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(71) Applicants: **MERCK SHARP & DOHME LLC** [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). **ARTIVA BIOTHERAPEUTICS, INC.** [US/US]; 5505 Morehouse Drive, STE 100, San Diego, CA 92121 (US).

(72) Inventors: **FRIDMAN, Arthur**; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). **HANDA, Masahisa**; 213 E. Grand Avenue, South San Francisco, CA 94080 (US). **HSIEH, Chung-Ming**; 33 Avenue Louis Pasteur, Boston, MA 02115 (US). **SINGH, Sujata**; 213 E. Grand Avenue, South San Francisco, CA 94080 (US). **TABRIZI-FARD, Sahba**; 213 E. Grand Avenue, South San Francis-

co, CA 94080 (US). **VANEGAS, Sandra, Isabel**; 213 E. Grand Avenue, South San Francisco, CA 94080 (US). **SO-MANCHI, Srinivas**; 12370 Carnel Country Rd, Unit 110, San Diego, CA 92130 (US). **FLYNN, Peter**; 2335 Oxford Avenue, Cardiff, CA 92007 (US). **RAYMON, Heather**; 2325 Avenida De La Playa, La Jolla, CA 92037 (US).

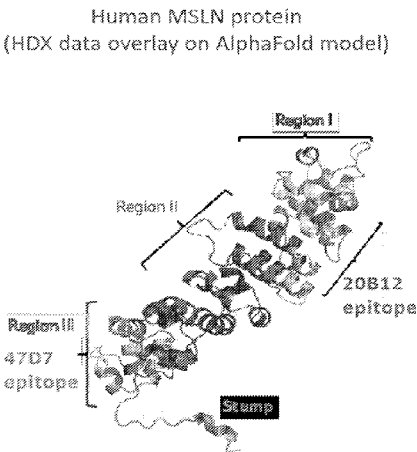
(74) Agent: **LIANG, Bing** et al.; JONES DAY, 250 Vesey Street, New York, NY 10281-1047 (US).

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(54) Title: CHIMERIC ANTIGEN RECEPTORS AND USE THEREOF

(57) Abstract: The present disclosure provides CARs comprising a human mesothelin (MSLN) binder, related polynucleotides and vectors, engineered cells, pharmaceutical compositions, and cell therapies.



45B6: Region 1, low affinity  
47D7: Region 3 (membrane proximal), high affinity  
20B12: Region 1-2 junction (tentative), lower affinity

FIG. 1A

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, ME, 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).

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## CHIMERIC ANTIGEN RECEPTORS AND USE THEREOF

### CROSS REFERENCE

**[0001]** This application claims priority to U.S. Provisional Application No. 63/508,195, filed on June 14, 2023, the entirety of which is incorporated herein by reference.

### SEQUENCE LISTING

**[0002]** This application contains a computer readable Sequence Listing which has been submitted in XML file format with this application, the entire content of which is incorporated by reference herein in its entirety. The Sequence Listing XML file submitted with this application is entitled “14463-045-228\_SEQLISTING.xml”, was created on May 31, 2024, and is 153,174 bytes in size.

### 1. FIELD

**[0003]** The present disclosure relates to chimeric antigen receptors (CAR) comprising an antigen-binding fragment of an anti-mesothelin antibody, engineered cells (e.g., Natural Killer cells) and methods of use thereof.

### 2. BRIEF SUMMARY OF THE INVENTION

**[0004]** The present disclosure provides a chimeric antigen receptor (CAR) comprising a polypeptide comprising (a) an extracellular antigen binding domain comprising a human mesothelin (MSLN) binder provided herein; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, in the present CAR, the human MSLN binder comprises a single chain Fv (scFv) that comprises a heavy chain variable domain (VH) and a light chain variable domain (VL). In some embodiments, the VL is N-terminal to the VH. In other embodiments, the VL is C-terminal to the VH. In some embodiments, the VL is joined to the VH via a flexible linker. In some specific embodiments, the flexible linker comprises the amino acid sequence of SEQ ID NO: 52. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 38 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 38. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 39 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 39. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 39 wherein the 1st amino acid residue of

SEQ ID NO: 39 is pyroglutamate. In some embodiments, the scFv comprises an amino acid sequence having the 2nd amino acid residue to the 241th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 241th amino acid residue of SEQ ID NO: 39 and the 1st amino acid residue of the amino acid sequence being pyroglutamate. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 40 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 40. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 40 wherein the 1st amino acid residue is pyroglutamate. In some embodiments, the scFv comprises an amino acid sequence having the 2nd amino acid residue to the 238th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 238th amino acid residue of SEQ ID NO: 40 and the 1st amino acid residue of the amino acid sequence being pyroglutamate. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 41 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 41. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 41 wherein the 1st amino acid residue of SEQ ID NO: 41 is pyroglutamate. In some embodiments, the scFv comprises an amino acid sequence having the 2nd amino acid residue to the 238th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 238th amino acid residue of SEQ ID NO: 41 and the 1st amino acid residue of the amino acid sequence being pyroglutamate. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 42 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 42. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 43 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 43. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 44 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 44.

**[0005]** In certain embodiments, in the CAR provided herein, the transmembrane domain is derived from a molecule selected from a group consisting of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152, and PD1. In some embodiments, the transmembrane domain is derived from a CD28 transmembrane domain. In some embodiments, the CD28 transmembrane domain comprises the amino acid sequence of SEQ ID NO: 54. In other embodiments, the CD28

transmembrane domain comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 54.

**[0006]** In certain embodiments, in the CAR provided herein, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell. In some embodiments, the primary intracellular signaling domain is derived from CD3-zeta (CD3ζ). In some embodiments, the primary intracellular signaling domain derived from CD3-zeta comprises the amino acid sequence of SEQ ID NO: 57. In some embodiments, the primary intracellular signaling domain derived from CD3-zeta comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 57.

**[0007]** In certain embodiments, in the CAR provided herein, the intracellular signaling domain further comprises a co-stimulatory signaling domain. In some embodiments, the co-stimulatory signaling domain is derived from a molecule selected from the group consisting of CD27, CD28, CD137, OX40, OX40L, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, ligands of CD83 and combinations thereof. In some embodiments, the co-stimulatory signaling domain comprises an OX40L intracellular signaling domain. In some embodiments, the OX40L intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 56. In other embodiments, the OX40L intracellular signaling domain comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 56. In some embodiments, the co-stimulatory signaling domain further comprises a CD28 intracellular signaling domain. In some embodiments, the CD28 intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 55. In some embodiments, the CD28 intracellular signaling domain comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 55.

**[0008]** In certain embodiments, the CAR provided herein further comprises a hinge domain located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the hinge domain is derived from CD8α.

**[0009]** In some embodiments, the hinge domain derived from CD8α comprises the amino acid sequence of SEQ ID NO: 60. In some embodiments, the hinge domain derived from CD8α comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 60. In other embodiments, the hinge domain comprises at least a portion of an IgG1 hinge domain. In some embodiments, the IgG1 hinge domain comprises the amino acid sequence of SEQ ID NO: 53. In some

embodiments, the IgG1 hinge domain comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 53.

**[0010]** In certain embodiments, the CAR provided herein further comprises a signal peptide located at the N-terminus of the polypeptide. In some embodiments, the signal peptide is derived from CD8 $\alpha$ . In some embodiments, the signal peptide derived from CD8 $\alpha$  comprises the amino acid sequence of SEQ ID NO: 51. In some embodiments, the signal peptide derived from CD8 $\alpha$  comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 51.

**[0011]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, or SEQ ID NO: 82. In some specific embodiments, the CAR provided herein comprises (i) the amino acid sequence of SEQ ID NO: 75 or an amino acid sequence having at least 85% identity to SEQ ID NO: 75; (ii) the amino acid sequence of SEQ ID NO: 76 or an amino acid sequence having at least 85% identity to SEQ ID NO: 76; (iii) the amino acid sequence of SEQ ID NO: 77; an amino acid sequence having at least 85% identity to SEQ ID NO: 77; the amino acid sequence of SEQ ID NO: 77 wherein the 1st amino acid residue of SEQ ID NO: 77 is pyroglutamate; or an amino acid sequence having the 2nd amino acid residue to the 674th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 674th amino acid residue of SEQ ID NO: 77 and the 1st amino acid residue of the amino acid sequence being pyroglutamate (iv) the amino acid sequence of SEQ ID NO: 78; an amino acid sequence having at least 85% identity to SEQ ID NO: 78; the amino acid sequence of SEQ ID NO: 78 wherein the 1st amino acid residue of SEQ ID NO: 78 is pyroglutamate; or an amino acid sequence having the 2nd amino acid residue to the 491st amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 491st amino acid residue of SEQ ID NO: 78 and the 1st amino acid residue of the amino acid sequence being pyroglutamate; (v) the amino acid sequence of SEQ ID NO: 79; an amino acid sequence having at least 85% identity to SEQ ID NO: 79; the amino acid sequence of SEQ ID NO: 79 wherein the 1st amino acid residue of SEQ ID NO: 79 is pyroglutamate; or an amino acid sequence having the 2nd amino acid residue to the 671th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 671th amino acid residue of SEQ ID NO: 79 and the 1st amino acid residue of the amino acid sequence being pyroglutamate (vi) the amino acid sequence of SEQ ID NO: 80; an amino acid sequence having at least 85% identity to SEQ ID NO: 80; the amino acid sequence of SEQ ID NO: 80 wherein the 1st amino acid residue

of SEQ ID NO: 80 is pyroglutamate; or an amino acid sequence having the 2nd amino acid residue to the 488th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 488th amino acid residue of SEQ ID NO: 80 and the 1st amino acid residue of the amino acid sequence being pyroglutamate(vii) the amino acid sequence of SEQ ID NO: 81; an amino acid sequence having at least 85% identity to SEQ ID NO: 81; the amino acid sequence of SEQ ID NO: 81 wherein the 1st amino acid residue of SEQ ID NO: 81 is pyroglutamate; or an amino acid sequence having the 2nd amino acid residue to the 671st amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 671st amino acid residue of SEQ ID NO: 81 and the 1st amino acid residue of the amino acid sequence being pyroglutamate or (viii) the amino acid sequence of SEQ ID NO: 82; an amino acid sequence having at least 85% identity to SEQ ID NO: 82; the amino acid sequence of SEQ ID NO: 82 wherein the 1st amino acid residue of SEQ ID NO: 82 is pyroglutamate; or an amino acid sequence having the 2nd amino acid residue to the 488th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 488th amino acid residue of SEQ ID NO: 82 and the 1st amino acid residue of the amino acid sequence being pyroglutamate.

