybrid vector refers to a vector or transfer plasmid comprising retroviral e.g., lentiviral, sequences for reverse transcription, replication, integration and/or packaging.

[0208] In some embodiments, the terms "lentiviral vector," "lentiviral expression vector" may be used to refer to lentiviral transfer plasmids and/or infectious lentiviral particles. Where reference is made herein to elements such as cloning sites, promoters, regulatory elements, heterologous nucleic acids, etc., it is to be understood that the sequences of these elements are present in RNA form in the lentiviral particles of the disclosure and are present in DNA form in the DNA plasmids of the disclosure. In one embodiment described herein, the expression vector is a lentivirus expression vector.

[0209] At each end of the provirus are structures called "long terminal repeats" or "LTRs." The term "long terminal repeat (LTR)" refers to domains of base pairs located at the ends of retroviral DNAs which, in their natural sequence context, are direct repeats and contain U3, Rand U5 regions. LTRs generally provide functions fundamental to the expression of retroviral genes (e.g., promotion, initiation and polyadenylation of gene transcripts) and to viral replication. The LTR contains numerous regulatory signals including transcriptional control elements, polyadenylation signals and sequences needed for replication and integration of the viral genome. The viral LTR is divided into three regions called U3, R, and U5. The U3 region contains the enhancer and promoter elements. The U5 region is the sequence between the primer binding site and the R region and contains the polyadenylation sequence. The R (repeat) region is flanked by the U3 and U5 regions. The LTR is composed of U3, R and U5 regions and appears at both the 5' and 3' ends of the viral genome. Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient packaging of viral RNA into particles (the Psi site).

[0210] As used herein, the term "packaging signal" or "packaging sequence" refers to sequences located within the retroviral genome which are required for insertion of the viral RNA into the viral capsid or particle, see e.g., Clever et al., 1995. J of Virology, Vol. 69, No. 4; pp. 2101–2109.

Several retroviral vectors use the minimal packaging signal (also referred to as the psi ['P] sequence) needed for encapsidation of the viral genome. Thus, as used herein, the terms "packaging sequence," "packaging signal," "psi" and the symbol "'P," are used in reference to the non–coding sequence required for encapsidation of retroviral RNA strands during viral particle formation.

[0211] In various embodiments, vectors comprise modified 5' LTR and/or 3' LTRs. Either or both of the LTR may comprise one or more modifications including, but not limited to, one or more deletions, insertions, or substitutions. Modifications of the 3' LTR are often made to improve the safety of lentiviral or retroviral systems by rendering viruses replication–defective. As used herein, the term "replication–defective" refers to virus that is not capable of complete, effective replication such that infective virions are not produced (e.g., replication–defective lentiviral progeny). The term "replication–competent" refers to wild–type virus or mutant virus that is capable of replication, such that viral replication of the virus is capable of producing infective virions (e.g., replication–competent lentiviral progeny).

[0212] "Self–inactivating" (SIN) vectors refers to replication–defective vectors, e.g., retroviral or lentiviral vectors, in which the right (3') LTR enhancer–promoter region, known as the U3 region, has been modified (e.g., by deletion or substitution) to prevent viral transcription beyond the first round of viral replication. This is because the right (3 ') LTR U3 region is used as a template for the left (5') LTR U3 region during viral replication and, thus, the viral transcript cannot be made without the U3 enhancer–promoter. In a further embodiment of the disclosure, the 3'LTR is modified such that the U5 region is replaced, for example, with an ideal poly(A) sequence. It should be noted that modifications to the LTRs such as modifications to the 3'LTR, the 5'LTR, or both 3' and 5'LTRs, are also contemplated herein.

[0213] An additional safety enhancement is provided by replacing the U3 region of the 5'LTR with a heterologous promoter to drive transcription of the viral genome during production of viral particles. Examples of heterologous promoters which may be used include, for example, viral simian virus 40 (SV40) (e.g., early or late), cytomegalovirus (CMV) (e.g., immediate early), Moloney murine leukemia virus (MoMLV), Rous sarcoma virus (RSV), and herpes simplex virus (HSV) (thymidine kinase) promoters. Typical promoters are able to drive high levels of transcription in a Tat–independent manner. This replacement reduces the possibility of recombination to generate replication–competent virus because there is no complete U3 sequence in the virus production system. In certain embodiments, the heterologous promoter has additional advantages in controlling the manner in which the viral genome is transcribed. For example, the heterologous promoter may be inducible, such that transcription of all or part of the viral genome will occur only when the induction factors are present. Induction factors include, but are not

limited to, one or more chemical compounds or the physiological conditions such as temperature or pH, in which the host cells are cultured.

[0214] In some embodiments, viral vectors comprise a TAR element. The term "TAR" refers to the "trans–activation response" genetic element located in the R region of lentiviral (e.g., HIV) LTRs. This element interacts with the lentiviral trans–activator (tat) genetic element to enhance viral replication.

[0215] The "R region" refers to the region within retroviral LTRs beginning at the start of the capping group (i.e., the start of transcription) and ending immediately prior to the start of the poly A tract. The R region is also defined as being flanked by the U3 and U5 regions. The R region plays a role during reverse transcription in permitting the transfer of nascent DNA from one end of the genome to the other.

[0216] As used herein, the term "FLAP element" refers to a nucleic acid whose sequence includes the central polypurine tract and central termination sequences (cPPT and CTS) of a includes the central polypurine tract and central termination sequences (cPPT and CTS) of a retrovirus, e.g., HIV–I or HIV–2. Suitable FLAP elements are described in U.S. Pat. No. 6,682,907 and in Zennou, et al., 2000, Cell, 101: 173. During HIV–I reverse transcription, central initiation of the plus–strand DNA at the central polypurine tract (cPPT) and central termination at the central termination sequence (CTS) lead to the formation of a three–stranded DNA structure: the HIV–I central DNA flap. While not wishing to be bound by any theory, the DNA flap may act as a cis–active determinant of lentiviral genome nuclear import and/or may increase the titer of the virus.

[0217] In one embodiment, retroviral or lentiviral transfer vectors comprise one or more export elements. The term "export element" refers to a cis–acting post–transcriptional regulatory element which regulates the transport of an RNA transcript from the nucleus to the cytoplasm of a cell. Examples of RNA export elements include, but are not limited to, the human immunodeficiency virus (HIV) rev response element (RRE) (see e.g., Cullen et al., 1991. J Virol. 65: 1053; and Cullen et al., 1991. Cell 58: 423), and the hepatitis B virus post–transcriptional regulatory element (HPRE). Generally, the RNA export element is placed within the 3' UTR of a gene, and may be inserted as one or multiple copies.

[0218] In other embodiments, expression of heterologous sequences in viral vectors is increased by incorporating post–transcriptional regulatory elements, efficient polyadenylation sites, and optionally, transcription termination signals into the vectors. A variety of posttranscriptional regulatory elements may increase expression of a heterologous nucleic acid at the protein, e.g., woodchuck hepatitis virus post–transcriptional regulatory element (WPRE; Zufferey et al., 1999, J Virol., 73:2886); the post–transcriptional regulatory element present in hepatitis B virus (HPRE) (Huang et al., Mol. Cell. Biol., 5:3864); and the like (Liu et al., 1995, Genes Dev., 9:1766).

[0219] In some embodiments, vectors may include regulatory oligonucleotides having transcriptional or translational regulatory activity. Such an oligonucleotide can be used in a variety of gene expression configurations for regulating control of expression. A transcriptional regulatory oligonucleotide, can increase (enhance) or decrease (silence) the level of expression of a recombinant expression construct. Regulatory oligonucleotides may selectively regulate expression in a context specific manner, including, for example, for conferring tissue specific, developmental stage specific, or the like expression of the polynucleotide, including constitutive or inducible expression. A regulatory oligonucleotide of the disclosure also can be a component of an expression vector or of a recombinant nucleic acid molecule comprising the regulatory oligonucleotide operatively linked to an expressible polynucleotide. A regulatory element can be of various lengths from a few nucleotides to several hundred nucleotides.

[0220] 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 "poly A site" or "poly A 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 may promote mRNA stability by addition of a poly A tail to the 3' end of the coding sequence and thus, contribute to increased translational efficiency. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. Illustrative examples of poly A signals that may be used in a vector of the disclosure, includes an ideal poly A sequence (e.g., AATAAA, ATTAAA, AGTAAA), a bovine growth hormone poly A sequence (BGHpA), a rabbit β–globin poly A sequence (rβgpA), or another suitable heterologous or endogenous poly A sequence known in the art.

[0221] Also described herein are “codon-optimized” nucleic acids. A "codon-optimized” nucleic acid refers to a nucleic acid sequence that has been altered such that the codons are optimal for expression in a particular system (such as a particular species or group of species). For example, a nucleic acid sequence can be optimized for expression in mammalian cells or in a particular mammalian species (such as human cells) by replacing at least one, more than one, or a significant number, of codons of the native sequence with codons that are more frequently or most frequently used in the genes of that species. Codon optimization does not alter the amino acid sequence of the encoded protein.

[0222] The codon-optimized nucleotide sequences can present improved properties related to expression efficacy. In some embodiments, the DNA sequence to be transcribed may be optimized

to facilitate more efficient transcription and/or translation. In some embodiments, the DNA sequence may be optimized regarding cis-regulatory elements (e.g., TATA box, termination signals, and protein binding sites), artificial recombination sites, chi sites, CpG dinucleotide content, negative CpG islands, GC content, polymerase slippage sites, and/or other elements relevant to transcription; the DNA sequence may be optimized regarding cryptic splice sites, mRNA secondary structure, stable free energy of mRNA, repetitive sequences, RNA instability domain, and/or other elements relevant to mRNA processing and stability; the DNA sequence may be optimized regarding codon usage bias, codon adaptability, internal chi sites, ribosomal binding sites (e.g., IRES), premature polyA sites, Shine-Dalgarno (SD) sequences, and/or other elements relevant to translation; and/or the DNA sequence may be optimized regarding codon context, codon-anticodon interaction, translational pause sites, and/or other elements relevant to protein folding.

[0223] The vectors may have one or more LTRs, wherein any LTR comprises one or more modifications, such as one or more nucleotide substitutions, additions, or deletions. The vectors may further comprise one of more accessory elements to increase transduction efficiency (e.g., a cPPT /FLAP), viral packaging (e.g., a Psi (Ψ) packaging signal, RRE), and/or other elements that increase therapeutic gene expression (e.g., poly (A) sequences), and may optionally comprise a WPRE or HPRE. The skilled artisan would appreciate that many other different embodiments may be fashioned from the existing embodiments of the disclosure.

[0224] A "host cell" includes cells transfected, infected, or transduced in vivo, ex vivo, or in vitro with a recombinant vector or a polynucleotide of the disclosure. Host cells may include packaging cells, producer cells, and cells infected with viral vectors. In some embodiments, host cells infected with viral vector of the disclosure are administered to a subject in need of therapy. In certain embodiments, the term "target cell" is used interchangeably with host cell and refers to transfected, infected, or transduced cells of a desired cell type. In some embodiments, the target cell is a T cell.

[0225] Large scale viral particle production is often necessary to achieve a reasonable viral titer. Viral particles are produced by transfecting a transfer vector into a packaging cell line that comprises viral structural and/or accessory genes, e.g., gag, pol, env, tat, rev, vif, vpr, vpu, vpx, or nef genes or other retroviral genes.

[0226] As used herein, the term "packaging vector" refers to an expression vector or viral vector that lacks a packaging signal and comprises a polynucleotide encoding one, two, three, four or more viral structural and/or accessory genes. Typically, the packaging vectors are included in a packaging cell, and are introduced into the cell via transfection, transduction or infection. Methods for transfection, transduction or infection are well known by those of skill in the art. A

retroviral/lentiviral transfer vector of the present disclosure may be introduced into a packaging cell line, via transfection, transduction or infection, to generate a producer cell or cell line. The packaging vectors of the present disclosure may be introduced into human cells or cell lines by common methods including, e.g., calcium phosphate transfection, lipofection or electroporation. In some embodiments, the packaging vectors are introduced into the cells together with a dominant selectable marker, such as neomycin, hygromycin, puromycin, blastocidin, zeocin, thymidine kinase, DHFR, Gln synthetase or ADA, followed by selection in the presence of the appropriate drug and isolation of clones. A selectable marker gene may be linked physically to genes encoding by the packaging vector, e.g., by IRES or self–cleaving viral peptides.

[0227] Viral envelope proteins (env) determine the range of host cells which may ultimately be infected and transformed by recombinant retroviruses generated from the cell lines. In the case of lentiviruses, such as HIV–1, HIV–2, SIV, FIV and EIV, the env proteins include gp41 and gp120. In some embodiments, the viral env proteins expressed by packaging cells of the disclosure are encoded on a separate vector from the viral gag and pol genes, as has been previously described.

[0228] Illustrative examples of retroviral–derived env genes which may be employed in the embodiments described herein include, but are not limited to: MLV envelopes, IOAI envelope, BAEV, FeLV–B, RDI 14, SSAV, Ebola, Sendai, FPV (Fowl plague virus), and influenza virus envelopes. Similarly, genes encoding envelopes from RNA viruses (e.g., RNA virus families of Picomaviridae, Calciviridae, Astroviridae, Togaviridae, Flaviviridae, Coronaviridae, Paramyxoviridae, Rhabdoviridae, Filoviridae, Orthomyxoviridae, Bunyaviridae, Arenaviridae, Reoviridae, Bimaviridae, Retroviridae) as well as from the DNA viruses (families of Hepadnaviridae, Circoviridae, Parvoviridae, Papovaviridae, Adenoviridae, Herpesviridae, Poxyiridae, and Iridoviridae) may be utilized. Representative examples include, FeLV, VEE, HFVW, WDSV, SFV, Rabies, ALV, BIV, BL V, EBV, CAEV, SNV, ChTL V, STLV, MPMV SMRV, RAV, FuSV, MH2, AEV, AMV, CTIO, and EIAV.

[0229] In other embodiments, envelope proteins for pseudotyping a virus of present disclosure include, but are not limited to any of the following virus: Influenza A such as H1N1, H1N2, H3N2 and H5Nl (bird flu), Influenza B, Influenza C virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis D virus, Hepatitis E virus, Rotavirus, any virus of the Norwalk virus group, enteric adenoviruses, parvovirus, Dengue fever virus, Monkey pox, Mononegavirales, Lyssavirus such as rabies virus, Lagos bat virus, Mokola virus, Duvenhage virus, European bat virus 1 & 2 and Australian bat virus, Ephemerovirus, Vesiculovirus, Vesicular Stomatitis Virus (VSV), Herpes viruses such as Herpes simplex virus types 1 and 2, varicella zoster, cytomegalovirus, Epstein–Barr virus (EBV), human herpesviruses (HHV), human herpesvirus type 6 and 8, Human immunodeficiency virus (HIV), papilloma virus, murine gamma herpes virus, Arenaviruses such

as Argentine hemorrhagic fever virus, Bolivian hemorrhagic fever virus, Sabia–associated hemorrhagic fever virus, Venezuelan hemorrhagic fever virus, Lassa fever virus, Machupo virus, Lymphocytic choriomeningitis virus (LCMV), Bunyaviridiae such as Crimean–Congo hemorrhagic fever virus, Hantavirus, hemorrhagic fever with renal syndrome causing virus, Rift Valley fever virus, Filoviridae (filovirus) including Ebola hemorrhagic fever and Marburg hemorrhagic fever, Flaviviridae including Kaysanur Forest disease virus, Omsk hemorrhagic fever virus, Tick–borne encephalitis causing virus and Paramyxoviridae such as Hendra virus and Nipah virus, variola major and variola minor (smallpox), alphaviruses such as Venezuelan equine encephalitis virus, eastern equine encephalitis virus, western equine encephalitis virus, SARS–associated coronavirus (SARS–Co V), West Nile virus, or any encephaliltis causing virus.