**[0012]** In another aspect, provided herein is a polypeptide that comprises the CAR provided herein linked to an IL-15 peptide via a self-cleaving peptide. In some embodiments, the self-cleaving peptide is capable of inducing ribosomal skipping between the CAR and the IL-15. In some embodiments, the IL-15 comprises the amino acid sequence of SEQ ID NO: 59 or a variant thereof. In some embodiments, the self-cleaving peptide comprises the amino acid sequence of SEQ ID NO: 58 or a variant thereof.

**[0013]** In another aspect, provided herein is a polynucleotide comprising a nucleic acid encoding the CAR or the polypeptide provided herein. In some embodiments, the polynucleotide comprises a nucleic acid encoding an IL-15. In some embodiments, the IL-15 comprises the amino acid sequence of SEQ ID NO: 59. In some embodiments, the IL-15 is encoded by a nucleic acid comprising the nucleic acid sequence of SEQ ID NO: 96. In some embodiments, the polynucleotide provided herein further comprises a nucleic acid encoding a self-cleaving peptide, optionally a T2A self-cleaving peptide. In some embodiments, the self-cleaving peptide comprises the sequence of SEQ ID NO: 58. In some embodiments, the self-cleaving peptide is encoded by the sequence of SEQ ID NO: 94.

**[0014]** In some embodiments, the polynucleotide provided herein encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, or SEQ ID NO: 68. In some specific embodiments, the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:

83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, or SEQ ID NO: 90.

**[0015]** In yet another aspect, provided herein is a vector comprising the polynucleotide provided herein. In some embodiments, the vector is a viral vector, and wherein optionally the viral vector is a retroviral vector or a lentiviral vector.

**[0016]** In yet another aspect, provided herein is an engineered cell comprising the polynucleotide or the vector provided herein. In certain embodiments, provided herein is an engineered cell expressing the chimeric antigen receptor encoded by the polynucleotide or the vector provided herein. In certain embodiments, provided herein is an engineered cell expressing the chimeric antigen receptor and the IL-15 encoded by the polynucleotide or the vector provided herein. In some embodiments, the engineered cell is a lymphocyte. In some embodiments, the lymphocyte is a natural killer (NK) cell. In some embodiments, the lymphocyte is a T cell. In some embodiments, the engineered cell is derived from a human cell. In some embodiments, the engineered cell is from a primary cell obtained from a subject. In some embodiments, the engineered cell is from a primary cell obtained from cord blood. In some embodiments, the engineered cell comprises a KIR-B haplotype. In some embodiments, the engineered cell expresses CD16 having the V/V polymorphism at F158.

**[0017]** Also provided herein is a population of cells comprising a plurality of the engineered cells provided herein. In some embodiments, in the population of cells, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the engineered cells comprise the CAR, the polynucleotide, or the vector provided herein. In some embodiments, in the population of engineered cells, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the engineered cells express the polyprotein, the polynucleotide, or the vector provided herein.

**[0018]** In yet another aspect, provided herein is a pharmaceutical composition comprising the population of cells provided herein. In some embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable excipient.

**[0019]** In yet another aspect, provided herein is a method of treatment comprising administering the engineered cell, the population of cells, or the pharmaceutical composition provided herein to a subject having a disease or condition associated with MSLN. In yet another aspect, provided herein is use of the engineered cell, the population of cells, or the pharmaceutical composition provided herein in the manufacture of a medicament for treating a disease or condition associated with MSLN. In yet another aspect, provide herein is use of the engineered cell, the population of cells, or the pharmaceutical composition provide herein in the

manufacture of a medicament for treating a disease or condition associated with MSLN. In some embodiments, the disease or condition associated with MSLN is cancer.

### 3. BRIEF DESCRIPTION OF THE DRAWINGS

[0020] **FIGs. 1A-1B** show that three anti-MSLN antibodies were selected for CAR-NK generation based on binding affinity and binding to different regions of MSLN.

[0021] **FIG. 2** illustrates exemplary CAR designs.

[0022] **FIGs. 3A-3C** show the binding of MSLN mAb-converted scFv-Fc constructs to MSLN-expressing CHO cells.

[0023] **FIGs. 4A-4D** show surface plasmon resonance (SPR) plots and calculated affinities of 45B6 Fab, 45B6 ScFv-Fc, and 47D7 ScFv-Fc for human mesothelin protein. **FIGs. 4A-4C** show SPR plots for 45B6 Fab, 45B6 ScFv-Fc, and 47D7 ScFv-Fc (respectively) against human mesothelin protein. **FIG. 4D** shows a table of analyzed data and calculated KD for the 45B6-derived molecules.

[0024] **FIGs. 5A-5C** show the epitope mapping of anti-MSLN antibodies 45B6, 20B12, and 47D7. **FIG. 5A** illustrates a differential heat map comparing hydrogen/deuterium exchange of recombinant mesothelin alone to that of recombinant mesothelin and antibody 45B6; **FIG. 5B** illustrates the H/D Difference Plot showing deuterium uptake protection upon antibody binding to human mesothelin protein: (top) 20B12 and (bottom) 47D7; **FIG. 5C** shows that H/D protection from all three antibodies were mapped onto mesothelin AlphaFold model (gray).

[0025] **FIG. 6** shows that 15B6 reduced soluble MSLN likely by blocking membrane MSLN shedding from cells.

[0026] **FIG. 7** shows the long-term cytotoxicity data of various clones.

[0027] **FIGs. 8A-8J** show the robust cytotoxic activity of the MSLN CAR-NK in long-term co-culture assays (HDX indicates hydrogen deuterium exchange).

[0028] **FIGs. 9A-9B** show the profile of NK cells activation and inhibitory receptors (MFI indicates mean fluorescence intensity).

[0029] **FIGs. 10A-10C** show the *in vivo* persistence of MSLN CAR-NK in naïve NOD scid gamma (NSG) mice.

[0030] **FIGs. 11A-11C** show the MSLN CAR-NK efficacy in NCI-N87 tumor model in tumor volume, persistence, and immunohistochemistry (IHC).

#### 4. DETAILED DESCRIPTION OF THE INVENTION

**[0031]** The present disclosure provides CARs, related polynucleotides and vectors, engineered cells (e.g., NK cells), pharmaceutical compositions, and cell therapies. In one aspect, provided herein is a CAR (see Section 4.2) comprising an extracellular antigen binding domain (see Section 4.2.1) that comprising human MSLN binder (see Section 4.2.1.1), an transmembrane domain (see Section 4.2.2), an intracellular signaling domain (see Section 4.2.3), a co-stimulatory signaling domain (see Section 4.2.4), optionally a hinge domain (see Section 4.2.5). In another aspect, provided herein is an nucleic acid molecule encoding the CAR (see Section 4.3). In another aspect, provided herein is an engineered immune effector cell (see Section 4.4). In yet another aspect, provided herein are procedures and methods related to expansion and stimulation of NK cells (see Section 4.5) and procedures and methods related to engineering the immune effector cells (see Section 4.6). In another aspect, provided herein are cryopreservation compositions and methods of cryopreserving (see Section 4.7) and pharmaceutical compositions (see Section 4.8). In yet another aspect, provided herein are CAR-NK cellular therapies (see Section 4.9) and combination therapies (see Section 4.10). The present disclosure is based on, among other things, the novel CARs that each comprise a human MSLN binder.

**[0032]** Mesothelin is a differentiation antigen whose expression in normal human tissues is limited to mesothelial cells lining the pleura, pericardium, and peritoneum (Chang & Pastan, Proc. Natl. Acad. Sci. USA 93: 136–140 (1996); Chang et al., Int. J. Cancer 50:373–381 (1992)). However, mesothelin is highly expressed in several human cancers, including virtually all mesotheliomas and pancreatic adenocarcinomas, and approximately 70% of ovarian cancers and 50% of lung adenocarcinomas (Ordonez, Mod Pathol. 16: 192–197 (2003); Argani et al., Clin. Cancer Res. 7: 3862–3868 (2001); Hassan et al., Appl. Immunohistochem Mol. Morphol. 13: 243–247 (2005); Ordonez, Am. J. Surg. Pathol. 27: 1418–1428 (2003)).

**[0033]** The mesothelin gene encodes a precursor protein of 71 kDa that is processed to a 31 kDa shed protein called megakaryocyte potentiating factor (MPF) and a 40 kDa fragment, mesothelin, that is attached to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor. MPF was isolated from the culture supernatant of a pancreatic cancer cell line and was so named because it stimulated the megakaryocyte colony-forming activity of interleukin-3 in mouse bone marrow cultures. The biologic function of mesothelin is not known; however, results of recent studies suggest that mesothelin may play a role in ovarian cancer metastasis by binding to MUC16/CA-125 (Rump et al., J. Biol. Chem. 279: 9190–9198 (2004)). A small amount of cell

bound mesothelin is shed into the serum and has been shown to be elevated in patients with mesothelioma and ovarian cancer (Hassan et al., Clin. Cancer Res. 12: 447–453 (2006)).

**[0034]** Mesothelin is a promising candidate for tumor-specific therapy given its limited expression in normal tissues and high expression in several cancers. These therapies include agents that target cell surface mesothelin or elicit an immune response against mesothelin. Agents that are in the clinic or about to enter clinical trials include CAT-5001, MORAb-009, and CRS-207 (Hassan & Ho, Eur. J. Cancer 44: 46-53 (2008)).

**[0035]** Therapeutic antibodies that bind to mesothelin may be useful for the treatment of cancer. Monoclonal antibodies (mAbs) both in monotherapy and in combination regimens has emerged as one of the fastest growing and most effective therapeutic strategies for the treatment of solid tumors and hematological diseases. Between 2015 and 2017, the U.S. Food and Drug Administration approved 27 therapeutic mAbs (Tsumoto et al., Immunotherapy 11: 119–127 (2019)) increasing the total of clinically used mAbs and biosimilars in 2017 to 57 and 11, respectively (Grilo & Mantalaris, Trends Biotechnol. 37: 9–16 (2019)). As of late 2019, numerous companies were supporting over 550 novel antibody therapeutics in early phase clinical trials, with approximately half of these against oncology targets (Kaplon et al., MAbs 12: 1–24 (2020)).

**[0036]** Targeted therapies, including the use of adoptive cell therapies such as chimeric antigen receptor T cells (CAR Ts), have revolutionized cancer treatment. These cell therapies may be autologous (CAR T cells manufactured using a patient's own T cells) or allogeneic (CAR T cells manufactured using T cells from healthy donors).