[0230] The terms "pseudotype" or "pseudotyping" as used herein, refer to a virus whose viral envelope proteins have been substituted with those of another virus possessing other characteristics. For example, HIV may be pseudotyped with vesicular stomatitis virus G–protein (VSV–G) envelope proteins, which allows HIV to infect a wider range of cells because HIV envelope proteins (encoded by the env gene) normally target the virus to CD4+ presenting cells.

[0231] As used herein, the term "packaging cell lines" is used in reference to cell lines that do not contain a packaging signal, but do stably or transiently express viral structural proteins and replication enzymes (e.g., gag, pol and env) which are necessary for the correct packaging of viral particles. Any suitable cell line may be employed to prepare packaging cells of the disclosure. Generally, the cells are mammalian cells. In another embodiment, the cells used to produce the packaging cell line are human cells. Suitable cell lines which may be used to produce the packaging cell line include, for example, CHO cells, BHK cells, MDCK cells, C3H 10Tl/2 cells, FLY cells, Psi–2 cells, BOSC 23 cells, P A317 cells, WEHI cells, COS cells, BSC 1 cells, BSC 40 cells, BMT 10 cells, VERO cells, W138 cells, MRC5 cells, A549 cells, HTI080 cells, 293 cells, 293T cells, B–50 cells, 3T3 cells, NIH3T3 cells, HepG2 cells, Saos–2 cells, Huh7 cells, HeLa cells, W163 cells, 211 cells, and 211A cells.

[0232] As used herein, the term "producer cell line" refers to a cell line which is capable of producing recombinant retroviral particles, comprising a packaging cell line and a transfer vector construct comprising a packaging signal. The production of infectious viral particles and viral stock solutions may be carried out using conventional techniques. Methods of preparing viral stock solutions are known in the art and are illustrated by, e.g., Y. Soneoka et al. (1995) Nucl. Acids Res. 23:628–633, and N. R. Landau et al. (1992) J Virol. 66:5110–5113. Infectious virus particles may be collected from the packaging cells using conventional techniques. For example, the infectious particles may be collected by cell lysis, or collection of the supernatant of the cell

culture, as is known in the art. Optionally, the collected virus particles may be purified if desired. Suitable purification techniques are well known to those skilled in the art.

[0233] The delivery of a gene(s) or other polynucleotide sequence using a retroviral or lentiviral vector by means of viral infection rather than by transfection is referred to as "transduction." In one embodiment, retroviral vectors are transduced into a cell through infection and provirus integration. In certain embodiments, a target cell, e.g., a T cell or NK cell, is "transduced" if it comprises a gene or other polynucleotide sequence delivered to the cell by infection using a viral or retroviral vector. In some embodiments, a transduced cell comprises one or more genes or other polynucleotide sequences delivered by a retroviral or lentiviral vector in its cellular genome.

[0234] Disclosed are host cells expressing one or more of the constructs of the disclosure. The host cells may be transduced with one or more viral vectors comprising nucleic acid sequences encoding one or more polypeptides expressing an engineered TCR and/or a CAR. Other methods relating to the use of viral vectors in gene therapy, which may be utilized according to certain embodiments of the present disclosure, may be found in, e.g., Kay, M.A. (1997) Chest 111(6 Supp.): 138S–142S; Ferry, N. and Heard, J.M. (1998) Hum. Gene Ther. 9:1975–81; Shiratory, Y. et al., (1999) Liver 19:265–74; Oka, K. et al., (2000) Curr. Opin. Lipidol. 11:179–86; Thule, P. M. and Liu, J.M. (2000) Gene Ther. 7:1744–52; Yang, N. S. (1992) Crit. Rev. Biotechnol. 12:335–56; Alt, M. (1995) J Hepatol. 23:746–58; Brody, S. L. and Crystal, R.G. (1994) Ann. NY Acad. Sci. 716:90–101; Strayer, D.S. (1999) Expert Opin. Investig. Drugs 8:2159–2172; Smith–Arica, J. R. and Bartlett, J. S. (2001) Curr. Cardiol. Rep. 3:43–49; and Lee, H. C. et al., (2000) Nature 408:483–8.

[0235] The compositions described herein may comprise one or more polynucleotides, polypeptides, vectors comprising same, and T cell composition and NK compositions, as contemplated herein. One embodiment described herein is a composition comprising a modified T cell that expresses a NKG2D CAR. Another embodiment described herein is a composition comprising a modified NK cell that expresses a NKG2D CAR. Compositions include, but are not limited to, pharmaceutical compositions. A "pharmaceutical composition" refers to a composition formulated in pharmaceutically–acceptable or physiologically–acceptable solutions for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy. It will also be understood that, if desired, the compositions of the present disclosure may be administered in combination with other agents as well, such as, e.g., cytokines, growth factors, hormones, small molecules, chemotherapeutics, pro–drugs, drugs, antibodies, or other various pharmaceutically–active agents. There is virtually no limit to other components that may also be included in the compositions, provided that the additional agents do not adversely affect the ability of the composition to deliver the intended therapy.

[0236] 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.

[0237] 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, com 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.

[0238] In one embodiment described herein, compositions of the present disclosure comprise an amount of modified T cells or NK cells contemplated herein. It may generally be stated that a pharmaceutical composition comprising the T cells or NK cells contemplated herein may be administered at a dosage of 10^2 to 10^{10} cells/kg body weight, 10^5 to 10^9 cells/kg body weight, 10^5 to 10^8 cells/kg body weight, 10^5 to 10^7 cells/kg body weight, 10^7 to 10^9 cells/kg body weight, or 10^7 to 10^8 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. T cells or NK cells modified to express an engineered NKG2D CAR 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) or lymphokines, cytokines, and/or chemokines (e.g., IFN–γ, IL–2, IL–7, IL–15, 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 engraftment and function of infused T cells.

[0239] Generally, compositions comprising the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised or immunosuppressed. In some, compositions comprising the modified T cells or NK cells contemplated herein are used in the treatment of cancers. The modified T cells or NK cells described herein may be administered either alone, or as a pharmaceutical composition in combination with carriers, diluents, excipients, and/or with other components such as IL–2, IL–7, and/or IL–15 or other cytokines or cell populations. In some embodiments, pharmaceutical compositions contemplated herein comprise an amount of genetically modified T cells or NK cells, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients.

[0240] Pharmaceutical compositions comprising modified T cells or NK cells contemplated herein may further comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions of the present disclosure may be formulated for parenteral administration, e.g., intravascular (intravenous or intra–arterial), intraperitoneal or intramuscular administration.

[0241] The liquid pharmaceutical compositions, whether they be solutions, suspensions or other like form, may include one or more of the following: sterile diluents such as water for injection, saline solution, such as physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation may be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Sterile injectable pharmaceutical composition are also included.

[0242] In some embodiments, compositions contemplated herein comprise an effective amount of an expanded modified T cell or NK cell composition, alone or in combination with one or more therapeutic agents. Thus, the T cell or NK cell compositions may be administered alone or in combination with other known cancer treatments, such as radiation therapy, chemotherapy, transplantation, immunotherapy, hormone therapy, photodynamic therapy, etc. The compositions may also be administered in combination with antibiotics and anti–viral agents. Such therapeutic agents may be accepted in the art as a treatment for a disease state as described herein, such as a cancer. In one embodiment the compositions contemplated herein may also be administered with

inhibitors of TGF–β, for example the small molecule inhibitor LY55299. Exemplary therapeutic agents contemplated include cytokines, growth factors, steroids, NSAIDs, DMARDs, anti–inflammatories, chemotherapeutics, radiotherapeutics, therapeutic antibodies, or other active and ancillary agents.

[0243] In certain embodiments, compositions comprising T cells or NK cells contemplated herein may be administered in conjunction with any number of chemotherapeutic agents. Illustrative examples of chemotherapeutic agents include but are not limited to alkylating agents such as thiotepa and cyclophosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine resume; nitrogen mustards such as chlorambucil, chlomaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6–diazo–5–oxo–L–norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti–metabolites such as methotrexate and 5–fluorouracil (5–FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6–mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6–azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5–FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti–adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2–ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2"trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara–C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol–Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (TAXOTERE®, Rhone–Poulenc Rorer, Antony,

France); chlorambucil; gemcitabine; 6– thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP–16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT–11; topoisomerase inhibitor RPS 2000; difluoromethylomithine (DMFO); retinoic acid derivatives such as Targretin™ (bexarotene), Panretin™ (alitretinoin); ONTAK™ (denileukin diftitox); esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti–hormonal agents that act to regulate or inhibit hormone action on tumors such as anti–estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)–imidazoles, 4–hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti–androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[0244] A variety of other therapeutic agents may be used in conjunction with the compositions described herein. In one embodiment, the composition comprising T cells is administered with an anti–inflammatory agent. Anti–inflammatory agents or drugs include, but are not limited to, steroids and glucocorticoids (including betamethasone, budesonide, dexamethasone, hydrocortisone acetate, hydrocortisone, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone), nonsteroidal anti–inflammatory drugs (NSAIDS) including aspirin, ibuprofen, naproxen, methotrexate, sulfasalazine, leflunomide, anti–TNF medications, cyclophosphamide and mycophenolate.

[0245] In some embodiments, NSAIDs are chosen from the group consisting of ibuprofen, naproxen, naproxen sodium, Cox–2 inhibitors such as VIOXX® (rofecoxib) and CELEBREX® (celecoxib), and sialylates. Exemplary analgesics are chosen from the group consisting of acetaminophen, oxycodone, tramadol or proporxyphene hydrochloride. Exemplary glucocorticoids are chosen from the group consisting of cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, or prednisone. Exemplary biological response modifiers include molecules directed against cell surface markers (e.g., CD4, CD5, etc.), cytokine inhibitors, such as the TNF antagonists (e.g., etanercept (ENBREL®), adalimumab (HUMIRA®) and infliximab (REMICADE®), chemokine inhibitors and adhesion molecule inhibitors. The biological response modifiers include monoclonal antibodies as well as recombinant forms of molecules. Exemplary disease-modifying anti–rheumatic drugs (DMARDs) include azathioprine, cyclophosphamide, cyclosporine, methotrexate, penicillamine, leflunomide, sulfasalazine, hydroxychloroquine, Gold (oral (auranofin) and intramuscular) and minocycline.

[0246] In other embodiments, the therapeutic antibodies suitable for combination with the CAR or TCR modified T cells or NK cells contemplated herein, include but are not limited to,

abagovomab, adecatumumab, afutuzumab, alemtuzumab, altumomab, amatuximab, anatumomab, arcitumomab, bavituximab, bectumomab, bevacizumab, bivatuzumab, blinatumomab, brentuximab, cantuzumab, catumaxomab, cetuximab, citatuzumab, cixutumumab, clivatuzumab, conatumumab, daratumumab, drozitumab, duligotumab, dusigitumab, detumomab, dacetuzumab, dalotuzumab, ecromeximab, elotuzumab, ensituximab, ertumaxomab, etaracizumab ,farietuzumab, ficlatuzumab, figitumumab, flanvotumab, futuximab, ganitumab, gemtuzumab, girentuximab, glembatumumab, ibritumomab, igovomab, imgatuzumab, indatuximab, inotuzumab, intetumumab, ipilimumab, iratumumab, labetuzumab, lexatumumab, lintuzumab ,lorvotuzumab, lucatumumab, mapatumumab, matuzumab, milatuzumab, minretumomab, mitumomab, moxetumomab, namatumab, naptumomab, necitumumab, nimotuzumab, nofetumomab, ocaratuzumab, ofatumumab, olaratumab, onartuzumab, oportuzumab, oregovomab, panitumumab, parsatuzumab, patritumab, pemtumomab, pertuzumab, pintumomab, pritumumab, racotumomab, radretumab, rilotumumab, rituximab, robatumumab, satumomab, sibrotuzumab, siltuximab, simtuzumab, solitomab, tacatuzumab, taplitumomab, tenatumomab, teprotumumab, tigatuzumab, tositumomab, trastuzumab, tucotuzumab, ublituximab, veltuzumab, vorsetuzumab, votumumab, zalutumumab, CC49 and 3F8.

[0247] In some embodiments, the compositions described herein are administered in conjunction with a cytokine. By "cytokine" as used herein is meant a generic term for proteins released by one cell population that act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, chemokines, and traditional polypeptide hormones. Included among the cytokines are growth hormones such as human growth hormone, N–methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor–alpha and–beta; mullerian–inhibiting substance; mouse gonadotropin–associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF–beta; platelet–growth factor; transforming growth factors (TGFs) such as TGF–alpha and TGF–beta; insulin–like growth factor–I and –II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon–alpha, –beta, and –gamma; colony stimulating factors (CSFs) such as macrophage–CSF (M–CSF); granulocyte–macrophage–CSF (GM–CSF); and granulocyte–CSF (G–CSF); interleukins (ILs) such as IL–1, IL–1α, IL–2, IL–3, IL–4, IL–5, IL–6, IL–7, IL–8, IL–9, IL–10, IL–11, IL–12; IL–15, a tumor necrosis factor such as TNF–α or TNF–β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes

proteins from natural sources or from recombinant cell culture, and biologically active equivalents of the native sequence cytokines.

[0248] Any cell may be used as a host cell for the polynucleotides, the vectors, or the polypeptides of the present disclosure. In some embodiments, the cell can be a prokaryotic cell, fungal cell, yeast cell, or higher eukaryotic cells such as a mammalian cell. Suitable prokaryotic cells include, without limitation, eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobactehaceae such as Escherichia, e.g., E. coli; Enterobacter; Erwinia; Klebsiella; Proteus; Salmonella, e.g., Salmonella typhimurium; Serratia, e.g., Serratia marcescans, and Shigella; Bacilli such as B. subtilis and B. licheniformis; Pseudomonas such as P. aeruginosa; and Streptomyces. In some embodiments, the cell is a human cell. In some embodiments, the cell is an immune cell. In some embodiments, the immune cell is selected from the group consisting of a T cell, a B cell, a tumor infiltrating lymphocyte (TIL), a TCR expressing cell, a natural killer (NK) cell, a dendritic cell, a granulocyte, an innate lymphoid cell, a megakaryocyte, a monocyte, a macrophage, a platelet, a thymocyte, and a myeloid cell. In one embodiment, the immune cell is a T cell. In another embodiment, the immune cell is an NK cell. In certain embodiments, the T cell is a tumor-infiltrating lymphocyte (TIL), autologous T cell, engineered autologous T cell (eACT™), an allogeneic T cell, a heterologous T cell, or any combination thereof. Unlike antibody therapies or standalone NKG2D CAR modified T cells, T cells (or any cells as described above).