**[0037]** CAR T cell manufacturing is a resource-intensive process that can result in failure to produce a viable autologous cell therapy for some patients. The average manufacturing time of 3 weeks that is needed for autologous CAR T cell products may be too long for critically ill patients. Finally, due to the complex nature of the manufacture and delivery of CAR-T cell product, which require close monitoring at top-tier cancer and medical centers, access to this treatment option may be out of reach, both financially and geographically, for most patients. Importantly, even for those patients who have access to this innovative treatment, CAR-T cell products confer a risk of serious and potentially deadly adverse effects. These adverse effects include cytokine release syndrome (CRS) and neurotoxicity, which can be difficult to manage or control.

**[0038]** Allogeneic CAR-T cell therapies, which utilize cells from healthy donors, may overcome some of the manufacturing and logistical challenges of autologous CAR-T cell therapies. However, these “off-the-shelf” CAR T cell therapies also have issues that include a

potentially higher risk of graft-versus-host disease (GVHD) and ineffectiveness due to rapid clearance by the patient's immune system.

**[0039]** Natural killer (NK) cells are cytolytic cells of the innate immune system with an intrinsic ability to lyse tumor cells and virus-infected cells. Prior antigen exposure is not required for NK cells to identify and lyse tumor cells. Natural killer cells have the inherent ability to bridge between innate immunity and engender a multi-clonal adaptive immune response resulting in long-term anticancer immune memory. Receptor engagement by NK cells drives effector function through degranulation of lytic granules, activation of programmed cell death receptors on target cells, and secretion of immune modulatory cytokines. The effector function of NK cells is governed through the balance of activating and inhibitory receptor signaling. Classically, NK cells are defined as CD56<sup>+</sup> and CD3<sup>-</sup> cells that are subdivided in to CD56<sup>bright</sup>CD16<sup>-</sup>, cytokine secreting cells and CD56<sup>dim</sup>CD16<sup>+</sup> cytolytic cells (Carson 1997, Cooper 2001).

**[0040]** Natural killer cells also engage tumor cells through antibody dependent cellular cytotoxicity (ADCC), a key component of the innate immune system. Antibody-coated target cells are killed by cells with Fc receptors that recognize the constant region of the bound antibody. Engagement of CD16 (FCγRIII) with antibody-opsonized tumor cells is sufficient to elicit cytotoxicity and cytokine release response by resting NK cells. Activated NK cells secrete cytokines and chemokines, such as interferon gamma (IFNγ); tumor necrosis factor alpha (TNFα); and macrophage inflammatory protein 1 (MIP1) that signal and recruit T cells to tumors. Through direct killing of tumor cells, NK cells also expose tumor antigens for recognition by the adaptive immune system. ADCC is recognized as a potent mechanism of NK cell action, particularly in combination with antibodies belonging to immunoglobulin G1 (IgG1) and IG3 subclasses. To initiate ADCC, NK cells engage with antibodies via the CD16 receptor.

**[0041]** Similar to T cells, allogeneic NK cells engineered to express CARs with anti-tumor activity may provide an important treatment option for cancer patients. NK cells do not suffer from some of the shortcomings of allogeneic CAR-T cells, which often retain expression of endogenous T cell receptors in addition to engineered chimeric antigen receptors. As a result, allogeneic CAR-NK cell treatments can be administered safely to patients without many of the risks associated with allogeneic T cell therapies, including GVHD. However, CAR-NK cells face many of the same challenges as other allogeneic cell therapies, including product sourcing, scalability, persistence, and dose-to-dose variability.

#### 4.1 Definitions

**[0042]** So that the disclosure may be more readily understood, certain technical and scientific terms are specifically defined below. Unless specifically defined elsewhere in this document, all other technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this disclosure belongs.

**[0043]** As used herein, including the appended claims, the singular forms of words such as “a,” “an,” and “the,” include their corresponding plural references unless the context clearly dictates otherwise.

**[0044]** As used herein, the term “about” refers to plus or minus up to 10% of the value it modifies (rounded up to the nearest whole number if the value is not sub-dividable, such as a number of molecules or nucleotides). The term “about”, when modifying the quantity (e.g., mg) of a substance or composition, a parameter of a substance or composition or a parameter used in characterizing a step in a method, or the like, refers to variation in the numerical quantity that can occur. For example, such variation can occur through typical measuring, handling and sampling procedures involved in the preparation, characterization and/or use of the substance or composition, through inadvertent error in these procedures, through differences in the manufacture, source, or purity of the ingredients employed to make or use the compositions or carry out the procedures.

**[0045]** As used herein, the term “mesothelin” or “MSLN” refers to a differentiation antigen whose expression in normal human tissues is limited to mesothelial cells lining the pleura, pericardium and peritoneum. MSLN is generated from a ~ 70 kDa precursor protein that is cleaved by endoprotease furin to release the N-terminal region MPF (megakaryocyte potentiating factor) and 40 kDa membrane bound mesothelin (GPI linked to the cell surface) (See PCT/US22/01253, which is incorporated by reference herein in its entirety). The amino acid sequence of human mesothelin precursor is set forth in SEQ ID NO: 1. The mature form comprises amino acids 296-622.

**[0046]** As used herein, the term “human MSLN” refers to the mature form of human mesothelin and the term “rhesus MSLN” refers to the mature form of Rhesus (*Macaca mulatta*) mesothelin. The amino acid sequences for the precursor and mature forms of rhesus MSLN are shown in SEQ ID NO: 2.

**[0047]** As used herein, the term “human MSLN binder” refers to an antibody or antigen binding fragment thereof that binds to soluble and/or membrane-bound human MSLN. A human MSLN binder includes but is not limited to a bivalent antibody tetramer (2H+2L), a monovalent

antibody (H+L), a bi-specific antibody that targets human MSLN and another target, a Fab fragment, a Fab' fragment, a F(ab')<sub>2</sub> fragment, an Fv region, and an scFv.

**[0048]** As used herein, the term “affinity” refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, “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 by common methods known in the art, including KinExA and surface plasmon resonance (SPR; Biacore™). Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

**[0049]** As used herein, the term “administration” and “treatment,” as it applies to an animal, human, experimental subject, cell, tissue, organ, or biological fluid, refers to contact of an exogenous pharmaceutical, therapeutic, diagnostic agent, or composition comprising a CAR as disclosed herein to the animal, human, subject, cell, tissue, organ, or biological fluid. Treatment of a cell encompasses contact of a reagent to the cell, as well as contact of a reagent to a fluid, where the fluid is in contact with the cell. “Administration” and “treatment” also means in vitro and ex vivo treatments, e.g., of a cell, by a reagent, diagnostic, binding compound, or by another cell. The term “subject” includes any organism, preferably an animal, more preferably a mammal (e.g., human, rat, mouse, dog, cat, rabbit). In a preferred embodiment, the term “subject” refers to a human.

**[0050]** As used herein, the term “amino acid” refers to a simple organic compound containing both a carboxyl (—COOH) and an amino (—NH<sub>2</sub>) group. Amino acids are the building blocks for proteins, polypeptides, and peptides. Amino acids occur in L-form and D-form, with the L-form in naturally occurring proteins, polypeptides, and peptides. Amino acids and their code names are set forth in the following chart.

| Amino acid    | Three letter code | One letter code |
|---------------|-------------------|-----------------|
| Alanine       | Ala               | A               |
| Arginine      | Arg               | R               |
| Asparagine    | Asn               | N               |
| Aspartic acid | Asp               | D               |
| Cysteine      | Cys               | C               |
| Glutamine     | Gln               | Q               |
| Glutamic acid | Glu               | E               |

| Amino acid    | Three letter code | One letter code |
|---------------|-------------------|-----------------|
| Glycine       | Gly               | G               |
| Histidine     | His               | H               |
| Isoleucine    | Ile               | I               |
| Leucine       | Leu               | L               |
| Lysine        | Lys               | K               |
| Methionine    | Met               | M               |
| Phenylalanine | Phe               | F               |
| Proline       | Pro               | P               |
| Serine        | Ser               | S               |
| Threonine     | Thr               | T               |
| Tryptophan    | Trp               | W               |
| Tyrosine      | Tyr               | Y               |
| Valine        | Val               | V               |

**[0051]** As used herein, the term “antibody” or “immunoglobulin” as used herein refers to a glycoprotein comprising at least two heavy chains (HCs) and two light chains (LCs) interconnected by disulfide bonds. Each HC is comprised of a heavy chain variable region or domain (VH) and a heavy chain constant region or domain. Each light chain is comprised of an LC variable region or domain (VL) and a LC constant domain. In certain naturally occurring IgG, IgD and IgA antibodies, the heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. In general, the basic antibody structural unit for antibodies is a Y-shaped tetramer comprising two HC/LC pairs (2H). Each tetramer includes two identical pairs of polypeptide chains, each pair having one LC (about 25 kDa) and HC chain (about 50-70 kDa) (H+L). Each HC:LC pair comprises one VH: one VL pair. The one VH:one VL pair may be referred to by the term “Fab”. Thus, each antibody tetramer comprises two Fabs, one per each arm of the Y-shaped antibody. An antibody provided herein may include post-translational modifications thereof, e.g., C-terminal Lysine clipping in the heavy chain, conversion of glutamine or glutamic acid to pyroglutamate, which may occur when recombinantly expressed in host cells (e.g., CHO cells), or during purification/storage.

**[0052]** The LC constant domain is comprised of one domain, CL. The human VH includes seven family members: VH1, VH2, VH3, VH4, VH5, VH6, and VH7; and the human VL includes 16 family members: V<sub>K</sub>1, V<sub>K</sub>2, V<sub>K</sub>3, V<sub>K</sub>4, V<sub>K</sub>5, V<sub>K</sub>6, V<sub>λ</sub>1, V<sub>λ</sub>2, V<sub>λ</sub>3, V<sub>λ</sub>4, V<sub>λ</sub>5, V<sub>λ</sub>6, V<sub>λ</sub>7, V<sub>λ</sub>8, V<sub>λ</sub>9, and V<sub>λ</sub>10. Each of these family members can be further divided into particular subtypes. The VH and VL can be further subdivided into regions of hypervariability, termed complementarity determining region (CDR) areas, interspersed with regions that are more

conserved, termed framework regions (FR). Each VH and VL is composed of three CDR regions and four FR regions, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. Numbering of the amino acids in a VH may be determined using the Kabat numbering scheme. See Béranger, et al., Ed. Ginetoux, Correspondence between the IMGT unique numbering for C-DOMAIN, the IMGT exon numbering, the Eu and Kabat numberings: Human IGHG, Created: 17/05/2001, Version: 08/06/2016, which is accessible at [www.imgt.org/IMGTScientificChart/Numbering/Hu\\_IGHGnber.html](http://www.imgt.org/IMGTScientificChart/Numbering/Hu_IGHGnber.html)).