[0249] Another embodiment described herein is a method of treating a cancer in a subject in need thereof comprising administering an effective amount, e.g., therapeutically effective amount of a composition comprising T cells or NK cells expressing TCR or CAR as described herein. The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

[0250] In other embodiments, methods comprising administering a therapeutically effective amount of modified T cells contemplated herein or a composition comprising the same, to a patient in need thereof, alone or in combination with one or more therapeutic agents, are provided. In certain embodiments, the cells of the disclosure are used in the treatment of patients at risk for developing a cancer. Thus, the present disclosure provides methods for the treatment or prevention of a cancer comprising administering to a subject in need thereof, a therapeutically effective amount of the modified T cells of the disclosure.

[0251] One of ordinary skill in the art would recognize that multiple administrations of the compositions of the disclosure may be required to affect the desired therapy. For example a composition may be administered 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more times over a span of 1

week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 1 year, 2 years, 5, years, 10 years, or more.

[0252] In certain embodiments, it may be desirable to administer activated T cells to a subject and then subsequently redraw blood (or have an apheresis performed), activate T cells therefrom according to the present disclosure, and reinfuse the patient with these activated and expanded T cells. This process may be carried out multiple times every few weeks. In certain embodiments, T cells may be activated from blood draws of from 10cc to 400cc. Not to be bound by theory, using this multiple blood draw/multiple reinfusion protocol may serve to select out certain populations of T cells.

[0253] The administration of the compositions contemplated herein may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. In some embodiments, compositions are administered parenterally. The phrases "parenteral administration" and "administered parenterally" as used herein refers to modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravascular, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intratumoral, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion. In one embodiment, the compositions contemplated herein are administered to a subject by direct injection into a tumor, lymph node, or site of infection.

[0254] In one embodiment, a subject in need thereof is administered an effective amount of a composition to increase a cellular immune response to a cancer in the subject. The immune response may include cellular immune responses mediated by cytotoxic T cells capable of killing infected cells, regulatory T cells, and helper T cell responses. Humoral immune responses, mediated primarily by helper T cells capable of activating B cells thus leading to antibody production, may also be induced. A variety of techniques may be used for analyzing the type of immune responses induced by the compositions of the present disclosure, which are well described in the art; e.g., 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, N.Y.

[0255] In the case of T cell–mediated killing, CAR–ligand binding initiates CAR signaling to the T cell, resulting in activation of a variety of T cell signaling pathways that induce the T cell to produce or release proteins capable of inducing target cell apoptosis by various mechanisms. These T cell–mediated mechanisms include (but are not limited to) the transfer of intracellular cytotoxic granules from the T cell into the target cell, T cell secretion of proinflammatory cytokines that may induce target cell killing directly (or indirectly via recruitment of other killer effector cells), and up regulation of death receptor ligands (e.g. FasL) on the T cell surface that

induce target cell apoptosis following binding to their cognate death receptor (e.g. Fas) on the target cell.

[0256] In embodiments described herein is a method of treating a subject diagnosed with a cancer, comprising removing T cells from the subject, genetically modifying said T cells with a vector comprising a nucleic acid encoding a NKG2D CAR as contemplated herein, thereby producing a population of modified T cells, and administering the population of modified T cells to the same subject.

[0257] In certain embodiments, the present disclosure also provides methods for stimulating an effector cell mediated immune modulator response to a target cell population in a subject comprising the steps of administering to the subject an immune effector cell population expressing a nucleic acid construct encoding a NKG2D CAR molecule.

[0258] The methods for administering the cell compositions described herein includes any method which is effective to result in reintroduction of ex vivo genetically modified immune effector cells that either directly express an engineered NKG2D CAR in the subject or on reintroduction of the genetically modified progenitors of immune effector cells that on introduction into a subject differentiate into mature immune effector cells that express the d NKG2D CAR molecule,. One method comprises transducing peripheral blood T cells ex vivo with a nucleic acid construct in accordance with the present disclosure and returning the transduced cells into the subject.

[0259] Although the foregoing disclosure has 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 of this disclosure 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 skilled in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

EXAMPLES

Example 1

NKG2D CAR construct design

[0260] As used in the following Examples engineered NKG2D chimeric antigen receptor constructs were designed and synthesized in a retroviral vector. A first construct, termed NKG2D CAR1 includes from N to C-terminus a signal peptide, a NKG2D extracellular domain, a CD8alpha hinge, a CD28 transmembrane domain, a 4-1BB intercellular domain, and a signaling domain comprising a CD3ζ signaling domain. The amino acid sequence of this chimeric antigen receptor is shown below:

MALPVTALLLPLALLLHAARPLFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKN
WYESQASCMSQNASLLKVYSKEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSP
NLLTIIEMQKGDCALYASSFKGYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSL
RPEACRPAAGGAVHTRGLDFACDFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKL
LYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNEL
NLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERR
RGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 53).

[0261] A second construct, termed NKG2D CAR2 includes from N to C-terminus a signal peptide, a NKG2D extracellular domain, a CD8alpha hinge, a CD28 transmembrane domain, a 4-1BB intercellular domain, and a signaling domain comprising a CD3ε signaling domain and a CD3ζ signaling domain. The amino acid sequence of this chimeric antigen receptor is shown below:

MALPVTALLLPLALLLHAARPLFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKN
WYESQASCMSQNASLLKVYSKEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSP
NLLTIIEMQKGDCALYASSFKGYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSL
RPEACRPAAGGAVHTRGLDFACDFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKL
LYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEKNRKAKAKPVTRGAGAGGRQRGQN
KERPPPVPNPDYEPIRKGQRDLYSGLLRVKFSRSADAPAYQQGQNQLYNELNLGRREEY
DVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG
LYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 54).

[0262] A third construct, termed NKG2D CAR3 comprises from N to C-terminus a NKG2D extracellular domain, a CD8 hinge, a CD8 transmembrane domain, a 4-1BB intercellular domain, and a signaling domain comprising a CD3ζ signaling domain. The amino acid sequence of this chimeric antigen receptor comprises the following amino acid sequence: LFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYS
KEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFK
GYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF
ACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFP
EEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGK
PRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH
MQALPPR (SEQ ID NO: 66). In one aspect, CAR3 is encoded by the following nucleotide sequence:

1 cgtgaggctc cggtgcccgt cagtgggcag agcgcacatc gcccacagtc cccgagaagt

61 tgggggggagg ggtcggcaat tgaaccggtg cctagagaag gtggcgcggg gtaaactggg

121 aaagtgatgt cgtgtactgg ctccgccttt ttcccgaggg tgggggagaa ccgtatataa

181 gtgcagtagt cgccgtgaac gttctttttc gcaacgggtt tgccgccaga acacaggtaa
241 gtgccgtgtg tggttcccgc gggcctggcc tctttacggg ttatggccct tgcgtgcctt
301 gaattacttc cacgcccctg gctgcagtac gtgattcttg atcccgagct tcgggttgga
361 agtgggtggg agagttcgag gccttgcgct taaggagccc cttcgcctcg tgcttgagtt
421 gaggcctggc ttgggcgctg gggccgccgc gtgcgaatct ggtggcacct tcgcgcctgt
481 ctcgctgctt tcgataagtc tctagccatt taaaattttt gatgacctgc tgcgacgctt
541 tttttctggc aagatagtct tgtaaatgcg ggccaagatc tgcacactgg tatttcggtt
601 tttggggccg cgggcggcga cggggcccgt gcgtcccagc gcacatgttc ggcgaggcgg
661 ggcctgcgag cgcggccacc gagaatcgga cgggggtagt ctcaagctgg ccggcctgct
721 ctggtgcctg gcctcgcgcc gccgtgtatc gccccgccct gggcggcaag gctggcccgg
781 tcggcaccag ttgcgtgagc ggaaagatgg ccgcttcccg gccctgctgc agggagctca
841 aaatggagga cgcggcgctc gggagagcgg gcgggtgagt cacccacaca aaggaaaagg
901 gcctttccgt cctcagccgt cgcttcatgt gactccacgg agtaccgggc gccgtccagg
961 cacctcgatt agttctcgag cttttggagt acgtcgtctt taggttgggg ggaggggttt
1021 tatgcgatgg agtttcccca cactgagtgg gtggagactg aagttaggcc agcttggcac
1081 ttgatgtaat tctccttgga atttgccctt tttgagtttg gatcttggtt cattctcaag
1141 cctcagacag tggttcaaag ttttttttctt ccatttcagg tgtcgtgaaa actaccccta
1201 aaagccaaag cgccgccacc atggctcttc ctgtgacagc tcttctgctg cccctggccc
1261 tgcttctgca tgctgctaga cctgagcaaa agttgatttc tgaggaagac ctcgccggca
1321 gtttattcaa ccaagaagtc caaattccct tgaccgaaag ttactgtggc ccatgtccta
1381 agaactggat atgttacaaa aataactgtt accaattctt cgatgaatct aagaattggt
1441 atgagagcca ggcttcttgt atgtctcaaa atgccagcct tcttaaagta tacagcaaag
1501 aggaccagga tttacttaaa ctggtgaagt catatcattg gatgggacta gtacacattc
1561 caacaaatgg atcttggcag tgggaagacg gctccattct ctcacccaac ctactaacaa
1621 taattgaaat gcagaaggga gactgtgcac tctatgcatc gagctttaaa ggctatatag
1681 aaaactgttc aactccaaat acatatattt gcatgcaaag gactgtgacc acgacgccag
1741 cgccgcgacc accaacaccg gcgcccacca tcgcgtcgca acccctgtcc ctgaggcctg
1801 aagcgtgccg gccagcggcg ggcggcgcag tgcacacgag agggctggac ttcgcctgtg
1861 atatctacat ctgggcgccc ttggccggga cttgtggggt ccttctcctg tcactggtta
1921 tcacccttta ctgcaaacgg ggcagaaaga aactcctgta tatattcaaa caaccattta
1981 tgagaccagt acaaactact caagaggaag atggctgtag ctgccgattt ccagaagaag
2041 aagaaggagg atgtgaactg agagtgaagt tcagcaggag cgcagacgcc cccgcgtacc
2101 agcaagggca gaaccagctc tataacgagc tcaatctagg acgaagagag gagtacgatg
2161 ttttggacaa gaggcgtggc cgggaccctg agatgggggg aaagccgaga aggaagaacc
2221 ctcaggaagg cctgtacaat gaactgcaga aagataagat ggcggaggcc tacagtgaga

2281 ttgggatgaa aggcgagcgc cggaggggca aggggcacga tggcctttac cagggtctca

2341 gtacagccac caaggacacc tacgacgccc ttcacatgca agctctgccc cctcgctga (SEQ ID NO: 67). CAR3 can be encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to (SEQ ID NO: 67). In certain aspects, the nucleic acid sequence of (SEQ ID NO: 67) may be modified to remove sequences encoding the Myc (human c-Myc proto-oncogene) epitope tag and linker between the Myc tag and the NKG2D extracellular domain. The nucleic acid sequence of (SEQ ID NO: 67) encodes for a CD8a signal peptide at positions 1221-1283, which signal peptide may be substituted for a different signal peptide.

[0263] A fourth construct, termed NKG2D CAR4 comprises from N to C-terminus a NKG2D extracellular domain, a CD8alpha hinge, a CD28 transmembrane domain, a 4-1BB intercellular domain, and a signaling domain comprising a CD3ζ signaling domain. The amino acid sequence of this chimeric antigen receptor comprises the following amino acid sequence: LFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYS KEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFK GYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACDFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGC SCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPE MGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDT YDALHMQALPPR (SEQ ID NO: 68). In one aspect, CAR4 is encoded by the following nucleotide sequence:

1 cgtgaggctc cggtgcccgt cagtgggcag agcgcacatc gcccacagtc cccgagaagt

61 tgggggggagg ggtcggcaat tgaaccggtg cctagagaag gtggcgcggg gtaaactggg

121 aaagtgatgt cgtgtactgg ctccgccttt ttcccgaggg tgggggagaa ccgtatataa

181 gtgcagtagt cgccgtgaac gttcttttttc gcaacgggtt tgccgccaga acacaggtaa

241 gtgccgtgtg tggttcccgc gggcctggcc tctttacggg ttatggccct tgcgtgcctt

301 gaattacttc cacgcccctg gctgcagtac gtgattcttg atcccgagct tcgggttgga

361 agtgggtggg agagttcgag gccttgcgct taaggagccc cttcgcctcg tgcttgagtt

421 gaggcctggc ttgggcgctg gggccgccgc gtgcgaatct ggtggcacct tcgcgcctgt

481 ctcgctgctt tcgataagtc tctagccatt taaaattttt gatgacctgc tgcgacgctt

541 tttttctggc aagatagtct tgtaaatgcg ggccaagatc tgcacactgg tatttcggtt

601 tttggggccg cgggcggcga cggggcccgt gcgtcccagc gcacatgttc ggcgaggcgg

661 ggcctgcgag cgcggccacc gagaatcgga cgggggtagt ctcaagctgg ccggcctgct

721 ctggtgcctg gcctcgcgcc gccgtgtatc gccccgccct gggcggcaag gctggcccgg

781 tcggcaccag ttgcgtgagc ggaaagatgg ccgcttcccg gccctgctgc agggagctca

841 aaatggagga cgcggcgctc gggagagcgg gcgggtgagt cacccacaca aaggaaaagg
901 gcctttccgt cctcagccgt cgcttcatgt gactccacgg agtaccgggc gccgtccagg
961 cacctcgatt agttctcgag cttttggagt acgtcgtctt taggttgggg ggaggggttt
1021 tatgcgatgg agtttcccca cactgagtgg gtggagactg aagttaggcc agcttggcac
1081 ttgatgtaat tctccttgga atttgccctt tttgagtttg gatcttggtt cattctcaag
1141 cctcagacag tggttcaaag ttttttttctt ccatttcagg tgtcgtgaaa actaccccta
1201 aaagccaaag cgccgccacc atggctcttc ctgtgacagc tcttctgctg cccctggccc
1261 tgcttctgca tgctgctaga cctgagcaaa agttgatttc tgaggaagac ctcgccggca
1321 gtttattcaa ccaagaagtc caaattccct tgaccgaaag ttactgtggc ccatgtccta
1381 agaactggat atgttacaaa aataactgtt accaattctt cgatgaatct aagaattggt
1441 atgagagcca ggcttcttgt atgtctcaaa atgccagcct tcttaaagta tacagcaaag
1501 aggaccagga tttacttaaa ctggtgaagt catatcattg gatgggacta gtacacattc
1561 caacaaatgg atcttggcag tgggaagacg gctccattct ctcacccaac ctactaacaa
1621 taattgaaat gcagaaggga gactgtgcac tctatgcatc gagctttaaa ggctatatag
1681 aaaactgttc aactccaaat acatatattt gcatgcaaag gactgtgacc acgacgccag
1741 cgccgcgacc accaacaccg gcgcccacca tcgcgtcgca acccctgtcc ctgaggcctg
1801 aagcgtgccg gccagcggcg ggcggcgcag tgcacacgag agggctggac ttcgcctgtg
1861 atttttgggt gctggtggtg gttggtggag tcctggcttg ctatagcttg ctagtaacag
1921 tggcctttat tattttctgg gtcaaacggg gcagaaagaa actcctgtat atattcaaac
1981 aaccatttat gagaccagta caaactactc aagaggaaga tggctgtagc tgccgatttc
2041 cagaagaaga agaaggagga tgtgaactga gagtgaagtt cagcaggagc gcagacgccc
2101 ccgcgtacca gcaagggcag aaccagctct ataacgagct caatctagga cgaagagagg
2161 agtacgatgt tttggacaag aggcgtggcc gggaccctga gatgggggga aagccgagaa
2221 ggaagaaccc tcaggaaggc ctgtacaatg aactgcagaa agataagatg gcggaggcct
2281 acagtgagat tgggatgaaa ggcgagcgcc ggaggggcaa ggggcacgat ggcctttacc
2341 agggtctcag tacagccacc aaggacacct acgacgccct tcacatgcaa gctctgcccc
2401ctcgctga (SEQ ID NO: 69)

CAR4 can be encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to (SEQ ID NO: 69). In certain aspects, the nucleic acid sequence of (SEQ ID NO: 69) may be modified to remove sequences encoding the Myc (human c-Myc proto-oncogene) epitope tag and linker between the Myc tag and the NKG2D extracellular domain. The nucleic acid sequence of (SEQ ID NO: 69) encodes for a CD8a signal peptide at positions 1221-1283, which signal peptide may be substituted for a different signal peptide.

[0264] A fifth construct, termed NKG2D CAR5 comprises from N to C-terminus a NKG2D extracellular domain, a CD8 hinge, a CD8 transmembrane domain, a 4-1BB intercellular domain, and a signaling domain comprising a CD3ε signaling domain and a CD3ζ signaling domain. The amino acid sequence of this chimeric antigen receptor comprises the following amino acid sequence:

LFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYS
KEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFK
GYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF
AIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEE
EEGGCELKNRKAKAKPVTRGAGAGGRQRGQNKERPPPVPNPDYEPIRKGQRDLYSGLN
QRRILRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRK
NPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQA
LPPR (SEQ ID NO: 70). In one aspect, CAR5 is encoded by the following nucleotide sequence:

1 cgcggaatga aagaccccac ctgtaggttt ggcaagctag cttaagtaac gccattttgc
61 aaggcatgga aaatacataa ctgagaatag agaagttcag atcaaggtta ggaacagaga
121 gacagcagaa tatgggccaa acaggatatc tgtggtaagc agttcctgcc ccggctcagg
181 gccaagaaca gatggtcccc agatgcggtc ccgccctcag cagtttctag agaaccatca
241 gatgtttcca gggtgcccca aggacctgaa atgaccctgt gccttatttg aactaaccaa
301 tcagttcgct tctcgcttct gttcgcgcgc ttctgctccc cgagctcaat aaaagagccc
361 acaacccctc actcggcgcg ccagtccttc gaagtagatc tttgtcgatc ctaccatcca
421 ctcgacacac ccgccagcgg ccgctgccaa gcttccgagc tctcgaatta attcacgccg
481 ccaccatggc tcttcctgtg acagctcttc tgctgcccct ggccctgctt ctgcatgctg
541 ctagacctga gcaaaagttg atttctgagg aagacctcgc cggcagttta ttcaaccaag
601 aagtccaaat tcccttgacc gaaagttact gtggcccatg tcctaagaac tggatatgtt
661 acaaaaataa ctgttaccaa ttcttcgatg aatctaagaa ttggtatgag agccaggctt
721 cttgtatgtc tcaaaatgcc agccttctta aagtatacag caaagaggac caggatttac
781 ttaaactggt gaagtcatat cattggatgg gactagtaca cattccaaca aatggatctt
841 ggcagtggga agacggctcc attctctcac ccaacctact aacaataatt gaaatgcaga
901 agggagactg tgcactctat gcatcgagct ttaaaggcta tatagaaaac tgttcaactc
961 caaatacata tatttgcatg caaaggactg tgaccaccac tcctgctcca agacctccta
1021 cccccgctcc tacaatcgcc agccaacctc tgagcctgag accggaggca tgcagacctg
1081 cggcaggggg agcagttcac acaagaggct tggacttcgc ttgcgacatc tacatctggg
1141 cccctctggc cggcacatgc ggagttcttc ttcttagcct ggtgatcacc ctgtactgca
1201 agagaggccg gaagaagctg ctgtacatct tcaagcagcc cttcatgaga cctgtgcaga
1261 ccacacagga ggaagacggc tgcagctgta gattccccga ggaagaggag ggcggctgtg

1321 agctgaagaa ccgcaaagca aaggcaaaac ccgtcacacg aggagcgggc gcagggggac
1381 gacaacgcgg tcagaataag gaacgcccgc ctccagtacc aaatccagat tatgaaccaa
1441 ttcggaaggg acaacgcgat ctctactccg gtctcaatca gaggcgaatt ctgagagtta
1501 agttcagcag gagcgccgac gcccctgcct accagcaagg acagaatcaa ctgtacaacg
1561 agctgaacct gggcagacgg gaggaatacg atgtgctgga caagaggaga ggcagagacc
1621 ccgagatggg cggcaaacct agaagaaaga acccccagga gggcctgtat aacgagctcc
1681 agaaggacaa gatggccgag gcctacagcg agatcggcat gaagggcgaa agaagaagag
1741 gcaagggcca cgacggcctc taccagggct taagcacagc tacaaaggac acctacgacg
1801 ccctgcacat gcaggccctg cccctagat ga (SEQ ID NO: 71)

CAR5 can be encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to (SEQ ID NO: 71). In certain aspects, the nucleic acid sequence of (SEQ ID NO: 71) may be modified to remove sequences encoding the Myc (human c-Myc proto-oncogene) epitope tag and linker between the Myc tag and the NKG2D extracellular domain. The nucleic acid sequence of (SEQ ID NO: 71) encodes for a CD8a signal peptide at positions 486-548, which signal peptide may be substituted for a different signal peptide.

[0265] A sixth construct, termed NKG2D CAR6 comprises from N to C-terminus a NKG2D extracellular domain, a CD8alpha hinge, a CD8 transmembrane domain, a 4-1BB intercellular domain, and a signaling domain comprising a CD3ε signaling domain and a CD3ζ signaling domain. The amino acid sequence of this chimeric antigen receptor comprises the following amino acid sequence:

LFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYS
KEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFK
GYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF
ACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFP
EEEEGGCEKNRKAKAKPVTRGAGAGGRQRGQNKERPPPVPNPDYEPIRKGQRDLYSGL
LRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQE
GLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

(SEQ ID NO: 72). In one aspect, CAR6 is encoded by the following nucleotide sequence:

1 cgcggaatga aagaccccac ctgtaggttt ggcaagctag cttaagtaac gccattttgc
61 aaggcatgga aaatacataa ctgagaatag agaagttcag atcaaggtta ggaacagaga
121 gacagcagaa tatgggccaa acaggatatc tgtggtaagc agttcctgcc ccggctcagg
181 gccaagaaca gatggtcccc agatgcggtc ccgccctcag cagtttctag agaaccatca
241 gatgtttcca gggtgcccca aggacctgaa atgaccctgt gccttatttg aactaaccaa
301 tcagttcgct tctcgcttct gttcgcgcgc ttctgctccc cgagctcaat aaaagagccc

361 acaacccctc actcggcgcg ccagtccttc gaagtagatc tttgtcgatc ctaccatcca
421 ctcgacacac ccgccagcgg ccgctgccaa gcttccgagc tctcgaatta attcacgccg
481 ccaccatggc tcttcctgtg acagctcttc tgctgcccct ggccctgctt ctgcatgctg
541 ctagacctga gcaaaagttg atttctgagg aagacctcgc cggcagttta ttcaaccaag
601 aagtccaaat tcccttgacc gaaagttact gtggcccatg tcctaagaac tggatatgtt
661 acaaaaataa ctgttaccaa ttcttcgatg aatctaagaa ttggtatgag agccaggctt
721 cttgtatgtc tcaaaatgcc agccttctta aagtatacag caaagaggac caggatttac
781 ttaaactggt gaagtcatat cattggatgg gactagtaca cattccaaca aatggatctt
841 ggcagtggga agacggctcc attctctcac ccaacctact aacaataatt gaaatgcaga
901 agggagactg tgcactctat gcatcgagct ttaaaggcta tatagaaaac tgttcaactc
961 caaatacata tatttgcatg caaaggactg tgaccacgac gccagcgccg cgaccaccaa
1021 caccggcgcc caccatcgcg tcgcaacccc tgtccctgag gcctgaagcg tgccggccag
1081 cggcgggcgg cgcagtgcac acgagagggc tggacttcgc ctgtgatatc tacatctggg
1141 cgcccttggc cgggacttgt ggggtccttc tcctgtcact ggttatcacc ctttactgca
1201 aacggggcag aaagaaactc ctgtatatat tcaaacaacc atttatgaga ccagtacaaa
1261 ctactcaaga ggaagatggc tgtagctgcc gatttccaga agaagaagaa ggaggatgtg
1321 aaaagaaccg aaaagcaaaa gccaagcctg ttacaagagg agcaggggca ggaggccgac
1381 agagagggca aaacaaagaa aggccccccgc ccgtcccaaa cccggattat gagccaatta
1441 ggaagggtca gagagacctg tattctgggc tcctgagagt gaagttcagc aggagcgcag
1501 acgccccccgc gtaccagcaa gggcagaacc agctctataa cgagctcaat ctaggacgaa
1561 gagaggagta cgatgttttg gacaagaggc gtggccggga ccctgagatg gggggaaagc
1621 cgagaaggaa gaaccctcag gaaggcctgt acaatgaact gcagaaagat aagatggcgg
1681 aggcctacag tgagattggg atgaaaggcg agcgccggag gggcaagggg cacgatggcc
1741 tttaccaggg tctcagtaca gccaccaagg acacctacga cgcccttcac atgcaagctc
1801 tgcccccctcg ctga (SEQ ID NO: 73)

CAR6 can be encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to (SEQ ID NO: 73). In certain aspects, the nucleic acid sequence of (SEQ ID NO: 73) may be modified to remove sequences encoding the Myc (human c-Myc proto-oncogene) epitope tag and linker between the Myc tag and the NKG2D extracellular domain. The nucleic acid sequence of (SEQ ID NO: 73) encodes for a CD8a signal peptide at positions 486-548, which signal peptide may be substituted for a different signal peptide.

[0266] A seventh construct, termed NKG2D CAR7 comprises from N to C-terminus a NKG2D extracellular domain, a CD8alpha hinge, a CD28 transmembrane domain, a 4-1BB intercellular domain, and a signaling domain comprising a CD3ε signaling domain and a CD3ζ signaling

domain. The amino acid sequence of this chimeric antigen receptor comprises the following amino acid sequence:

LFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYS
KEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFK
GYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF
ACDFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGC
SCRFPEEEEGGCEKNRKAKAKPVTRGAGAGGRQRGQNKERPPPVPNPDYEPIRKGQRD
LYSGLLRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQ
ALPPR (SEQ ID NO: 74). In one aspect, CAR7 is encoded by the following nucleotide sequence:

1 cgcggaatga aagaccccac ctgtaggttt ggcaagctag cttaagtaac gccattttgc
61 aaggcatgga aaatacataa ctgagaatag agaagttcag atcaaggtta ggaacagaga
121 gacagcagaa tatgggccaa acaggatatc tgtggtaagc agttcctgcc ccggctcagg
181 gccaagaaca gatggtcccc agatgcggtc ccgccctcag cagtttctag agaaccatca
241 gatgtttcca gggtgcccca aggacctgaa atgaccctgt gccttatttg aactaaccaa
301 tcagttcgct tctcgcttct gttcgcgcgc ttctgctccc cgagctcaat aaaagagccc
361 acaacccctc actcggcgcg ccagtccttc gaagtagatc tttgtcgatc ctaccatcca
421 ctcgacacac ccgccagcgg ccgctgccaa gcttccgagc tctcgaatta attcacgccg
481 ccaccatggc tcttcctgtg acagctcttc tgctgcccct ggccctgctt ctgcatgctg
541 ctagacctga gcaaaagttg atttctgagg aagacctcgc cggcagttta ttcaaccaag
601 aagtccaaat tcccttgacc gaaagttact gtggcccatg tcctaagaac tggatatgtt
661 acaaaaataa ctgttaccaa ttcttcgatg aatctaagaa ttggtatgag agccaggctt
721 cttgtatgtc tcaaaatgcc agccttctta aagtatacag caaagaggac caggatttac
781 ttaaactggt gaagtcatat cattggatgg gactagtaca cattccaaca aatggatctt
841 ggcagtggga agacggctcc attctctcac ccaacctact aacaataatt gaaatgcaga
901 agggagactg tgcactctat gcatcgagct ttaaaggcta tatagaaaac tgttcaactc
961 caaatacata tatttgcatg caaaggactg tgaccacgac gccagcgccg cgaccaccaa
1021 caccggcgcc caccatcgcg tcgcaacccc tgtccctgag gcctgaagcg tgccggccag
1081 cggcgggcgg cgcagtgcac acgagagggc tggacttcgc ctgtgatttt tgggtgctgg
1141 tggtggttgg tggagtcctg gcttgctata gcttgctagt aacagtggcc tttattattt
1201 tctgggtcaa acggggcaga aagaaactcc tgtatatatt caaacaacca tttatgagac
1261 cagtacaaac tactcaagag gaagatggct gtagctgccg atttccagaa gaagaagaag
1321 gaggatgtga aaagaaccga aaagcaaaag ccaagcctgt tacaagagga gcaggggcag
1381 gaggccgaca gagagggcaa aacaaagaaa ggccccccgcc cgtcccaaac ccggattatg
1441 agccaattag gaagggtcag agagacctgt attctgggct cctgagagtg aagttcagca

1501 ggagcgcaga cgccccgcg taccagcaag ggcagaacca gctctataac gagctcaatc

1561 taggacgaag agaggagtac gatgttttgg acaagaggcg tggccgggac cctgagatgg

1621 ggggaaagcc gagaaggaag aaccctcagg aaggcctgta caatgaactg cagaaagata

1681 agatggcgga ggcctacagt gagattggga tgaaaggcga gcgccggagg ggcaaggggc

1741 acgatggcct ttaccagggt ctcagtacag ccaccaagga cacctacgac gcccttcaca

1801 tgcaagctct gcccctcgc tga (SEQ ID NO: 75)

CAR7 can be encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to (SEQ ID NO: 75). In certain aspects, the nucleic acid sequence of (SEQ ID NO: 75) may be modified to remove sequences encoding the Myc (human c-Myc proto-oncogene) epitope tag and linker between the Myc tag and the NKG2D extracellular domain. The nucleic acid sequence of (SEQ ID NO: 75) encodes for a CD8a signal peptide at positions 486-548, which signal peptide may be substituted for a different signal peptide.