**[0053]** The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system. Typically, the numbering of the amino acids in the heavy chain constant domain begins with number 118, which is in accordance with the Eu numbering scheme. The Eu numbering scheme is based upon the amino acid sequence of human IgG1 (Eu), which has a constant domain that begins at amino acid position 118 of the amino acid sequence of the IgG1 described in Edelman et al., Proc. Natl. Acad. Sci. USA. 63: 78-85 (1969), and is shown for the IgG1, IgG2, IgG3, and IgG4 constant domains in Béranger et al., op. cit.

**[0054]** The variable regions of the heavy and light chains contain a binding domain comprising the CDRs that interacts with an antigen. A number of methods are available in the art for defining CDR sequences of antibody variable domains (see Dondelinger et al., Frontiers in Immunol. 9: Article 2278 (2018)). The common numbering schemes include the following.

**[0055]** Kabat numbering scheme is based on sequence variability and is the most commonly used (See Kabat et al. Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991) (defining the CDR regions of an antibody by sequence); Chothia numbering scheme is based on the location of the structural loop region (See Chothia & Lesk, J. Mol. Biol. 196: 901-917 (1987); Al-Lazikani et al., J. Mol. Biol. 273: 927-948 (1997)); AbM numbering scheme is a compromise between the two used by Oxford Molecular's AbM antibody modelling software (see Karu et al., ILAR Journal 37: 132–141 (1995); Contact numbering scheme is based on an analysis of the available complex crystal structures (See [www.bioinf.org.uk](http://www.bioinf.org.uk): Prof. Andrew C.R. Martin's Group; Abhinandan & Martin, Mol. Immunol. 45:3832–3839 (2008)).

**[0056]** IMGT (ImMunoGeneTics) numbering scheme is a standardized numbering system for all the protein sequences of the immunoglobulin superfamily, including variable domains from antibody light and heavy chains as well as T cell receptor chains from different species and

counts residues continuously from 1 to 128 based on the germ-line V sequence alignment (see Giudicelli et al., Nucleic Acids Res. 25:206–11 (1997); Lefranc, Immunol Today 18:509(1997); Lefranc et al., Dev Comp Immunol. 27:55–77 (2003)).

**[0057]** The following general rules disclosed in [www.bioinf.org.uk](http://www.bioinf.org.uk): Prof. Andrew C.R. Martin's Group and reproduced in **Table 1** below may be used to define the CDRs in an antibody sequence that includes those amino acids that specifically interact with the amino acids comprising the epitope in the antigen to which the antibody binds. There are rare examples where these generally constant features do not occur; however, the Cys residues are the most conserved feature.

**Table 1. General rules that may be used to define the CDRs in an antibody sequence**

| Loop | Kabat                                       | AbM           | Chothia <sup>1</sup> | Contact <sup>2</sup> | IMGT      |
|------|---------------------------------------------|---------------|----------------------|----------------------|-----------|
| L1   | L24--L34                                    | L24--L34      | L24--L34             | L30--L36             | L27--L32  |
| L2   | L50--L56                                    | L50--L56      | L50--L56             | L46--L55             | L50--L52  |
| L3   | L89--L97                                    | L89--L97      | L89--L97             | L89--L96             | L89--L97  |
| H1   | H31--H35B<br>(Kabat Numbering) <sup>3</sup> | H26--<br>H35B | H26--H32..34         | H30--H35B            | H26--H35B |
| H1   | H31--H35<br>(Chothia Numbering)             | H26--H35      | H26--H32             | H30--H35             | H26--H33  |
| H2   | H50--H65                                    | H50--H58      | H52--H56             | H47--H58             | H51--H56  |
| H3   | H95--H102                                   | H95--<br>H102 | H95--H102            | H93--H101            | H93--H102 |

<sup>1</sup>Some of these numbering schemes (particularly for Chothia loops) vary depending on the individual publication examined.

<sup>2</sup>Any of the numbering schemes can be used for these CDR definitions, except the Contact numbering scheme uses the Chothia or Martin (Enhanced Chothia) definition.

<sup>3</sup>The end of the Chothia CDR-H1 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 H35A nor H35B is present, the loop ends at H32

If only H35A is present, the loop ends at H33

If both H35A and H35B are present, the loop ends at H34

**[0058]** The entire nucleotide sequence of the heavy chain and light chain variable regions are commonly numbered according to Kabat while the three CDRs within the variable region may be defined according to any one of the aforementioned numbering schemes.

**[0059]** In general, the state of the art recognizes that in many cases, the CDR3 region of the heavy chain is the primary determinant of antibody specificity, and examples of specific antibody generation based on CDR3 of the heavy chain alone are known in the art (e.g., Beiboer et al., J. Mol. Biol. 296: 833-849 (2000); Klimka et al., British J. Cancer 83: 252-260 (2000); Rader et al., Proc. Natl. Acad. Sci. USA 95: 8910-8915 (1998); Xu et al., Immunity 13: 37-45 (2000).

**[0060]** As used herein, the term “Fc domain”, or “Fc” as used herein is the crystallizable fragment domain or region obtained from an antibody that comprises the CH2 and CH3 domains of an antibody. In an antibody, the two Fc domains are held together by two or more disulfide bonds and by hydrophobic interactions of the CH3 domains. The Fc domain may be obtained by digesting an antibody with the protease papain. Typically, amino acids in the Fc domain are numbered according to the Eu numbering convention (See Edelman et al., Biochem. 63: 78-85 (1969)).

**[0061]** As used herein, the term “antigen” as used herein refers to any foreign substance which induces an immune response in the body.

**[0062]** As used herein, the term “antigen binding fragment” refers to a polypeptide or polypeptides comprising a fragment of a full-length antibody, which retains the ability to specifically bind to the antigen bound by the full length antibody, and/or to compete with the full length antibody for specifically binding to the antigen. Examples of antigen binding fragments include but are not limited to Fab fragment, Fab' fragment, F(ab')<sub>2</sub> fragment, Fv region, and scFv.

**[0063]** As used herein, the term “Fab fragment” refers to an antigen binder comprising one antibody light chain and the CH1 and VH of one antibody heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule. A “Fab fragment” can be the product of papain cleavage of an antibody.

**[0064]** As used herein, the term “Fab' fragment” refers to an antigen binder comprising one antibody light chain and a portion or fragment of one antibody heavy chain that contains the VH and the CH1 domain up to a region between the CH1 and CH2 domains, such that an interchain disulfide bond can be formed between the two heavy chains of two Fab' fragments to form a F(ab')<sub>2</sub> molecule.

**[0065]** As used herein, the term “F(ab')<sub>2</sub> fragment” refers to an antigen binder comprising two antibody light chains and two heavy chains containing the VH and the CH1 domain up to a region between the CH1 and CH2 domains, such that an interchain disulfide bond is formed between the two heavy chains. An F(ab')<sub>2</sub> fragment thus is composed of two Fab' fragments that are held together by a disulfide bond between the two heavy chains. An “F(ab')<sub>2</sub> fragment” can be the product of pepsin cleavage of an antibody.

**[0066]** As used herein, the term “Fv region” refers to an antigen binder comprising the variable regions from both the heavy and light chains of an antibody but lacks the constant regions.

**[0067]** As used herein, the term “scFv” or “single-chain variable fragment” refers to a fusion protein comprising a VH and VL fused or linked together by a short linker peptide of ten to about 25 amino acids. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. This protein retains the specificity of the original immunoglobulin, despite removal of the constant regions and the introduction of the linker.

**[0068]** The antibody fragments can be obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies. Antigen-binding fragments can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins.

**[0069]** As used herein, the term “chimeric antigen receptor” (CAR) refers to a recombinant polypeptide comprising at least an extracellular domain that binds specifically to an antigen or a target, a transmembrane domain and an intracellular signaling domain. Engagement of the extracellular domain of the CAR with the target antigen on the surface of a target cell results in clustering of the CAR and delivers an activation stimulus to the CAR-containing cell. CARs redirect the specificity of immune effector cells and trigger proliferation, cytokine production, phagocytosis and/or production of molecules that can mediate cell death of the target antigen-expressing cell in a major histocompatibility (MHC)-independent manner.

**[0070]** As used herein, the term “extracellular antigen binding domain,” “extracellular domain,” or “extracellular ligand binding domain” when used in reference to a CAR refers to the part of a CAR that is located outside of the cell membrane and is capable of binding to an antigen, target or ligand.

**[0071]** As used herein, the term “hinge region” or “hinge domain” when used in reference to a CAR refers to the part of a CAR that connects two adjacent domains of the CAR protein, e.g., the extracellular domain and the transmembrane domain.

**[0072]** As used herein, the term “transmembrane domain” refers to the portion of a CAR that extends across the cell membrane and anchors the CAR to cell membrane.

**[0073]** As used herein, the term “intracellular T cell receptor-activating signaling domain”, “cytoplasmic signaling domain,” or “intracellular signaling domain” refers to the part of a CAR that is located inside of the cell membrane and is capable of transducing an effector signal.

**[0074]** As used herein, the term “immune cell” or “immune effector cell” refers to a cell that is involved in an immune response, e.g., in the promotion of an immune effector response. Examples of immune cells include T cells, B cells, natural killer (NK) cells, mast cells, and myeloid-derived phagocytes. According to particular embodiments, the engineered immune cells are T cells, and are referred to as CAR-T cells because they are engineered to express CARs of the disclosure. According to particular embodiments, the engineered immune cells are NK cells, and are referred to as CAR-NK cells because they are engineered to express CARs of the disclosure.

**[0075]** As used herein, the term “engineered cell” refers to a cell that has been genetically modified by the addition of extra genetic material in the form of DNA or RNA to the total genetic material of the cell. According to embodiments herein, the engineered cells have been genetically modified to express a human MSLN-targeting CAR according to the disclosure.

**[0076]** As used herein, the term “engineered immune cell” refers to an immune cell, also referred to as an immune effector cell, that has been genetically modified by the addition of extra genetic material in the form of DNA or RNA to the total genetic material of the cell. According to embodiments herein, the engineered immune cells have been genetically modified to express a human MSLN-targeting CAR according to the disclosure.

**[0077]** As used herein, the term “stimulatory molecule” refers to a molecule expressed by a T cell that provides the primary cytoplasmic signaling sequence(s) that regulate primary activation of the T cell receptor (TCR) complex in a stimulatory way for at least some aspect of the T cell signaling pathway. Stimulatory molecules comprise two distinct classes of cytoplasmic signaling sequence, those that initiate antigen-dependent primary activation (referred to as “primary signaling domains”), and those that act in an antigen-independent manner to provide a secondary of co-stimulatory signal (referred to as “co-stimulatory signaling domains”).