[0267] An eight construct, termed NKG2D CAR8 comprises from N to C-terminus a NKG2D extracellular domain, a CD8alpha hinge, a CD28 transmembrane domain, a 4-1BB intercellular domain, and a signaling domain comprising a CD3ε signaling domain and a CD3ζ signaling domain. The amino acid sequence of this chimeric antigen receptor comprises the following amino acid sequence:

LFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYS
KEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFK
GYIENCSTPNTYICMQRTVTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF
ACDFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGC
SCRFPEEEEGGCEKNRKAKAKPVTRGAGAGGRQRGQNKERPPPVPNPDYEPIRKGQRD
LYSGLNQRRILRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMG
GKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD
ALHMQALPPR (SEQ ID NO: 76). In one aspect, CAR8 is encoded by the following nucleotide sequence:

1 cgcggaatga aagaccccac ctgtaggttt ggcaagctag cttaagtaac gccattttgc

61 aaggcatgga aaatacataa ctgagaatag agaagttcag atcaaggtta ggaacagaga

121 gacagcagaa tatgggccaa acaggatatc tgtggtaagc agttcctgcc ccggctcagg

181 gccaagaaca gatggtcccc agatgcggtc ccgccctcag cagtttctag agaaccatca

241 gatgtttcca gggtgcccca aggacctgaa atgaccctgt gccttatttg aactaaccaa

301 tcagttcgct tctcgcttct gttcgcgcgc ttctgctccc cgagctcaat aaaagagccc

361 acaacccctc actcggcgcg ccagtccttc gaagtagatc tttgtcgatc ctaccatcca

421 ctcgacacac ccgccagcgg ccgctgccaa gcttccgagc tctcgaatta attcacgccg

481 ccaccatggc tcttcctgtg acagctcttc tgctgcccct ggccctgctt ctgcatgctg
541 ctagacctga gcaaaagttg atttctgagg aagacctcgc cggcagttta ttcaaccaag
601 aagtccaaat tcccttgacc gaaagttact gtggcccatg tcctaagaac tggatatgtt
661 acaaaaataa ctgttaccaa ttcttcgatg aatctaagaa ttggtatgag agccaggctt
721 cttgtatgtc tcaaaatgcc agccttctta aagtatacag caaagaggac caggatttac
781 ttaaactggt gaagtcatat cattggatgg gactagtaca cattccaaca aatggatctt
841 ggcagtggga agacggctcc attctctcac ccaacctact aacaataatt gaaatgcaga
901 agggagactg tgcactctat gcatcgagct ttaaaggcta tatagaaaac tgttcaactc
961 caaatacata tatttgcatg caaaggactg tgaccacgac gccagcgccg cgaccaccaa
1021 caccggcgcc caccatcgcg tcgcaacccc tgtccctgag gcctgaagcg tgccggccag
1081 cggcgggcgg cgcagtgcac acgagagggc tggacttcgc ctgtgatttt tgggtgctgg
1141 tggtggttgg tggagtcctg gcttgctata gcttgctagt aacagtggcc tttattattt
1201 tctgggtcaa acggggcaga aagaaactcc tgtatatatt caaacaacca tttatgagac
1261 cagtacaaac tactcaagag gaagatggct gtagctgccg atttccagaa gaagaagaag
1321 gaggatgtga aaagaaccgc aaagcaaagg caaaacccgt cacacgagga gcgggcgcag
1381 ggggacgaca acgcggtcag aataaggaac gcccgcctcc agtaccaaat ccagattatg
1441 aaccaattcg gaagggacaa cgcgatctct actccggtct caatcagagg cgaattctga
1501 gagtgaagtt cagcaggagc gcagacgccc ccgcgtacca gcaagggcag aaccagctct
1561 ataacgagct caatctagga cgaagagagg agtacgatgt tttggacaag aggcgtggcc
1621 gggaccctga gatgggggga aagccgagaa ggaagaaccc tcaggaaggc ctgtacaatg
1681 aactgcagaa agataagatg gcggaggcct acagtgagat tgggatgaaa ggcgagcgcc
1741 ggaggggcaa ggggcacgat ggcctttacc agggtctcag tacagccacc aaggacacct
1801 acgacgccct tcacatgcaa gctctgcccc ctcgctga

(SEQ ID NO: 77)

CAR8 can be encoded by a nucleic acid having at least 75% sequence identity (such as, at least 75%, at least 80%, at least 90%, at least 95%, or 100% identity; e.g., 85–90%, 85–95%, 85–100%, 90–95%, 90–100%, or 95–100%) to (SEQ ID NO: 77). In certain aspects, the nucleic acid sequence of (SEQ ID NO: 77) may be modified to remove sequences encoding the Myc (human c-Myc proto-oncogene) epitope tag and linker between the Myc tag and the NKG2D extracellular domain. The nucleic acid sequence of (SEQ ID NO: 77) encodes for a CD8a signal peptide at positions 486-548, which signal peptide may be substituted for a different signal peptide.

Example 2

Transduction Efficiency

[0268] Retroviral vectors were used for all T-cell transductions. An engineered TCR targeting the HPV16+ $E7_{11-19}$ epitope and was used for singular or co-transduction with the NKG2D CAR constructs (Jin B.Y. et al., 2018 JCI Insight). Previously cryopreserved PBMCs obtained from healthy donors were thawed and activated with anti-CD3 for 48 hours in Optimizer T cell media supplemented with 300 IU of IL-2. The PBMCs were transduced with retrovirus containing the engineered TCR targeting the HPV16+ $E7_{11-19}$ epitope (referred to in this Example, and the Examples below, as HPV-TCR), the NKG2D CAR, or both constructs. Transduced T cells were expanded for 10 days in Optimizer T cell media supplemented with IL-7 (10 ng/mL), IL-15 (10 ng/mL), and Akt VIII inhibitor (AKTi, 1 μM). Transduction efficiency was measured by flow cytometry at day 8 and day 15 post T cell activation. Antibodies to detect TCR transduction efficiency include anti-mouse TCRβ clone H57-597 (BioLegend) and antibodies to detect NKG2D transduction efficiency include anti-human CD314 clone 1D11 (BioLegend). All flow cytometry data was collected on LSR–Fortessa (BD LSR Fortessa™) with BD FACSDiva™ software and data was analyzed using FlowJo (all from BD Sciences). All antibody staining was performed at 4°C in PBS containing 1% BSA.

[0269] Table 4 shows the expression level (measured as mean fluorescence intensity (MFI) and % positive cells) of transduced and non-transduced T cells after day 8 and 15 of culture by flow cytometry. High transduction efficiency was observed in all constructs. A small NKG2D+ only population is present in the HPV-TCR + NKG2D CAR1 and HPV-TCR + NKG2D CAR2 groups.

Table 4: Expression level and percentage of transgene in transduced and non-transduced PBMCs gated on live CD3+ T cells

Experimental Group	mTCRβ MFI		% positive		NKG2D MFI		% positive	
	Day 8	Day 15	Day 8	Day 15	Day 8	Day 15	Day 8	Day 15
Non-transduced T cells	21	18	0.1%	0.1%	1026	1145	23.5%	20.1%
HPV-TCR only	55481	59065	82.5%	84.5%	1284	1354	21.6%	21.1%
NKG2D CAR1 only	14	33	0.4%	0.6%	25733	22040	96.6%	97.4%
NKG2D CAR2 only	10	25	0.5%	0.3%	16637	15327	91.7%	86.8%
HPV-TCR + NKG2D CAR 1	48476	50264	70.1%	71.4%	22635	20930	97.5%	94.7%
HPV-TCR + NKG2D CAR 2	46045	48190	68.8%	69.5%	17188	16418	92.9%	86.3%

[0270] Table 19 shows the expression level (as mean fluorescence intensity (MFI) and % positive cells) of transduced and non-transduced T cells after day 7/8 of culture based on Myc staining and expression measurement by flow cytometry. High transduction efficiency was observed in all constructs.

Table 19: Expression level and percentage of transgene in transduced and non-transduced T-cells from two donors

	Donor 1	Donor 2
	%Myc	%Myc
CAR3	98.6	96.9
CAR6	99.7	99.6
CAR5	80.1	62.1
CAR4	99.2	98.7
CAR7	98.8	98.4
CAR8	98.9	98.6
NTD	0.15	0.2

Example 3

Expansion and Viability

[0271] Cell expansion was measured by the Vi-Cell XR Cell Counter (Beckman Coulter) and cell viability was measured by staining cells with Live/Dead Blue (ThermoFisher) in PBS for 20 minutes on ice and analyzed by flow cytometry at day 8 and day 15 post T cell activation (Table 5). Transformed T cell was activated as described in Example 2. Table 5 shows the expansion and cell viability of transduced and non-transduced T cells cultured with Optimizer media supplemented with IL-7, IL-15, and AKTi. NKG2D CAR1 only, NKG2D CAR2 only, HPV-TCR + NKG2D CAR1 and HPV-TCR + NKG2D CAR2 displayed greater levels of proliferation than HPV-TCR only or non-transduced T cells.

Table 5: Fold expansion and viability of transduced and non-transduced PBMCs gated on CD3+ T cells

Experimental Group	Viability		Fold expansion
	Day 8	Day 15	Day 8
Non-transduced T cells	98.2%	96.5%	51x
HPV-TCR only	94.2%	88.9%	42x

NKG2D CAR1 only	85.1%	74.3%	81x
NKG2D CAR2 only	89.4%	88.8%	74x
HPV-TCR + NKG2D CAR1	90.4%	78.5%	75x
HPV-TCR + NKG2D CAR2	88.6%	83.0%	68x

Example 4

Product Attribute Determination

[0272] Memory phenotype and CD4/CD8 ratio was measured by flow cytometry at day 8 post T cell activation (Table 6). Antibodies used to assess memory phenotype include anti-human CD45RA clone HI100 (BioLegend), anti-human CD45RO clone UCHL1 (BioLegend), anti-human CCR7 clone G043H7 (BioLegend), anti-human CD62L clone DREG-56 (BioLegend). Antibodies used to assess CD4/CD8 ratio include anti-human CD3 clone SK7 (BioLegend), anti-human CD4 clone RPA-T4 (BioLegend), and anti-human CD8 clone SK1 (BioLegend). Transformed T cells were activated as described in Example 2. Memory phenotype was assessed as follows: naïve T cells (CD45RA+ CD45RO- CCR7+ CD62L+); central memory T cells (CD45RA- CD45RO+ CCR7+ CD62L+); effector memory T cells (CD45RA- CD45RO+ CCR7- CD62L-); terminal effector T cells (CD45RA+ CD45RO- CCR7- CD62L-). All flow cytometry data was collected on LSR–Fortessa (BD LSR Fortessa™) with BD FACSDiva™ software and data was analyzed using FlowJo (all from BD Sciences). All antibody staining was performed at 4°C in PBS containing 1% BSA.

[0273] Shown in Table 6 is the CD4/CD8 ratio and memory phenotype of transduced and non-transduced CD3+ T cells cultured with TC Media with supplemental IL-2 after day 8. There were no significant changes in the CD4/CD8 ratio between transduced T cells and non-transduced T cells. Non-transduced and HPV-TCR only transduced T cells exhibited a larger naïve T cell compartment than T cells transduced with NKG2D CAR1 only, NKG2D CAR2 only, HPV-TCR + NKG2D CAR1, or HPV-TCR + NKG2D CAR2. In addition, Non-transduced and HPV-TCR only transduced T cells exhibited a smaller memory T cell compartment than T cells transduced with NKG2D CAR1 only, NKG2D CAR2 only, HPV-TCR + NKG2D CAR1, or HPV-TCR + NKG2D CAR2.

Table 6: CD4/CD8 ratio and memory phenotype of transduced and non-transduced PBMCs gated on live CD3+ T cells

Experimental Group	CD4/CD8 ratio		Memory phenotype			
	CD4	CD8	Naïve	TCM	TEM	TE
Non-transduced	51.8%	48.2%	22.7%	46.0%	14.0%	5.3%
HPV-TCR only	45.7%	54.3%	24.3%	45.0%	15.0%	5.8%
NKG2D CAR1 only	46.8%	53.2%	3.5%	63.0%	28.1%	9.4%
NKG2D CAR2 only	44.7%	55.3%	8.5%	56.4%	25.7%	5.4%
HPV-TCR + NKG2D CAR1	48.1%	51.9%	3.1%	61.6%	28.2%	7.1%
HPV-TCR + NKG2D CAR2	47.2%	52.8%	7.2%	57.4%	28.5%	6.9%

Example 5

Cytotoxicity

[0274] HPV-TCR transduced T cells have been previously shown to effectively eliminate HPV16+ tumor cell lines and produce high levels of IFNγ. Sequencing of patient tumors from a Phase I clinical trial with the HPV-TCR (Nagarsheth N.B. et al., 2021 Nat Med) suggest that tumors are highly heterogeneous and can exhibit mutations in MHC-I antigen processing and presentation pathways, thereby rendering them resistant to HPV-TCR engineered adoptive TCR-T therapy. To model the patient tumor data, a HPV16+ tumor cell line (CaSki) was engineered to lack A*02:01 by knocking out β2M (β2MKO) using a CRISPR-Cas9 system (Vakulskas CA et al., 2018 Nature). Additionally, the SiHa HPV16+ tumor cell line, which normally lacks HLA-A*02:01, was engineered to overexpress A*02:01 using a retroviral vector. The CaSki and SiHa tumor cell lines were chosen due to high and low E7 expression, respectively. Killing of these tumor cell lines with or without HLA-A*02:01 intact with non-transduced and transduced PBMCs was assessed using the xCELLigence RTCA MP (Agilent Technologies) platform the various effector-to-target (E:T) ratios shown in Table 7 and 8.

[0275] Table 7 shows the results of transduced T cells (see Example 2) that were sorted at day 8 and then co-cultured at various E:T ratios with WT or β2MKO CaSki cells at day 10 in a 96 well xCELLigence plate. Impedance values (IV) were measured 72 hours later. Control IV represents impedance value of non-transduced T cells, and Experimental IV represents impedance value of either HPV-TCR only, NKG2D CAR1 only, NKG2D CAR2 only, HPV-TCR + NKG2D CAR1, or HPV-TCR + NKG2D CAR2. Percent cytotoxicity was calculated by the following equation:

$$\%\ \boldsymbol{Cytotoxicity} = \mathbf{1} - (\boldsymbol{Control\ IV} - \boldsymbol{Experimental\ IV}) * \mathbf{100}$$

Table 7: Killing of WT and β2MKO CaSki cells by transduced and non-transduced PBMCs at different E:T ratios

Experimental Group	WT CaSki			β2MKO CaSki		
	3:1 ratio	1:1 ratio	1:3 ratio	3:1 ratio	1:1 ratio	1:3 ratio
Non-transduced	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
HPV-TCR only	102.5%	101.5%	96.1%	13.6%	11.2%	7.5%
NKG2D CAR1 only	103.0%	101.4%	96.7%	102.6%	101.6%	97.0%
NKG2D CAR2 only	103.2%	104.0%	101.3%	104.4%	98.1%	93.5%
HPV-TCR + NKG2D CAR1	105.1%	102.3%	98.4%	102.4%	103.8%	96.5%
HPV-TCR + NKG2D CAR2	101.8%	99.3%	89.2%	101.6%	100.3%	89.8%

[0276] Table 8 shows the results of transduced T cells (see Example 2) that were sorted at day 8 and then co-cultured at various E:T ratios with WT or A*02:01 expressing SiHa cells at day 10 in a 96-well xCELLigence E-plate. Impedance values (IV) were measured 72 hours later and percent cytotoxicity was calculated is described above.

Table 8: Killing of SiHa cells by transduced and non-transduced PBMCs

Experimental Group	WT SiHa			A*02 SiHa		
	3:1 ratio	1:1 ratio	1:3 ratio	3:1 ratio	1:1 ratio	1:3 ratio
Non-transduced	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
HPV-TCR only	13.7%	8.9%	2.1%	99.5%	78.3%	57.1%
NKG2D CAR1 only	102.5%	99.5%	94.1%	102.1%	101.1%	84.1%
NKG2D CAR2 only	103.6%	97.4%	91.1%	99.8%	93.5%	75.3%
HPV-TCR + NKG2D CAR1	101.5%	99.8%	88.6%	104.2%	100.8%	87.0%
HPV-TCR + NKG2D CAR2	102.3%	93.6%	77.5%	102.2%	98.5%	79.4%

HPV-TCR transduced T cells successfully eliminated WT CaSki and A*02 SiHa cell lines, but were unable to eliminate β2M KO CaSki or WT SiHa cell lines. Importantly, NKG2D CAR transduced T cells and NKG2D CAR co-transduced with HPV-TCR T cells were able to successfully kill CaSki and SiHa cell lines, independent of their HLA-A*02:01 status.

Example 6

Cytokine Production

[0277] Cytokine production was measured by retrieving supernatants from the xCELLigence 96-well plates used in example 5 after 24 hours of culture. The cytokine levels were measured using a Meso Sector S 600 platform (Institute for Biopharmaceutical Research, Inc.). IFNγ production from HPV-TCR + NKG2D CAR T cells was similar to HPV-TCR transduced cells when co-cultured with WT or A*02 SiHa cells. However, IFNγ production from HPV-TCR + NKG2D CAR T cells increased compared to HPV-TCR transduced T cells when co-cultured with β2MKO CaSki or WT SiHa cells, which correlated with cytotoxicity data (see Table 9 and Table 10). These data showed that NKG2D CAR T cells secrete nominal levels of IFNγ when HPV-TCR transduced T cells eliminate target cells, but produce sufficient IFNγ when HPV-TCR transduced T cells did not eliminate target cells. Interestingly, NKG2D CAR1 and HPV-TCR + NKG2D CAR1 transduced T cells produce greater levels of IFNγ than NKG2D CAR2 or HPV-TCR + NKG2D CAR2 transduced T cells.

[0278] Table 9 shows the results of transduced T cells that were sorted at day 8 and then co-cultured at various E:T ratios with WT or β2MKO CaSki cells at day 10 in a 96-well xCELLigence E-plate. At 24 hours, media supernatants were extracted and processed for determining IFNγ concentration (pg/mL) using the Meso Sector S 600 platform according to the manufacturer's directions.

Table 9: IFNγ production of transduced and non-transduced PBMCs co-cultured with CaSki cells

Experimental Group	WT CaSki			β2MKO CaSki		
	3:1 ratio	1:1 ratio	1:3 ratio	3:1 ratio	1:1 ratio	1:3 ratio
HPV-TCR only	1721	641	273	130	90	40
NKG2D CAR1 only	4486	2931	1053	5024	2820	1306
NKG2D CAR2 only	2836	1063	403	1989	777	262
HPV-TCR + NKG2D CAR1	3721	1369	523	4191	2067	895
HPV-TCR + NKG2D CAR2	1467	561	212	1567	622	192

[0279] Table 10 shows the results of transduced T cells that were sorted at day 8 and then co-cultured at various E:T ratios with WT or A*02 SiHa cells at day 10 in a 96-well xCELLigence E-plate. At 24 hours, media supernatants were extracted and processed for determining IFNγ

concentration (pg/mL) using the Meso Sector S 600 platform according to the manufacturer's directions.

Table 10: IFNγ production of transduced and non-transduced PBMCs co-cultured with SiHa cells

Experimental Group	WT SiHa			A*02 SiHa		
	3:1 ratio	1:1 ratio	1:3 ratio	3:1 ratio	1:1 ratio	1:3 ratio
HPV-TCR only	30	12	3	155	75	45
NKG2D CAR1 only	7538	3968	1970	7271	4145	1380
NKG2D CAR2 only	4638	1300	342	4034	1554	560
HPV-TCR + NKG2D CAR1	5563	2522	1149	6518	3223	1296
HPV-TCR + NKG2D CAR2	3240	901	232	3223	938	284

Example 7

Long Term Expansion and Exhaustion of Transduced T Cells

[0280] Repeated antigen stimulation in a long-term killing assay functionally exhausts the T cells and is believed to correlate with *in vivo* efficacy. To complete a serial antigen stimulation assay, target cells (WT CaSki, β2MKO CaSki, WT SiHa, or A*02 SiHa) were added to transduced T cells (see Example 2) every 2-3 days. At the fifth stimulation, cytotoxicity of either WT CaSki, β2MKO CaSki, WT SiHa, or A*02 SiHa was assessed at the various E:T ratios shown in Tables 11 and 12 using the xCELLigence RTCA MP platform. Despite the addition of the NKG2D CAR, HPV-TCR + NKG2D CAR transduced T cells successfully eliminated its targets after serial stimulation and exhibited high levels of expansion compared to HPV-TCR only transduced T cells (see Table 11, Table 12).

[0281] Table 11 shows the results of transduced T cells that were sorted at day 8 and then co-cultured at a 3:1 E:T ratio with WT CaSki cells at day 10. WT CaSki cells were then added every 2-3 days. At the fifth stimulation, T cells were co-cultured at various E:T ratios with WT or β2MKO CaSki cells in a 96 well xCELLigence plate. Impedance values (IV) were measured 72 hours later and cytotoxicity was calculated as described above.

Table 11: Killing of CaSki cells by transduced and non-transduced PBMCs after 5 rounds of antigen stimulation

Experimental Group	WT CaSki			β2MKO CaSki		
	3:1 ratio	1:1 ratio	1:3 ratio	3:1 ratio	1:1 ratio	1:3 ratio
HPV-TCR only	86.8%	44.2%	12.5%	11.2%	4.1%	-3.3%
NKG2D CAR1 only	100.4%	99.6%	75.6%	102.4%	90.1%	57.1%
NKG2D CAR2 only	101.1%	92.5%	44.9%	98.2%	81.3%	36.1%
HPV-TCR + NKG2D CAR1	N.D	N.D.	N.D.	N.D.	N.D.	N.D.
HPV-TCR + NKG2D CAR2	102.4%	97.1%	62.4%	95.0%	73.2%	26.7%

[0282] Table 12 shows the results of transduced T cells that were sorted at day 8 and then co-cultured at a 3:1 E:T ratio with WT CaSki cells at day 10. WT CaSki cells were then added every 2-3 days. At the fifth stimulation, T cells were co-cultured at various E:T ratios with WT or A*02 SiHa cells in a 96 well xCELLigence plate. Impedance values (IV) were measured 72 hours later and cytotoxicity was calculated cytotoxicity was calculated as described above.

Table 12: Killing of SiHa cells by transduced and non-transduced PBMCs after 5 rounds of antigen stimulation

Experimental Group	WT SiHa			A*02 SiHa		
	3:1 ratio	1:1 ratio	1:3 ratio	3:1 ratio	1:1 ratio	1:3 ratio
HPV-TCR only	4.7%	-2.1%	-6.2%	9.1%	-2.2%	-5.4%
NKG2D CAR1 only	99.7%	82.5%	40.9%	98.8%	87.1%	39.2%
NKG2D CAR2 only	92.5%	80.4%	23.6%	94.1%	65.2%	21.7%
HPV-TCR + NKG2D CAR1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
HPV-TCR + NKG2D CAR2	91.8%	78.1%	29.0%	90.5%	70.1%	19.6%

Example 8

Cytotoxicity and Cytokine Production of NKG2D CAR and NKG2D CAR Co-expressed with HPV-TCR Co-cultured with HPV16+ Tumor Cell lines

[0283] To address safety of the NKG2D based CARs, NKG2D CAR1 only, NKG2D CAR2 only, HPV-TCR + NKG2D CAR1, and HPV-TCR + NKG2D CAR2 transduced T cells, were co-cultured with either the C33A cell line, which is largely NKG2D ligand negative, and primary normal cervical epithelial cells at various E:T ratios as shown in Table 13 and Table 14. Primary normal cervical epithelial cells were chosen due to higher NKG2D ligand expression. Cytotoxicity data from the xCELLigence RTCA MP platform and IFNγ production from the Meso Sector S 600 platform indicated low reactivity to both C33A and primary normal cervical epithelial cells. These data are suggestive that NKG2D CAR2 has a strong safety profile and is unlikely to exhibit off-target effects.

[0284] Table 13 shows the results of transduced T cells that were sorted at day 8 and then co-cultured at various E:T ratios with C33A or primary normal cervical epithelial cells at day 10 in a 96-well xCELLigence E-plate. At 24 hours, media supernatants were extracted and processed for determining IFNγ concentration (pg/mL) using the Meso Sector S 600 platform.

Table 13: Killing of C33A and primary normal cervical epithelial cells by transduced and non-transduced PBMCs

Experimental Group	C33A			Primary normal cervical		
	3:1 ratio	1:1 ratio	1:3 ratio	3:1 ratio	1:1 ratio	1:3 ratio
Non-transduced T cells	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
HPV-TCR only	7.6%	4.5%	-4.3%	3.3%	3.0%	-8.1%
NKG2D CAR1 only	30.1%	1.6%	-4.5%	17.2%	1.8%	-5.4%
NKG2D CAR2 only	9.5%	2.5%	-6.2%	7.5%	2.7%	-2.9%
HPV-TCR + NKG2D CAR1	21.0%	8.5%	-6.4%	12.2%	4.5%	-5.6%
HPV-TCR + NKG2D CAR2	9.7%	5.9%	-5.4%	6.0%	1.1%	-4.3%

[0285] Table 14 shows the results of transduced T cells that were sorted at day 8 and then co-cultured at various E:T ratios with C33A cell line or primary normal cervical epithelial cells at day 10 in a 96-well xCELLigence E-plate. At 24 hours, media supernatants were extracted and processed for determining IFNγ concentration (pg/mL) using the Meso Sector S 600 platform.

Table 14: IFNγ production of transduced and non-transduced PBMCs co-cultured with either C33A cell line or primary normal cervical epithelial cells

Experimental Group	C33A			Primary normal cervical		
	3:1 ratio	1:1 ratio	1:3 ratio	3:1 ratio	1:1 ratio	1:3 ratio
Non-transduced T cells	50	48	47	53	49	37
HPV-TCR only	67	54	61	60	53	49
NKG2D CAR1 only	549	251	109	378	204	74
NKG2D CAR2 only	134	85	62	104	74	52
HPV-TCR + NKG2D CAR1	309	159	65	268	73	37
HPV-TCR + NKG2D CAR2	121	84	67	142	73	48

Example 9

In vivo efficacy of NKG2D CAR against HPV16+ tumors in a mouse xenograft model

[0286] To determine the in vivo efficacy of HPV-TCR only, NKG2D CAR only, and HPV-TCR + NKG2D CAR in eliminating both WT and β2MKO HPV16+ solid tumors, 5E6 WT CaSki and 5E6 β2MKO CaSki cells were implanted subcutaneously on the left and right flanks, respectively, of 6–8-week-old female NSG mice (NOD.Cg- Prkdcscid Il2rgtm1Wjl/ SzJ). Five study groups were included: vehicle control (PBS), non-transduced T cells (NTD), HPV-TCR only, NKG2D CAR2 only, and HPV-TCR + NKG2D CAR2. 20 X 10^6 T cells per mouse were adoptively transferred at day 6, when the tumor volume of WT CaSki cells had reached approximately 70 mm^3 and the β2MKO CaSki cells had reached approximately 30 mm^3. No IL-2 supplementation was added. Tumors were measured every 3-4 days using digital calipers and mouse weight was recorded. Peripheral blood was collected 24-hours post T cell adoptive transfer, and then every week afterward. Persistence of T cells in the peripheral blood was characterized through flow cytometry. Antibodies to assess T cell persistence in the peripheral blood include anti-mouse CD45 clone 30-F11 (BioLegend), anti-human CD45 clone HI30 (BioLegend), anti-human CD4 clone RPA-T4, (BioLegend), anti-human CD8 clone SK1 (BioLegend), anti-human CD279 clone EH12.2H7 (BioLegend), anti-human HLA-DR clone L243 (BioLegend), anti-mouse TCRβ clone H57-597 (BioLegend), anti-human CD314 clone 1D11 (Biolegend), and L/D Blue (ThermoFisher). All flow cytometry data was collected on LSR–Fortessa (BD LSR Fortessa™) with BD FACSDiva™ software and data was analyzed using FlowJo (all from BD Sciences). All antibody staining was performed at 4°C in PBS containing 1% BSA. The study duration was 62 days. Overall, the HPV-TCR was able to control tumor growth of WT CaSki cells on the left flank, but did not show any sign of tumor control of the β2MKO CaSki cells implanted on the right flank.