As used herein, the term “immune cell” or “immune effector cell” refers to a cell that is involved in an immune response, e.g., in the promotion of an immune effector response. Examples of

immune cells include T cells, B cells, natural killer (NK) cells, mast cells, and myeloid-derived phagocytes. According to particular embodiments, the engineered immune cells are T cells, and are referred to as *CAR-T* cells because they are engineered to express CARs comprising an scFv disclosed herein that targets human MSLN; or the engineered immune cells are NK cells, and are referred to as *CAR-NK* cells because they are engineered to express CARs comprising an scFv disclosed herein that targets human MSLN.

**[0078]** As used herein, the term “isolated” antibodies or antigen-binding fragments thereof are at least partially free of other biological molecules from the cells or cell cultures in which they are produced. Such biological molecules include nucleic acids, proteins, lipids, carbohydrates, or other material such as cellular debris and growth medium. An isolated antibody or antigen-binding fragment may further be at least partially free of expression system components such as biological molecules from a host cell or of the growth medium thereof. Generally, the term “isolated” is not intended to refer to a complete absence of such biological molecules or to an absence of water, buffers, or salts or to components of a pharmaceutical formulation that includes the antibodies or fragments.

**[0079]** As used herein, the term “monoclonal antibody” refers to a population of substantially homogeneous antibodies, i.e., the antibody molecules comprising the population are identical in amino acid sequence except for possible naturally occurring mutations that may be present in minor amounts. In contrast, conventional (polyclonal) antibody preparations typically include a multitude of different antibodies having different amino acid sequences in their variable domains that are often specific for different epitopes. The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present disclosure may be made by the hybridoma method first described by Kohler et al., *Nature* 256: 495 (1975) or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The “monoclonal antibodies” may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature* 352: 624-628 (1991), and Marks et al., *J. Mol. Biol.* 222: 581-597 (1991), for example. See also Presta, J. *Allergy Clin. Immunol.* 116: 731 (2005).

**[0080]** As used herein, the term “gene” is used broadly to refer to any segment of nucleic acid associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. For example, “gene” refers to a nucleic acid fragment that expresses mRNA, functional RNA, or specific protein, including regulatory

sequences. “Genes” also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins. “Genes” can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters. Genes include both naturally occurring nucleotide sequences encoding a molecule of interest and synthetically derived nucleotide sequences encoding a molecule of interest, for example, complementary DNA (cDNA) obtained from a messenger RNA (mRNA) nucleotide sequence.

**[0081]** As used herein, the term “polynucleotides” discussed herein form part of the present disclosure. A “polynucleotide”, “polynucleic acid”, “nucleic acid “ or “nucleic acid molecule”, used interchangeably, include DNA and RNA, single- or double-stranded. Polynucleotides e.g., encoding an immunoglobulin chain or component of the antibody display system of the present disclosure, may, in an embodiment of the disclosure, be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'-non-coding regions, and the like.

**[0082]** Polynucleotides e.g., encoding an immunoglobulin chain or component of the antibody display system of the present disclosure, may be operably associated with a promoter. A “promoter” or “promoter sequence” is, in an embodiment of the disclosure, a DNA regulatory region capable of binding an RNA polymerase in a cell (e.g., directly or through other promoter-bound proteins or substances) and initiating transcription of a coding sequence. A promoter sequence is, in general, bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at any level. Within the promoter sequence may be found a transcription initiation site (conveniently defined, for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. The promoter may be operably associated with other expression control sequences, including enhancer and repressor sequences or with a nucleic acid of the disclosure. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and 5,168,062), the SV40 early promoter region (Benoist, et al., *Nature* 290: 304-310 (1981)), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., *Cell* 22: 787-797 (1980)), the herpes thymidine kinase promoter (Wagner et al., *Proc. Natl. Acad. Sci. USA* 78: 1441-1445 (1981)), the regulatory sequences of the metallothionein gene (Brinster et al., *Nature* 296: 39-42 (1982)); prokaryotic expression

vectors such as the  $\beta$ -lactamase promoter (Villa-Komaroff et al., Proc. Natl. Acad. Sci. USA 75: 3727-3731 (1978)), or the tac promoter (DeBoer et al., Proc. Natl. Acad. Sci. USA 80: 21-25 (1983)); see also "Useful proteins from recombinant bacteria" in Scientific American 242: 74-94 (1980); and promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter or the alkaline phosphatase promoter.

**[0083]** As used herein, the terms "vector", "cloning vector" and "expression vector" include a vehicle (e.g., a plasmid) by which a DNA or RNA sequence can be introduced into a host cell so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence. Polynucleotides encoding an immunoglobulin chain or component of the antibody display system of the present disclosure may, in an embodiment of the disclosure, be in a vector.

**[0084]** As used herein, the terms "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that not all progeny will have precisely identical DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

**[0085]** As used herein, the term "control sequences" or "regulatory sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for expression in eukaryotes, for example, include a promoter, operator or enhancer sequences, transcription termination sequences, and polyadenylation sequences for expression of a messenger RNA encoding a protein and a ribosome binding site for facilitating translation of the messenger RNA.

**[0086]** As used herein, a nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence, e.g., a regulatory sequence. For example, DNA for a pre-sequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient

restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

**[0087]** As used herein, the term “encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a “nucleotide sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

**[0088]** As used herein, the term “expression” as used herein is defined as the transcription and/or translation of a particular nucleotide sequence.

**[0089]** As used herein, the term “treat” or “treating” means to administer a therapeutic agent, such as a composition containing any of the human MSLN binders of the present disclosure, topically, subcutaneously, intramuscular, intradermally, or systemically to an individual in need. The amount of a therapeutic agent that is effective to treat cancer or proliferative disease in the individual may vary according to factors such as the injury or disease state, age, and/or weight of the individual, and the ability of the therapeutic agent to elicit a desired response in the individual. Whether the therapeutic objective has been achieved can be assessed by the individual and/or any clinical measurement typically used by physicians or other skilled healthcare providers to assess the severity or progression status of the treatment. Thus, the terms denote that a beneficial result has been or will be conferred on a human or animal individual in need.

**[0090]** As used herein, the term “treatment,” as it applies to a human or veterinary individual, refers to therapeutic treatment, as well as diagnostic applications. “Treatment” as it applies to a human or veterinary individual, encompasses contact of the antibodies or antigen binding fragments of the present disclosure to a human or animal subject.

**[0091]** An “effective amount” is an amount sufficient to effect beneficial or desired results. For example, a therapeutic amount is one that achieves the desired therapeutic effect. This

amount can be the same or different from a prophylactically effective amount, which is an amount necessary to prevent onset of disease or disease symptoms. An effective amount can be administered in one or more administrations, applications or dosages. A therapeutically effective amount of a therapeutic compound (i.e., an effective dosage) depends on the therapeutic compounds selected. The compositions can be administered from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the therapeutic compounds described herein can include a single treatment or a series of treatments. As used herein, the term “therapeutically effective amount” refers to a quantity of a specific substance sufficient to achieve a desired effect in an individual being treated. For instance, this may be the amount necessary to inhibit or reduce the severity of a disease or disorder in an individual.

**[0092]** As used herein, the term “combination therapy” refers to treatment of a human or animal individual comprising administering a first therapeutic agent and a second therapeutic agent consecutively or concurrently to the individual. In general, the first and second therapeutic agents are administered to the individual separately and not as a mixture; however, there may be embodiments where the first and second therapeutic agents are mixed prior to administration.

**[0093]** The terms “host cell,” “host cell line,” and “host cell culture” are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include “engineered cells,” “transformants,” and “transformed cells,” which include the primary engineered (e.g., transformed) cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein. As appropriate, the host cells can be stably or transiently transfected with a polynucleotide encoding a fusion protein, as described herein.

**[0094]** As used herein, “sequence identity” or “identity” refers to the degree to which the amino acid residues of two polypeptides or nucleic acids are the same (*i.e.*, identical) at the equivalent positions when a query sequence is optimally aligned over the length of a reference sequence, introducing gaps as necessary if the reference and query sequence do not have the same length to achieve a maximum percent identity (called a “global alignment”). For this disclosure, a reference sequence is always an amino acid or nucleic acid sequence specifically

disclosed herein, and a query sequence is any other sequence aligned to the reference sequence. Sequence identity is expressed as a percent sequence identity (% sequence identity or % identity). The global alignment and calculation of the percent identity is performed over a contiguous block of residue positions for the entire reference sequence. If the query sequence is longer than the reference sequence, then the percent identity is determined only using the residue positions of the reference sequence. Any residues of the query sequence with positions beyond the first and last positions of the reference sequence after an initial global alignment are removed and the reference and query sequences are re-aligned to determine percent identity. A query sequence has 100% identity to a reference sequence when the residues of both sequences are identical at the equivalent positions over the length of the reference sequence. Similarly, a query sequence has 50% identity to a reference sequence when 50% of the residues of the two amino acid sequences are identical at the equivalent positions over the length of the reference sequence.

**[0095]** A global alignment can be created using the global alignment tool “Needle” from the online European Molecular Biology Open Software Suite (EMBOSS) ([www.ebi.ac.uk/Tools/psa/emboss\\_needle/](http://www.ebi.ac.uk/Tools/psa/emboss_needle/)) or the global alignment tool “BLAST® » Global Alignment” from the National Center for Biotechnology Information (NCBI) ([blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE\\_TYPE=BlastSearch&PROG\\_DEFAULTS=on&BLAST\\_INIT=GlobalAln&BLAST\\_SPEC=GlobalAln&BLAST\\_PROGRAMS=blastn](http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&PROG_DEFAULTS=on&BLAST_INIT=GlobalAln&BLAST_SPEC=GlobalAln&BLAST_PROGRAMS=blastn)). Both of these global alignment tools incorporate the Needleman–Wunsch algorithm (Needleman, S.B. & Wunsch, C.D. [“A general method applicable to the search for similarities in the amino acid sequences of two proteins.” *J. Mol. Biol.* 48:443-453 (1970)]. A global alignment of nucleotide sequences using BLAST Global Alignment in this disclosure uses the following default parameters: match score = 2; mismatch score = -3; Gap Cost Existence score = 5; Gap Cost Extension Score = 2. A global alignment of protein sequences in this disclosure using BLAST Global Alignment uses the following default parameters: Gap Cost Existence = 11; Gap Cost Extension = 1. A global alignment of nucleotide sequences using EMBOSS Needle in this disclosure uses the following default parameters: Output Format = pair; Matrix = DNAMfull; Gap Open = 10; Gap Extend = 0.5; End Gap Penalty = false; End Gap Open = 10; End Gap Extend = 0.5. A global alignment of protein sequences using EMBOSS Needle in this disclosure uses the following default parameters: Output Format = pair; Matrix = BLOSUM62; Gap Open = 10; Gap Extend = 0.5; End Gap Penalty = false; End Gap Open = 10; End Gap Extend = 0.5.