In contrast, NKG2D CAR2 only and HPV-TCR + NKG2D CAR2 successfully controlled tumor growth of both WT and β2MKO CaSki cells (see Table 15, Table 16). Furthermore, weight of tumor-bearing mice did not fluctuate significantly for the first several weeks post adoptive T cell transfer, until the mice had to come off the study due to tumor size. (see Table 17). Despite no observable tumors in all mice receiving NKG2D CAR2 only or HPV-TCR + NKG2D CAR2 T cells, most of the mice demonstrated relapse in tumor growth, suggesting a lack of persistence of the transduced T cells. This is further supported by the absence of transduced T cells in the peripheral blood three weeks after adoptive T cell transfer (see Table 18).

Table 15: Tumor volume of WT CaSki cells over time

Days post tumor inoculation	6	8	11	13	15	18	20	22	25	27	29	32	34
Vehicle	77	78	97	107	126	251	316	343	394	526	607	625	665
	85	87	96	111	180	301	402	440	501	541	652	704	793
	72	75	76	86	158	166	232	331	406	463	531	633	663
	63	70	80	89	168	191	208	239	253	326	398	496	503
	63	67	71	93	148	236	327	342	452	500	537	549	676
	90	97	100	108	111	134	210	271	350	392	463	536	551
	64	67	72	78	121	169	217	302	328	368	488	500	613
	66	72	73	75	111	199	248	384	420	506	536	646	683
NTD	88	98	106	112	148	288	345	460	591	695	708	758	760
	68	72	76	84	105	256	340	468	555	660	705	759	880
	70	74	74	80	90	241	250	281	305	363	398	564	747
	67	73	79	91	129	255	312	543	573	599	616	647	684
	64	74	76	78	79	120	148	162	200	291	429	459	264
	79	87	91	104	116	309	331	443	529	581	716	737	878
	72	77	86	88	114	295	319	356	435	527	567	623	635
	63	65	79	85	90	242	253	330	434	489	490	492	499
HPV-TCR only	73	76	79	33	32	16	11	12	34	64	112	232	324
	63	77	79	43	35	23	16	30	57	77	125	280	384
	68	72	81	56	33	29	25	26	70	91	102	125	144

	64	73	74	45	41	21	15	17	22	83	151	182	265
	89	94	99	57	47	15	23	46	69	156	264	382	430
	67	72	73	42	29	20	19	34	48	71	88	162	248
	65	71	75	30	26	15	13	28	33	66	84	236	254
	66	74	76	27	14	10	9	13	34	48	47	132	207
	69	75	84	54	50	39	34	51	93	117	191	229	489
	67	70	73	19	17	13	20	32	75	106	146	342	371
NKG2D CAR2 only	74	79	92	69	65	53	66	81	126	350	355	541	953
	70	77	79	44	25	20	25	57	129	157	245	276	444
	75	82	86	40	19	16	24	31	67	95	147	205	224
	63	72	76	19	13	11	14	25	39	90	98	104	275
	78	83	90	57	27	23	30	68	182	239	237	332	616
	63	75	79	44	40	15	40	77	135	215	269	351	362
	64	73	77	33	29	15	28	57	80	111	118	240	307
	69	78	82	48	24	17	21	28	63	118	137	216	420
	63	67	70	27	18	11	12	39	53	154	162	296	381
	64	70	75	33	26	11	36	39					
HPV-TCR + NKG2D CAR2	67	70	72	35	19	17	17	42	90	155	215	276	350
	66	74	77	29	20	15	19	32	68	107	227	240	390
	66	75	79	32	27	24	20	23	53	58	79	198	304
	77	81	86	26	25	13	9	28	41	116	186	297	358
	72	78	81	33	23	16	17	43	102	161	213	260	483
	81	89	94	28	23	14	17	22	28	55	110	291	345

	67	71	75	34	17	13	21	36	87	158	286	365	448
	64	70	72	26	22	12	14	19	33	57	79	166	198
	66	70	74	38	21	11	25	32	75	94	187	287	392
	65	67	69	25	20	8	15	27	55	68	80	168	320
Days post tumor inoculation	**36**	**39**	**41**	**43**	**46**	**48**	**50**	**53**	**55**	**57**	**60**	**62**	
Vehicle	717	796	884	923									
	926	1217	1306										
	742	831	1034	1149									
	610	780	985										
	739	757	923										
	582	693	981	1039	1090								
	685	857	982										
	833	881	1152										
NTD	704												
	913	798	535										
	863	425	394	438	384								
	802	711	569	347	279	211	166	165					
	238	153	123	111	101	84	60						
	715												
	624	464	296										
	261												

	390	426	632	328	211	163	127	176	176	177	160	97	
	435	440	549	826	827	883							
	221	210	376	472	513	533	600	372	158	165	164		
	590	547	633	601	391	380	384	407	409	268	289	213	
	579	621	779	962									
	375	412	497	635	649	744	651	504	479	489	424	185	
	340	355	451	628	607	635	802	834					
	291	365	474	558	733	647	868	413	280	282	277	181	
HPV-TCR only	523	517	754	855									
	402	565	721	938	877	755	691	668	669	799			
	466	760	1139	1569	1992								
	471	524	755	983	1013	1119	1083	933	1098	1189			
	278	416	736	868	1024	1110	1262	1068	1095	1160	915	944	
	363	481	581	827	1038	970	901	846	846	913	994		
	935	1044	1332	1724	2306								
	450	808	1103	1278	1316	1329	1638	1698					
	430	610	726	785	847	890	1045	1087	1109	1121	1194		
NKG2D CAR2 only	496	564	762	870	1185	1048	1077	1138	1339				
	461	572	714	756	771	853	934	1118	1154	1235			
HPV-TCR + NKG2D CAR2	419	513	783	847	1158	1174	1209	1542					
	495	632	917	1114	1320	1102	1262	1176					
	457	484	458	569	631	670	807	1475					

425	594	816	1028	1020	1068	1082	1162					
647	805	676	865	978	994	1017	887	890	924	1096		
450	517	552	572	807	916	939	1008	965	1016	1079	607	
550	814	1165	1376	1494	2014							
374	415	605	823	941	1034	1312	1932					
611	773	1163	1347	1585								
496	693	559	678	795	912	1165	1411	1339				

Table 16: Tumor volume of β2MKO CaSki cells over time

Days post tumor inoculation	6	8	11	13	15	18	20	22	25	27	29	32	34
Vehicle	34	36	51	58	105	197	281	354	400	487	561	669	689
	28	29	39	44	98	128	206	234	344	371	382	484	501
	23	30	41	51	79	182	238	300	323	385	409	417	454
	22	31	38	44	79	114	198	223	242	297	341	511	613
	31	36	48	54	89	196	273	369	400	459	532	676	762
	63	73	79	99	155	215	310	357	364	441	511	504	586
	44	47	52	58	101	247	254	342	423	522	539	690	748
	64	66	71	115	131	313	432	551	574	681	676	822	880
NTD	35	43	48	55	75	161	169	209	259	312	343	487	494
	35	38	57	63	92	169	213	323	413	455	492	584	667
	19	25	33	40	76	78	83	123	130	192	201	330	375
	31	40	48	54	77	110	134	173	242	322	349	371	381
	45	48	53	65	85	240	242	250	349	399	419	449	270
	46	47	55	59	107	217	231	284	373	460	472	401	408
	20	27	31	33	45	136	158	165	279	319	334	356	377
	50	58	62	67	114	290	298	404	407	473	539	517	327
HPV-TCR only	41	49	66	72	143	350	367	414	450	530	609	633	680
	37	40	49	63	120	266	374	470	520	554	586	707	816
	14	21	32	44	71	224	230	308	335	413	506	738	758

	25	30	37	51	102	211	243	293	336	372	454	563	629
	59	63	69	81	137	239	299	483	542	556	581	657	638
	21	29	30	45	90	225	298	401	537	624	688	855	904
	24	28	38	60	113	207	281	390	515	570	612	771	863
	27	31	53	72	82	210	274	315	351	413	521	579	661
	17	22	35	53	67	216	248	337	347	431	425	501	688
	32	40	44	112	208	216	366	498	727	772	795	945	965
NKG2D	58	64	68	30	0	0	0	0	0	0	0	0	0
	36	47	50	25	0	0	0	0	0	0	0	0	0
	26	36	38	22	0	0	0	0	0	0	0	0	0
	49	54	57	27	0	0	0	0	0	0	0	0	0
	44	49	51	29	0	0	0	0	0	0	0	0	0
	35	40	43	26	12	0	0	0	0	0	0	0	0
	21	30	34	24	0	0	0	0	0	0	0	0	0
	40	42	45	26	0	0	0	0	0	18	25	29	56
	29	33	43	26	0	0	0	0	0	0	0	0	0
CAR2 only	38	43	46	30	19	0	0	0					
	32	41	42	29	0	0	0	0	0	0	0	0	0
	33	40	43	27	0	0	0	0	0	17	15	49	56
	15	23	30	18	0	0	0	0	0	0	0	0	0
HPV-TCR	68	74	77	32	15	4	4	4	11	23	22	58	114
+ NKG2D	28	37	39	17	0	0	0	0	0	0	0	25	84
CAR2	25	31	32	23	0	0	0	0	0	0	0	0	0

	35	39	46	25	7	0	0	0	0	18	31	96
	32	35	38	24	0	0	0	0	0	12	39	62
	22	26	40	23	0	0	0	0	18	18	72	86
	37	42	46	27	0	0	0	0	0	0	0	0
Days post tumor inoculation	**36**	**39**	**41**	**43**	**46**	**48**	**50**	**53**	**55**	**60**	**62**	
Vehicle	774	836	1083	1187								
	624	767	1007									
	607	668	805	1035								
	759	937	1037									
	933	1020	1300									
	624	703	806	953	1080							
	776	902	1019									
	942	968	1141									
NTD	488											
	685	618	522									
	436	404	256	236	151							
	383	295	272	282	155	154	166	100				
	259	125	85	77	74	59	60					
	297											
	397	346	423									
	145											

HPV-TCR only	832	837	980	879	533	409	416	392	249	297	186	117	
	813	862	915	1017	1152	1222							
	805	643	865	1020	949	981	768	668	427	357	282		
	763	758	762	829	793	671	680	552	558	578	201	154	
	734	869	1029	1204									
	756	781	839	944	1000	944	952	962	589	534	444	296	
	939	883	1035	1209	1121	1164	1183	1312					
	758	868	878	882	903	218	519	721	792	565	697	590	
	866	869	994	1163									
	699	565	774	1036	1039	1083	1094	1246	1319	1376			
NKG2D CAR2 only	0	89	167	179	304								
	0	90	174	195	236	280	457	632	856	1045			
	0	55	126	139	147	160	213	299	388	442	598	648	
	0	55	73	91	179	212	334	523	711	885	1178		
	0	54	88	122	189								
	90	94	115	160	180	211	354	418					
	0	33	98	151	171	224	308	525	657	860	1167		
	138	172	234	279	387	398	538	753	1025				
HPV-TCR + NKG2D CAR2	0	84	159	231	275	321	530	717	827	1094			
	123	134	177	181	248	264	356	488					
	172	172	224	233	317	428	604	892					
	54	112	162	188	227	283	450	598					

140	217	351	403	479	526	630	961					
189	195	272	365	407	480	609	687	758	895	1015		
0	60	74	148	157	172	222	459	669	750	753	750	
129	153	206	238	258	311							
168	171	172	195	264	364	541	792					
189	234	250	295	430								
0	100	150	194	221	294	442	508	712				

Table 17: Weight of tumor-bearing mice over time

Days post tumor inoculation	6	8	11	13	15	18	20	22	25	27	29	32	34
Vehicle	0.0	-3.9	-1.7	-5.6	-3.9	-0.9	-3.9	-0.4	0.4	1.3	4.3	4.8	3.9
	0.0	3.1	3.6	3.1	7.1	12.5	12.1	9.4	10.3	9.4	11.6	12.5	10.7
	0.0	-0.9	0.0	-1.8	0.9	2.7	0.4	4.0	4.9	4.5	6.7	4.9	3.1
	0.0	0.8	-1.5	-3.1	0.8	5.0	0.4	-0.4	-0.8	0.8	1.2	2.7	-0.4
	0.0	-0.8	3.3	1.6	4.1	9.8	9.4	11.9	9.8	8.2	10.7	11.1	9.0
	0.0	-6.8	-6.0	-12.0	-8.8	-5.2	-6.8	-5.6	##	-12.9	##	-5.6	-8.8
	0.0	-2.7	-0.4	-4.0	-1.8	5.4	0.4	2.7	5.4	4.9	5.4	7.1	1.8
	0.0	-0.4	2.3	2.7	2.3	6.8	5.7	7.2	7.6	8.4	8.7	8.7	7.6
NTD	0.0	-0.8	2.3	-1.1	4.2	6.8	3.8	6.8	8.3	3.8	6.0	3.4	-8.3
	0.0	-0.8	-2.1	-3.7	0.8	-0.8	-4.5	-2.5	-1.2	-4.1	-2.5	3.3	-1.2
	0.0	1.4	5.2	0.9	4.2	10.3	6.6	8.0	8.9	8.5	8.9	10.8	-2.3
	0.0	-3.8	1.7	-1.7	0.0	3.8	1.3	-2.5	-0.8	-3.8	-1.7	3.8	-1.7
	0.0	-0.4	0.4	-6.1	-2.7	1.1	0.4	0.4	-3.8	-6.8	-6.4	-5.3	-8.7
	0.0	-5.0	-2.1	-6.2	-3.7	1.2	-3.3	0.4	0.4	0.8	-1.2	-10.8	-16.2
	0.0	-3.2	-2.4	-3.2	-1.2	3.6	2.8	4.7	3.2	2.4	2.0	-2.0	-8.3
	0.0	-2.8	-4.0	-4.8	-2.0	-1.2	-3.2	-3.2	-2.0	-3.6	-2.4	-7.2	-13.7
HPV-TCR only	0.0	4.0	4.9	1.3	3.1	6.7	-2.7	-1.8	1.3	3.1	4.5	5.4	3.1
	0.0	-1.6	0.4	-3.1	-0.8	2.0	-2.7	-1.2	-0.8	-3.9	-2.7	-1.2	-3.5
	0.0	-2.5	-2.1	-5.4	-5.0	3.3	-2.5	0.0	0.8	-0.8	2.5	5.0	0.4