**[0096]** As used herein the term “conservative amino acid substitution” refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid

of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. (1987) *Molecular Biology of the Gene*, The Benjamin/Cummings Pub. Co., p. 224 (4th Ed.)). In addition, substitutions of structurally or functionally similar amino acids are less likely to disrupt biological activity. Exemplary conservative substitutions are set forth in Table 2 below.

**Table 2: Exemplary Conservative Amino Acid Substitutions**

| Original residue | Conservative substitution |
|------------------|---------------------------|
| Ala (A)          | Gly; Ser                  |
| Arg (R)          | Lys; His                  |
| Asn (N)          | Gln; His                  |
| Asp (D)          | Glu; Asn                  |
| Cys (C)          | Ser; Ala                  |
| Gln (Q)          | Asn                       |
| Glu (E)          | Asp; Gln                  |
| Gly (G)          | Ala                       |
| His (H)          | Asn; Gln                  |
| Ile (I)          | Leu; Val                  |
| Leu (L)          | Ile; Val                  |
| Lys (K)          | Arg; His                  |
| Met (M)          | Leu; Ile; Tyr             |
| Phe (F)          | Tyr; Met; Leu             |
| Pro (P)          | Ala                       |
| Ser (S)          | Thr                       |
| Thr (T)          | Ser                       |
| Trp (W)          | Tyr; Phe                  |
| Tyr (Y)          | Trp; Phe                  |
| Val (V)          | Ile; Leu                  |

Function-conservative variants of the antibodies of the invention are also contemplated by the present invention. “Function-conservative variants,” as used herein, refers to antibodies or fragments in which one or more amino acid residues have been changed without altering a desired property, such as an antigen affinity and/or specificity. Such variants include, but are not limited to, replacement of an amino acid with one having similar properties, such as the conservative amino acid substitutions of Table 1.

## 4.2 Chimeric Antigen Receptors (CARs)

[0097] The present disclosure provides a chimeric antigen receptor (CAR) comprising a polypeptide comprising (a) an extracellular antigen binding domain comprising a human mesothelin (MSLN) binder provided herein; (b) a transmembrane domain; and (c) an intracellular signaling domain.

### 4.2.1 Extracellular antigen binding domain

[0098] In one aspect, the extracellular antigen binding domain comprises a human MSLN binder.

#### 4.2.1.1 Human MSLN Binders

[0099] The human MSLN binders of the present disclosure are chimeric or fully human antibodies or antigen binding fragments thereof that specifically bind human MSLN. The human MSLN binders comprise a heavy chain variable domain (VH) and a light chain variable domain (VL).

[0100] These human MSLN binders comprise six complementarity determining regions (CDRs) comprising a particular combination of three CDRs from a VH and three CDRs from the VL that pairs with the VH. The CDR sequences may be defined according to any numbering scheme useful for defining CDR sequences including but not limited to the Kabat, Chothia, AbM, ImMunoGeneTics (IMGT), or Contact numbering scheme. Guidance for defining the CDR sequences may be found in the general rules disclosed in [www.bioinf.org.uk](http://www.bioinf.org.uk): Prof. Andrew C.R. Martin's Group and reproduced in **Table 1**.

[0101] In a particular embodiment, the CDRs are defined by Kabat or IMGT. The CDR amino acid sequences shown in **Tables 2-4** are set forth according to the Kabat numbering scheme for identifying CDR amino acid sequences.

**Table 3. Amino acid sequences of human MSLN binders SV017.47D7.1C2 (VH and VL CDRs as defined by Kabat)**

|    | CDR1                 | CDR2                            | CDR3                   |
|----|----------------------|---------------------------------|------------------------|
| VH | SYWMH (SEQ ID NO: 5) | RNSDGTSTVYADSVKG (SEQ ID NO: 6) | GRGIMTY (SEQ ID NO: 7) |
|    | CDR1                 | CDR2                            | CDR3                   |

|                                                                                                                                                                      |                               |                           |                           |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|---------------------------|---------------------------|
| VL                                                                                                                                                                   | RASQDIGSWLT<br>(SEQ ID NO: 8) | AASSLQS<br>(SEQ ID NO: 9) | QQYKSYPLT (SEQ ID NO: 10) |
| <sup>±</sup> human MSLN binders SV017.13B12.1E1, SV017.50G3.1B1, and SV017.51F4.1A1 comprise CDRs having the same amino acid sequences as the CDRs of SV017.47D7.1C2 |                               |                           |                           |

**Table 4. Amino acid sequences of human MSLN binder SV018.20B12.1D1 (VH and VL CDRs as defined by Kabat)**

|    | CDR1                           | CDR2                                 | CDR3                         |
|----|--------------------------------|--------------------------------------|------------------------------|
| VH | SYYSWS<br>(SEQ ID NO: 13)      | YIYYSGSTNYPNPSLKS<br>(SEQ ID NO: 14) | TGYIPMDV<br>(SEQ ID NO: 15)  |
|    | CDR1                           | CDR2                                 | CDR3                         |
| VL | RASQSIGSNLH<br>(SEQ ID NO: 16) | YVSQSFS<br>(SEQ ID NO: 17)           | HQSRSLPYT<br>(SEQ ID NO: 18) |

**Table 5. Amino acid sequences of human MSLN binder SV018.45B6.1G1 (VH and VL CDRs as defined by Kabat)**

|    | CDR1                            | CDR2                                 | CDR3                         |
|----|---------------------------------|--------------------------------------|------------------------------|
| VH | TYGIS<br>(SEQ ID NO: 21)        | WITAYNRNTNYAQKLQG<br>(SEQ ID NO: 22) | VGDSDAFDI<br>(SEQ ID NO: 23) |
|    | CDR1                            | CDR2                                 | CDR3                         |
| VL | RTSQSVFNSFLS<br>(SEQ ID NO: 24) | GTSTRAT<br>(SEQ ID NO: 25)           | QQDYKLPLT<br>(SEQ ID NO: 26) |

**[0102]** A particular CDR amino acid sequence determined using any one of the schemes for identifying CDR amino acid sequences (*See Table 1*) have more or less amino acids than that of CDR amino acid sequences identified according to any other numbering scheme but the CDR amino acid sequences will overlap to some extent. Thus, the CDR amino acid sequences defined according to Kabat are not to be construed as limiting and any human MSLN binder in which the CDR amino acid sequences have been identified by another numbering scheme will fall within the scope of the human MSLN binders of the present disclosure provided the amino acid sequences for such human MSLN binders comprise the six CDR amino acid sequences as identified by Kabat. For all human MSLN binders disclosed herein unless indicated otherwise, the amino acids comprising the variable domains as a whole are numbered according to the Kabat numbering scheme independently of how the amino acids comprising the CDR are defined. The heavy chain constant domains are numbered according to the Eu numbering scheme.

**[0103]** In particular embodiments of the disclosure, the human MSLN binder comprises (a) a VH domain comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 5, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 6, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 7; and (b) a VL domain comprising

a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 8, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 10, wherein the CDR sequences are defined by the Kabat numbering scheme.

**[0104]** In particular embodiments of the disclosure, the human MSLN binder comprises (a) a VH domain comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 13, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 14, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 15; and (b) a VL domain comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 17, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 18, wherein the CDR sequences are defined by the Kabat numbering scheme.

**[0105]** In particular embodiments of the disclosure, the human MSLN binder comprises (a) a VH domain comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 22, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23; and (b) a VL domain comprising a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 24, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26, wherein the CDR sequences are defined by the Kabat numbering scheme.

**[0106]** In further embodiments, the mesothelin binder comprises a VH domain comprising the amino acid sequence set forth in SEQ ID NO: 3, SEQ ID NO: 27, SEQ ID NO: 28, or SEQ ID NO: 29 and a VL domain comprising the amino acid sequence set forth in SEQ ID NO: 4.

**[0107]** In further embodiments, the mesothelin binder comprises a VH domain comprising the amino acid sequence set forth in SEQ ID NO: 11 and a VL domain comprising the amino acid sequence set forth in SEQ ID NO: 12.

**[0108]** In further embodiments, the mesothelin binder comprises a VH domain comprising the amino acid sequence set forth in SEQ ID NO: 19 and a VL domain comprising the amino acid sequence set forth in SEQ ID NO: 20.

**[0109]** In particular embodiments, the VH and VL disclosed herein are expressed as an scFv in which the VL and VH domains are linked together by a peptide linker. The peptide linker joins the carboxyl terminus of one variable region domain to the amino terminus of the other variable domain without compromising the fidelity of the VH–VL pairing and antigen-binding sites. Thus, in the scFv, the C-terminus of a VL is linked by a peptide linker to the N-terminus of

a VH or the C-terminus of a VH is linked by a peptide linker to the N-terminus of a VL. Peptide linkers for linking the variable domains can vary from 10 to 25 amino acids in length and are typically, but not always, composed of hydrophilic amino acids such as glycine (G) and serine (S) having the structure G4S, for example, (G4S)<sub>n</sub>, wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 (SEQ ID NO: 95). Peptide linkers of shorter lengths (0–4 amino acids) have also been used; however, scFv bearing shorter linkers may form multimers. Generally, the (G4S)<sub>3</sub> (SEQ ID NO: 52) peptide comprising three repeating G4S units is used as an scFv peptide linker (See for example, Leath et al., *Int. J. Oncol.* 24:765–771 (2004); Holliger et al., *Proc. Natl. Acad. Sci. USA* 90:6444–6448 (1993); Iliades et al., *FEBS Lett.* 409:437–441 (1997)).

**[0110]** Exemplary scFvs include the structure VL-(G4S)<sub>n</sub>-VH or VH-(G4S)<sub>n</sub>-VL wherein (a) VL comprises the amino acid sequence set forth in SEQ ID NO: 4 and VH comprises the amino acid sequence set forth in SEQ ID NO: 3; (b) VL comprises the amino acid sequence set forth in SEQ ID NO: 4 and VH comprises the amino acid sequence set forth in SEQ ID NO: 27; (c) VL comprises the amino acid sequence set forth in SEQ ID NO: 4 and VH comprises the amino acid sequence set forth in SEQ ID NO: 28; (d) VL comprises the amino acid sequence set forth in SEQ ID NO: 4 and VH comprises the amino acid sequence set forth in SEQ ID NO: 29; (e) VL comprises the amino acid sequence set forth in SEQ ID NO: 12 and VH comprises the amino acid sequence set forth in SEQ ID NO: 11; or (f) VL comprises the amino acid sequence set forth in SEQ ID NO: 20 and VH comprises the amino acid sequence set forth in SEQ ID NO: 19, wherein n is 1, 2, 3, 4, or 5.

**[0111]** Exemplary scFvs comprise the structure VL-(G4S)<sub>n</sub>-VH or VH-(G4S)<sub>n</sub>-VL wherein (a) the VH domain comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 5, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 6, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 7; and (b) the VL domain comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 8, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 10, wherein the CDR sequences are defined by the Kabat numbering scheme.

**[0112]** Exemplary scFvs comprise the structure VL-(G4S)<sub>n</sub>-VH or VH-(G4S)<sub>n</sub>-VL wherein (a) the VH domain comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 13, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 14, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 15; and (b) the VL domain comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, a CDR2

comprising the amino acid sequence set forth in SEQ ID NO: 17, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 18, wherein the CDR sequences are defined by the Kabat numbering scheme.

**[0113]** Exemplary scFvs comprise the structure VL-(G4S)<sub>n</sub>-VH or VH-(G4S)<sub>n</sub>-VL wherein (a) the VH domain comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 21, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 22, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 23; and (b) a VL domain comprises a CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 24, a CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 25, and a CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 26, wherein the CDR sequences are defined by the Kabat numbering scheme.

**[0114]** Exemplary scFvs comprise the structure VL-(G4S)<sub>n</sub>-VH or VH-(G4S)<sub>n</sub>-VL wherein the VL comprises the amino acid sequence set forth in SEQ ID NO: 4 and the VH comprises the amino acid sequence set forth in SEQ ID NO: 3; wherein n is 1, 2, 3, 4, or 5.

**[0115]** Exemplary scFvs comprise the structure VL-(G4S)<sub>n</sub>-VH or VH-(G4S)<sub>n</sub>-VL wherein the VL comprises the amino acid sequence set forth in SEQ ID NO: 4 and the VH comprises the amino acid sequence set forth in SEQ ID NO: 27; wherein n is 1, 2, 3, 4, or 5.

**[0116]** Exemplary scFvs comprise the structure VL-(G4S)<sub>n</sub>-VH or VH-(G4S)<sub>n</sub>-VL wherein the VL comprises the amino acid sequence set forth in SEQ ID NO: 4 and the VH comprises the amino acid sequence set forth in SEQ ID NO: 28; wherein n is 1, 2, 3, 4, or 5.

**[0117]** Exemplary scFvs comprise the structure VL-(G4S)<sub>n</sub>-VH or VH-(G4S)<sub>n</sub>-VL wherein the VL comprises the amino acid sequence set forth in SEQ ID NO: 4 and the VH comprises the amino acid sequence set forth in SEQ ID NO: 29; wherein n is 1, 2, 3, 4, or 5.

**[0118]** Exemplary scFvs comprise the structure VL-(G4S)<sub>n</sub>-VH or VH-(G4S)<sub>n</sub>-VL wherein the VL comprises the amino acid sequence set forth in SEQ ID NO: 12 and the VH comprises the amino acid sequence set forth in SEQ ID NO: 11; wherein n is 1, 2, 3, 4, or 5.

**[0119]** Exemplary scFvs comprise the structure VL-(G4S)<sub>n</sub>-VH or VH-(G4S)<sub>n</sub>-VL wherein the VL comprises the amino acid sequence set forth in SEQ ID NO: 20 and the VH comprises the amino acid sequence set forth in SEQ ID NO: 19; wherein n is 1, 2, 3, 4, or 5.

**[0120]** In some embodiments, provided herein is a CAR comprising a polypeptide comprising: an extracellular antigen binding domain comprising a human MSLN binder; a transmembrane domain; and an intracellular signaling domain; wherein the human MSLN binder comprises a VH comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising

the amino acid sequence of SEQ ID NO: 3, 27, 28, or 29 and a VL comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 4. In some embodiments, the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 5, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 6, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 7; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 8, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 9, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 10.

**[0121]** In some embodiments, the human MSLN binder provided in the present CAR comprises a VH comprising the amino acid sequence of SEQ ID NO: 3 and a VL comprising the amino acid sequence of SEQ ID NO: 4. In some embodiments, the human MSLN binder provided in the present CAR comprises a VH comprising the amino acid sequence of SEQ ID NO: 27 and a VL comprising the amino acid sequence of SEQ ID NO: 4. In some embodiments, the human MSLN binder provided in the present CAR comprises a VH comprising the amino acid sequence of SEQ ID NO: 28 and a VL comprising the amino acid sequence of SEQ ID NO: 4. In some embodiments, the human MSLN binder provided in the present CAR comprises a VH comprising the amino acid sequence of SEQ ID NO: 29 and a VL comprising the amino acid sequence of SEQ ID NO: 4. In some embodiments, the human MSLN binder in the present CAR comprises a VH comprising an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 27, SEQ ID NO: 28, or SEQ ID NO: 29; and a VL comprising an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 4, and, wherein the VH comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 3, 27, 28, or 29 and the VL comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 4; or the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 5, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 6, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 7; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 8, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 9, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 10.

**[0122]** In some embodiments, provided herein is a CAR comprising a polypeptide comprising: an extracellular antigen binding domain comprising a human MSLN binder; a transmembrane domain; and an intracellular signaling domain; wherein the human MSLN binder comprises a VH comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 11 and a VL comprising a CDR 1, a CDR 2 and a CDR

3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 12. In some embodiments, the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 13, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 14, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 15; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 16, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 17, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 18. In some embodiments, the human MSLN binder in the present CAR comprises a VH comprising the amino acid sequence of SEQ ID NO: 11 and a VL comprising the amino acid sequence of SEQ ID NO: 12. In some embodiments, the human MSLN binder in the present CAR comprises a VH comprising an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 11; and a VL comprising an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 12, and wherein the VH comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 11 and the VL comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 12; or the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 13, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 14, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 15; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 16, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 17, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 18.

**[0123]** In some embodiments, provided herein is a CAR comprising a polypeptide comprising: an extracellular antigen binding domain comprising a human MSLN binder; a transmembrane domain; and an intracellular signaling domain; wherein the human MSLN binder comprises a VH comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 19 and a VL comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 20. In some embodiments, the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 21, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 22, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 23; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 24, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 25, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 26. In some embodiments, the human MSLN binder in the present CAR comprises a VH comprising the amino acid sequence of SEQ ID NO: 19 and a VL comprising the amino acid sequence of SEQ ID NO: 20. In some embodiments, the human MSLN binder in the present CAR comprises a VH

comprising an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 19; and a VL comprising an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 20, and wherein the VH comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 19 and the VL comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 20; or the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 21, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 22, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 23; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 24, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 25, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 26.

**[0124]** In some embodiments, provided herein is a CAR comprising a polypeptide comprising: an extracellular antigen binding domain comprising a human MSLN binder; a transmembrane domain; and an intracellular signaling domain; wherein the human MSLN binder comprises a VH comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 30 and a VL comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 31. In some embodiments, the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 32, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 33, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 34; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 35, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 36, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 37. In some embodiments, the human MSLN binder in the present CAR comprises a VH comprising the amino acid sequence of SEQ ID NO: 30 and a VL comprising the amino acid sequence of SEQ ID NO: 31. In some embodiments, the human MSLN binder in the present CAR comprises a VH comprising an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 30; and a VL comprising an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 31, and wherein the VH comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 30 and the VL comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 31; or the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 32, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 33, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 34; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 35, a CDR 2 comprising the amino acid

sequence of SEQ ID NO: 36, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 37.

**[0125]** In some embodiments, provided herein is a CAR comprising: (a) an extracellular domain comprising an scFv that specifically binds to human MSLN; (b) a transmembrane domain; and (c) an intracellular signaling domain. In some embodiments, the scFv comprises a VH and a VL. In some embodiments, the VL region is N-terminal to the VH region. In other embodiments, the VL region is C-terminal to the VH region. In some embodiments, the VL region is joined to the VH region via a flexible linker. In some specific embodiments, the flexible linker comprises the amino acid sequence of SEQ ID NO: 52. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 38 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 38. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 39 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 39. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 39 wherein the 1st amino acid residue SEQ ID NO: 39 is pyroglutamate. In some embodiments, the scFv comprises an amino acid sequence having the 2nd amino acid residue to the 241th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 241th amino acid residue of SEQ ID NO: 39 and the 1st amino acid residue of the amino acid sequence being pyroglutamate. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 40 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 40. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 40 wherein the 1st amino acid residue SEQ ID NO: 40 is pyroglutamate. In some embodiments, the scFv comprises an amino acid sequence having the 2nd amino acid residue to the 238th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 238th amino acid residue of SEQ ID NO: 40 and the 1st amino acid residue of the amino acid sequence being pyroglutamate. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 41 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 41. In some embodiments, the scFv comprises an amino acid sequence of SEQ ID NO: 41 wherein the 1st amino acid residue of SEQ ID NO: 41 is pyroglutamate. In some embodiments, the scFv comprises an amino acid sequence having the 2nd amino acid residue to the 238th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 238th amino acid residue of SEQ ID NO: 41 and the 1st amino

acid residue of the amino acid sequence being pyroglutamate. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 42 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 42. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 43 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 43. In some embodiments, the scFv comprises the amino acid sequence of SEQ ID NO: 44 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 44.

#### 4.2.2 *Transmembrane Domain*

**[0126]** The CARs of the present disclosure comprise a transmembrane domain that can be directly or indirectly fused to the extracellular antigen binding domain. The transmembrane domain may be derived either from a natural or from a synthetic source. As used herein, a “transmembrane domain” refers to any protein structure that is thermodynamically stable in a cell membrane, preferably an eukaryotic cell membrane. Transmembrane domains compatible for use in the CARs described herein may be obtained from a naturally occurring protein. Alternatively, it can be a synthetic, non-naturally occurring protein segment, e.g., a hydrophobic protein segment that is thermodynamically stable in a cell membrane.

**[0127]** Transmembrane domains are classified based on the three dimensional structure of the transmembrane domain. For example, transmembrane domains may form an alpha helix, a complex of more than one alpha helix, a beta-barrel, or any other stable structure capable of spanning the phospholipid bilayer of a cell. Furthermore, transmembrane domains may also or alternatively be classified based on the transmembrane domain topology, including the number of passes that the transmembrane domain makes across the membrane and the orientation of the protein. For example, single-pass membrane proteins cross the cell membrane once, and multi-pass membrane proteins cross the cell membrane at least twice (e.g., 2, 3, 4, 5, 6, 7 or more times). Membrane proteins may be defined as Type I, Type II or Type III depending upon the topology of their termini and membrane-passing segment(s) relative to the inside and outside of the cell. Type I membrane proteins have a single membrane-spanning region and are oriented such that the N-terminus of the protein is present on the extracellular side of the lipid bilayer of the cell and the C-terminus of the protein is present on the cytoplasmic side. Type II membrane proteins also have a single membrane-spanning region but are oriented such that the C-terminus of the protein is present on the extracellular side of the lipid bilayer of the cell and the N-terminus of the protein is present on the cytoplasmic side. Type III membrane proteins have

multiple membrane- spanning segments and may be further sub-classified based on the number of transmembrane segments and the location of N- and C-termini.