	0.0	0.4	3.5	0.9	6.5	10.4	5.6	6.1	5.6	3.5	8.2	11.3	7.4
	0.0	-3.2	0.7	-2.8	-1.4	1.1	-2.1	0.7	-0.7	-1.1	1.8	2.1	-1.1
	0.0	3.0	5.1	1.7	4.7	10.3	6.0	10.3	10.3	8.1	9.8	7.7	3.8
	0.0	0.0	2.1	-0.4	4.2	5.4	2.9	5.4	6.3	1.7	5.0	9.6	5.9
	0.0	2.0	2.0	0.0	0.0	2.4	0.4	0.8	3.2	3.6	1.6	5.2	2.4
	0.0	3.4	3.1	5.3	3.1	5.0	0.4	2.7	4.6	2.3	4.2	6.5	0.4
	0.0	-3.4	-4.5	-7.2	-4.5	-2.6	-2.3	-3.4	-2.3	-6.4	-4.2	0.4	-3.4
NKG2D	0.0	-3.2	-1.6	-6.0	-0.4	0.4	-3.2	-5.2	-3.2	-1.2	3.2	6.4	2.0
	0.0	2.7	6.4	4.5	9.5	9.1	9.5	12.7	14.1	17.3	15.5	16.8	13.2
	0.0	0.5	3.3	-2.8	3.3	5.2	0.5	3.3	-0.9	-0.9	1.9	3.3	-0.5
	0.0	-4.5	-4.5	-7.0	-2.9	-2.9	-6.1	-1.2	-2.5	-5.3	-4.5	-3.3	-3.7
	0.0	-0.4	2.2	1.8	4.0	4.0	-0.4	0.9	0.4	-1.3	2.7	5.4	3.1
	0.0	1.6	3.6	2.0	1.6	5.6	1.6	4.0	2.8	2.4	1.6	6.0	2.4
	0.0	-3.7	1.2	0.0	6.6	6.2	1.2	1.7	3.3	-0.4	2.1	3.7	0.4
	0.0	-3.7	-4.1	-4.8	1.5	-3.0	-5.9	-5.9	-9.3	-9.6	-8.1	-8.9	-8.9
	0.0	2.9	4.1	5.4	10.4	10.0	5.8	6.2	6.2	2.5	6.6	5.0	3.3
CAR2 only	0.0	-0.8	0.4	-1.6	1.6	3.3	0.8	-0.4					
	0.0	0.8	2.5	1.6	2.9	7.0	4.9	6.6	5.3	5.7	5.7	7.0	3.7
	0.0	0.4	0.9	-3.4	0.4	0.9	-2.2	-2.6	1.7	-1.7	-0.9	-0.4	-4.7
	0.0	-9.0	-9.0	-12.1	-10.2	-7.4	-11.3	-10.2	-9.8	-11.7	-11.3	-9.0	-9.8
HPV-TCR	0.0	-4.0	-0.9	-7.1	-1.8	4.0	1.3	4.5	2.2	1.8	3.6	4.9	3.6
+ NKG2D	0.0	0.8	3.8	0.0	4.6	8.0	5.9	8.4	7.2	7.2	6.8	4.6	4.6
CAR2	0.0	-2.8	2.8	-0.9	1.4	2.3	-0.5	0.5	-1.4	-0.5	2.3	-1.9	-3.8

	0.0	2.7	-2.2	0.0	-0.4	0.0	1.8	2.7	2.2	0.4	4.9	-0.9	-1.3
	0.0	0.4	1.9	0.4	1.5	3.8	-3.4	-6.1	-8.0	-11.1	-8.0	-6.9	-8.0
	0.0	-1.9	1.9	1.1	1.1	5.3	1.1	0.0	-1.9	-1.1	3.4	1.1	-2.7
	0.0	-1.6	-2.4	-3.6	-1.6	2.0	-3.6	-4.0	-4.4	-7.5	-4.0	-5.6	-6.7
Days post tumor inoculation	**36**	**39**	**41**	**43**	**46**	**48**	**50**	**53**	**55**	**57**	**60**	**62**	
	3.5	3.5	3.5	5.6									
	7.6	14.3	12.5										
	7.6	8.9	8.9	10.7									
	0.8	8.5	4.2										
	7.0	11.1	12.7										
	-6.0	-2.4	-3.6	-0.8	1.6								
	0.4	-0.4	0.4										
Vehicle	3.0	3.4	2.3										
	-15.8												
	-4.5	-14.0	-23.6										
	0.9	0.0	-3.3	-2.8	-18.3								
	-2.1	-2.5	-5.9	-9.7	-15.7	-16.1	-18.6	-21.2					
	-14.4	-11.7	-13.6	-14.4	-17.4	-17.0	-21.6						
	###												
	-13.4	-15.0	-21.7										
NTD	-20.1												

	5.4	13.0	8.1	5.8	4.0	6.7	5.8	1.3	2.2	1.8	0.4	-4.5	
	-3.5	1.6	1.6	2.3	-2.3	-2.0							
	-1.3	1.7	3.3	1.3	-0.4	-0.8	-0.8	-7.5	-10.5	-15.1	-21.3		
	7.4	12.1	12.1	10.8	10.0	5.6	3.9	-1.3	-3.5	-7.4	-8.7	-12.1	
	-2.1	1.4	1.4	3.9									
	2.1	3.4	0.4	0.0	3.0	2.1	-5.1	-2.6	-1.7	-6.0	-3.8	-2.6	
	5.0	9.2	9.6	5.9	9.6	7.9	9.2	9.2					
	-0.8	4.8	2.0	5.6	0.8	3.2	6.4	-1.6	4.0	2.4	3.2	2.0	
HPV-TCR only	0.0	3.1	-2.3	-3.1									
	-4.9	0.8	-1.5	1.1	1.9	-2.6	0.8	-1.5	0.8	2.3			
	-2.4	2.8	3.2	5.6	8.8								
	12.3	14.1	20.5	20.9	17.7	19.1	20.0	18.2	22.7	24.1			
	2.3	8.0	6.1	7.5	10.3	12.7	12.7	9.4	12.7	15.0	18.3	16.4	
	-1.2	0.8	-1.6	-1.2	1.2	-0.8	1.6	-0.4	0.0	2.9	5.7		
	-0.1	6.3	4.0	9.9	12.6								
	-0.4	5.2	2.4	6.8	4.0	6.0	8.0	7.6					
	0.0	2.1	2.5	3.3	4.1	5.0	5.8	7.0	7.9	11.2	14.9		
	-8.9	-4.8	-5.2	-3.7	-3.0	-4.4	-2.6	-2.2	2.2				
NKG2D	2.9	8.7	8.3	9.5	4.6	3.7	10.0	11.2	12.4	14.1			
CAR2 only													
HPV-TCR	4.9	6.6	8.6	11.1	13.1	13.9	13.1	12.3					
+ NKG2D	-4.3	3.4	3.9	9.9	8.2	5.2	5.6	4.7					
CAR2	-8.6	-6.3	-9.0	-7.8	-5.9	-1.2	-0.4	1.6					

5.4	5.8	6.7	9.4	8.0	11.2	10.7	10.7					
2.1	4.2	2.5	6.3	4.6	8.0	8.9	6.3	8.4	8.4	11.0		
-2.3	0.0	-0.5	-1.4	1.9	3.3	5.2	1.9	7.0	4.7	8.9	4.2	
0.0	4.9	3.1	4.9	8.5	9.4							
-7.6	-5.7	-6.9	-3.4	-2.7	1.5	3.1	3.1					
-4.2	-1.1	0.4	1.1	3.8								
-6.0	-1.6	-4.8	-1.6	-0.4	1.2	4.4	5.6	4.8				

Table 18: Percentage of adoptively transferred T cells in peripheral blood

Percent hCD45 in peripheral lymphocytes						
Days post tumor inoculation	**1**	**7**	**14**	**21**	**28**	**35**
	0.00	0.01	0.03	0.02	0.02	0.01
	0.00	0.04	0.04	0.02	0.02	0.02
	0.06	0.00	0.02	0.02	0.01	0.01
	0.01	0.00	0.02	0.02	0.01	0.01
	0.10	0.00	0.03	0.02	0.00	0.01
	0.02	0.01	0.03	0.02	0.00	0.00
	0.01	0.09	0.03	0.02	0.01	0.00
Vehicle	0.19	0.00	0.02	0.01	0.00	5.03
	8.03	8.63	2.56	9.25	41.80	78.20
	7.47	0.36	2.24	4.62	19.80	43.20
	9.44	8.83	1.95	4.58	16.70	29.90
	5.45	6.13	2.09	4.55	11.50	28.10
	6.90	6.54	0.73	8.59	25.30	27.20
	4.61	5.04	1.48	5.09	53.60	38.00
	7.35	6.04	1.26	6.15	29.80	26.60
NTD	4.77	3.45	1.94	14.80	40.00	0.00
	4.14	1.77	0.16	0.09	1.33	0.45
	3.26	1.24	0.17	0.15	0.16	0.43
	4.21	2.11	0.33	0.51	0.00	18.60
	3.32	4.59	0.43	0.62	0.00	2.02
	3.62	2.92	0.16	0.29	0.00	1.53
	3.26	1.97	0.15	0.11	0.69	1.99
	3.24	2.41	0.16	0.58	1.80	0.58
	4.23	2.46	0.17	0.62	0.49	5.95
	4.36	4.46	0.20	2.52	0.38	0.47
HPV-TCR only	3.18	2.72	0.12	0.22	0.24	0.02
	0.43	1.35	0.04	0.07	0.01	0.50
	0.60	1.92	0.09	1.28	1.39	0.01
NKG2D CAR2 only	0.67	3.19	0.07	0.06	0.02	4.89
	0.65	3.15	0.09	0.16	0.65	0.01

	1.06	1.43	0.13	0.06	0.04	0.10
	0.75	2.70	0.05	0.12	0.03	0.02
	0.73	3.39	0.10	0.09	0.02	0.01
	0.86	2.98	0.10	0.07	0.02	0.02
	0.00	1.21	0.05	0.05	0.02	0.02
	0.65	1.75	0.09	0.07	0.03	0.00
	0.42	1.91	0.06	0.02	0.03	0.01
	0.32	1.99	0.06	0.03	0.00	0.03
	0.40	1.84	0.10	0.06	0.02	0.81
	0.37	1.81	0.13	0.37	0.40	0.02
	0.32	2.10	0.06	0.02	0.01	0.10
	0.25	1.62	0.05	0.01	0.01	0.01
	0.24	1.32	0.09	0.05	0.01	0.22
	0.26	2.71	0.10	0.02	0.01	0.33
HPV-TCR +	0.30	2.76	0.05	0.26	0.08	0.02
NKG2D CAR2	0.30	3.12	0.07	0.05	0.02	10.90

[0287] In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

1. A chimeric antigen receptor (CAR), comprising:
a NKG2D ecto domain;
a transmembrane domain;
a 4-1BB costimulatory domain; and
a signaling domain comprising a CD3-zeta signaling domain.

2. The CAR of claim 1, further comprising:
a CD8-alpha hinge domain.

3. The CAR of claim 2, wherein the CD8-alpha hinge domain comprises the amino acid sequence according to SEQ ID NO: 15.

4. The CAR of any one of claims 1-3, wherein the NKG2D ecto domain comprises the amino acid sequence according to SEQ ID NO: 3.

5. The CAR of any one of claims 1-4, wherein the transmembrane domain further comprises a CD28 transmembrane domain.

6. The CAR of claim 5, wherein the CD28 transmembrane domain comprises the amino acid sequence according to SEQ ID NO: 21.

7. The CAR of any one of claims 1-6, wherein the 4-1BB costimulatory domain comprises the amino acid sequence according to SEQ ID NO: 33 or SEQ ID NO: 63.

8. The CAR of any one of claims 1-7, wherein the CD3zeta signaling domain comprises the amino acid sequence according to SEQ ID NO: 27.

9. The CAR of any one of claims 1-8, wherein the signaling domain further comprises a CD3-epsilon signaling domain.

10. The CAR of claim 9, wherein the CD3-epsilon signaling domain comprises the amino acid sequence according to SEQ ID NO: 31 or SEQ ID NO: 61.

11. The CAR of any one of claims 1-10, comprising an amino acid sequence having at least 90% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, and SEQ ID NO: 76.

12. A nucleic encoding the CAR of any one of claims 1-11.

13. The nucleic acid of claim 12, comprising a nucleotide sequence having at least 90% sequence identity to a nucleotide sequence selected from the group consisting of SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, and SEQ ID NO: 77.

14. A recombinant vector comprising the nucleic acid of claim 12 or 13.

15. The recombinant vector of claim 14 or the nucleic acid of claim 12 or 13, wherein the recombinant vector or nucleic acid further comprises a nucleic acid encoding an engineered T cell receptor (TCR).

16. The recombinant vector of claim 14 or the nucleic acid of claim 12 or 13, wherein the recombinant vector or nucleic acid further comprises a nucleic acid encoding a second CAR that is specific for a tumor antigen.

17. The recombinant vector or nucleic acid of claim 15 or 16, where the tumor antigen comprises HPV–16 E6 and HPV–16 E7, alpha folate receptor, 5T4, αvβ6 integrin, BCMA, B7–H3, B7–H6, CAIX, CD19, CD20, CD22, CD28, CD30, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD137 (4–1BB), CD138, CD171, CEA, CSPG4, CLL-1, CS1, EGFR, EGFR family including ErbB2 (HERII), EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, FAP, fetal AchR, FRa, Flt3, GD2, GD3, Glypican–3 (GPC3), HLA–Al + MAGEI, HLA–A2 + MAGE1, HLAA3 + MAGE1, HLA–Al + NY–ES0–1, HLA–A2 + NY–ES0–1, HLA–A3 + NY–ES0–1, IL–llRα, IL–13Rα2, Lambda, Lewis–Y, Kappa, Mesothelin, Mucl, Muc16, NCAM, NKG2D Ligands, NYE-S0–1, PRAME, PSCA, PSMA, RORI, SSX, Survivin, TACI, TAG72, TEMs, or VEGFRII.

18. A host cell transformed with the nucleic acid or recombinant vector of any one of claims 12-17.

19. A host cell transformed with the nucleic acid of claim 12 or 13 or recombinant vector of claim 14 and a nucleic acid or recombinant vector encoding an engineered T cell receptor (TCR) or a second CAR that is specific for a tumor antigen.

20. The host cell of claim 19, wherein the tumor antigen comprises HPV–16 E6 and HPV–16 E7, alpha folate receptor, 5T4, αvβ6 integrin, BCMA, B7–H3, B7–H6, CAIX, CD19, CD20, CD22, CD28, CD30, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD137 (4–1BB), CD138, CD171, CEA, CSPG4, CLL-1, CS1, EGFR, EGFR family including ErbB2 (HERII), EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, FAP, fetal AchR, FRa, Flt3, GD2, GD3, Glypican–3 (GPC3), HLA–Al + MAGEI, HLA–A2 + MAGE1, HLAA3 + MAGE1, HLA–Al + NY–ES0–1, HLA–A2 + NY–ES0–1, HLA–A3 + NY–ES0–1, IL–llRα, IL–13Rα2, Lambda, Lewis–Y, Kappa, Mesothelin, Mucl, Muc16, NCAM, NKG2D Ligands, NYE-S0–1, PRAME, PSCA, PSMA, RORI, SSX, Survivin, TACI, TAG72, TEMs, or VEGFRII.

21. The host cell of any one of claims 18-20, where the host cell comprises an iPSC, a T cell, or a NK cell.

22. A pharmaceutical composition comprising the host cell of claim 21.

23. A method of treating disease in a patient in need of thereof, comprising administering the host cell of claim 21 or the pharmaceutical composition of claim 22 to the patient.

24. The method of claim 23, wherein the host cell is allogeneic to the patient.