**[0128]** In some embodiments, the transmembrane domain of the CAR described herein is derived from a Type I single-pass membrane protein. In some embodiments, transmembrane domains from multi-pass membrane proteins may also be compatible for use in the CARs described herein. Multi-pass membrane proteins may comprise a complex (at least 2, 3, 4, 5, 6, 7 or more) alpha helices or a beta sheet structure. In some embodiments, the N-terminus and the C-terminus of a multi-pass membrane protein are present on opposing sides of the lipid bilayer, e.g., the N-terminus of the protein is present on the cytoplasmic side of the lipid bilayer and the C-terminus of the protein is present on the extracellular side.

**[0129]** In some embodiments, the transmembrane domain of the CAR comprises a transmembrane domain derived from the transmembrane domain of an alpha, beta or zeta chain of a T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, KIRDS2, OX40, CD2, CD27, LFA-1 (CD11a, CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRFl), CD160, CD19, IL-2R beta, IL-2R gamma, IL-7R a, ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRT AM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, PAG/Cbp, NKp44, NKp30, NKp46, NKG2D, and/or NKG2C. In some embodiments, the transmembrane domain is derived from a molecule selected from the group consisting of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152 and PD1.

**[0130]** Transmembrane domains for use in the CARs described herein can also comprise at least a portion of a synthetic, non-naturally occurring protein segment. In some embodiments, the transmembrane domain is a synthetic, non-naturally occurring alpha helix or beta sheet. In some embodiments, the protein segment is at least approximately 20 amino acids, e.g., at least 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more amino acids. Examples of synthetic transmembrane domains are known in the art, for example in U.S. Patent No. 7,052,906 and PCT Publication No. WO 2000/032776, the relevant disclosures of which are incorporated by reference herein.

**[0131]** The transmembrane domain provided herein may comprise a transmembrane region and a cytoplasmic region located at the C-terminal side of the transmembrane domain. The

cytoplasmic region of the transmembrane domain may comprise three or more amino acids and, in some embodiments, helps to orient the transmembrane domain in the lipid bilayer. In some embodiments, one or more cysteine residues are present in the transmembrane region of the transmembrane domain. In some embodiments, one or more cysteine residues are present in the cytoplasmic region of the transmembrane domain. In some embodiments, the cytoplasmic region of the transmembrane domain comprises positively charged amino acids. In some embodiments, the cytoplasmic region of the transmembrane domain comprises the amino acids arginine, serine, and lysine.

**[0132]** In some embodiments, the transmembrane region of the transmembrane domain comprises hydrophobic amino acid residues. In some embodiments, the transmembrane domain of the CAR provided herein comprises an artificial hydrophobic sequence. For example, a triplet of phenylalanine, tryptophan and valine may be present at the C terminus of the transmembrane domain. In some embodiments, the transmembrane region comprises mostly hydrophobic amino acid residues, such as alanine, leucine, isoleucine, methionine, phenylalanine, tryptophan, or valine. In some embodiments, the transmembrane region is hydrophobic. In some embodiments, the transmembrane region comprises a poly-leucine-alanine sequence. The hydrophathy, or hydrophobic or hydrophilic characteristics of a protein or protein segment, can be assessed by any method known in the art, for example the Kyte and Doolittle hydrophathy analysis.

**[0133]** In certain embodiments, in the CAR provided herein, the transmembrane domain is derived from a molecule selected from a group consisting of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152, and PD1. According to some embodiments, the transmembrane domain is a transmembrane domain from molecules such as CD8, CD2, and GMCSFR and the like. In some embodiments, the transmembrane domain is derived from a CD28 transmembrane domain. In some embodiments, the CD28 transmembrane domain comprises the amino acid sequence of SEQ ID NO: 54. In other embodiments, the CD28 transmembrane domain comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 54.

#### *4.2.3 Intracellular signaling domain*

**[0134]** The CARs of the present disclosure comprise an intracellular signaling domain. The intracellular signaling domain is responsible for activation of at least one of the normal effector functions of the immune effector cell expressing the CARs. The term “effector function” refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines. Thus the term “cytoplasmic signaling domain” refers to the portion of a protein which transduces the effector function signal

and directs the cell to perform a specialized function. While usually the entire cytoplasmic signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the cytoplasmic signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The term cytoplasmic signaling domain is thus meant to include any truncated portion of the cytoplasmic signaling domain sufficient to transduce the effector function signal.

**[0135]** In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell. In some embodiments, the CAR comprises an intracellular signaling domain consisting essentially of a primary intracellular signaling domain of an immune effector cell. “Primary intracellular signaling domain” refers to cytoplasmic signaling sequence that acts in a stimulatory manner to induce immune effector functions. In some embodiments, the primary intracellular signaling domain contains a signaling motif known as immunoreceptor tyrosine-based activation motif, or ITAM. An “ITAM,” as used herein, is a conserved protein motif that is generally present in the tail portion of signaling molecules expressed in many immune cells. The motif may comprises two repeats of the amino acid sequence YxxL/I separated by 6-8 amino acids, wherein each x is independently any amino acid, producing the conserved motif YxxL/Ix(6-8)YxxL/I. ITAMs within signaling molecules are important for signal transduction within the cell, which is mediated at least in part by phosphorylation of tyrosine residues in the ITAM following activation of the signaling molecule. ITAMs may also function as docking sites for other proteins involved in signaling pathways. Exemplary ITAM-containing primary cytoplasmic signaling sequences include those derived from CD3ζ, FcR gamma (FCER1G), FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d.

**[0136]** In certain embodiments, in the CAR provided herein, the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell. In some embodiments, the primary intracellular signaling domain is derived from CD3-zeta (CD3ζ). In some embodiments, the primary intracellular signaling domain derived from CD3-zeta comprises the amino acid sequence of SEQ ID NO: 57. In some embodiments, the primary intracellular signaling domain derived from CD3-zeta comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 57.

**[0137]** Any suitable intracellular signaling domain can be used in a CAR provided herein. In particular embodiments, the entire intracellular signaling domain is used. In other particular embodiments, a truncated portion of the signaling domain that transduces the effector signal is

used. According to particular embodiments, the intracellular signaling domain generates a signal that promotes an immune effector function of the CAR-expressing cell, e.g., a CAR-T cell, including, but not limited to, proliferation, activation and/or differentiation, or killing of target cells in the case of CAR-NK cells. In particular embodiments, the signal promotes, e.g., cytolytic activity, helper activity, and/or cytokine secretion of the CAR-T cell. The intracellular signaling domain may comprise a functional signaling domain derived from CD3 $\zeta$ , TCR $\zeta$ , FcR $\gamma$ , FcR $\beta$ , CD3 $\beta$ , CD3 $\delta$ , CD3 $\epsilon$ , CD16, CD22, CD27, CD28, CD30, CD79a, CD79b, CD134 (also known as TNFRSF4 or OX-40), 4-1BB (CD137), CD278 (also known as ICOS), Fc $\epsilon$ RI, DAP10, DAP12, ITAM domains or CD66d, and the like. According to particular embodiments, the intracellular signaling domain comprises a primary signaling domain, e.g., CD3 $\zeta$ , and one or more co-stimulatory signaling domains, e.g., 4-BB.

#### 4.2.4 Co-stimulatory signaling domain

**[0138]** Many immune effector cells require co-stimulation, in addition to stimulation of an antigen-specific signal, to promote cell proliferation, differentiation and survival, as well as to activate effector functions of the cell. In some embodiments, the CAR comprises at least one co-stimulatory signaling domain. The term “co-stimulatory signaling domain,” as used herein, refers to at least a portion of a protein that mediates signal transduction within a cell to induce an immune response such as an effector function. The co-stimulatory signaling domain of the chimeric receptor described herein can be a cytoplasmic signaling domain from a co-stimulatory protein, which transduces a signal and modulates responses mediated by immune cells, such as T cells, NK cells, macrophages, neutrophils, or eosinophils. “Co-stimulatory signaling domain” can be the cytoplasmic portion of a co-stimulatory molecule. The term “co-stimulatory molecule” refers to a cognate binding partner on an immune cell (such as T cell) that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the immune cell, such as, but not limited to, proliferation and survival.

**[0139]** In some embodiments, the intracellular signaling domain comprises a single co-stimulatory signaling domain. In some embodiments, the intracellular signaling domain comprises two or more (such as about any of 2, 3, 4, or more) co-stimulatory signaling domains. In some embodiments, the intracellular signaling domain comprises two or more of the same co-stimulatory signaling domains. In some embodiments, the intracellular signaling domain comprises two or more co-stimulatory signaling domains from different co-stimulatory proteins, such as any two or more co-stimulatory proteins described herein. In some embodiments, the intracellular signaling domain comprises a primary intracellular signaling domain (such as cytoplasmic signaling domain of CD3 $\zeta$ ) and one or more co-stimulatory signaling domains. In

some embodiments, the one or more co-stimulatory signaling domains and the primary intracellular signaling domain (such as cytoplasmic signaling domain of CD3  $\zeta$ ) are fused to each other via optional peptide linkers. The primary intracellular signaling domain, and the one or more co-stimulatory signaling domains may be arranged in any suitable order. In some embodiments, the one or more co-stimulatory signaling domains are located between the transmembrane domain and the primary intracellular signaling domain (such as cytoplasmic signaling domain of CD3  $\zeta$ ). Multiple co-stimulatory signaling domains may provide additive or synergistic stimulatory effects.

**[0140]** Activation of a co-stimulatory signaling domain in a host cell (e.g., an immune cell) may induce the cell to increase or decrease the production and secretion of cytokines, phagocytic properties, proliferation, differentiation, survival, and/or cytotoxicity. The co-stimulatory signaling domain of any co-stimulatory molecule may be compatible for use in the CARs described herein. The type(s) of co-stimulatory signaling domain is selected based on factors such as the type of the immune effector cells in which the effector molecules would be expressed (e.g., T cells, NK cells, macrophages, neutrophils, or eosinophils) and the desired immune effector function (e.g., ADCC effect). Examples of co-stimulatory signaling domains for use in the CARs can be the cytoplasmic signaling domain of co-stimulatory proteins, including, without limitation, members of the B7/CD28 family (e.g., B7-1/CD80, B7-2/CD86, B7-H1/PD-L1, B7-H2, B7-H3, B7-H4, B7-H6, B7-H7, BTLA/CD272, CD28, CTLA-4, Gi24/VISTA/B7-H5, ICOS/CD278, PD-1, PD-L2/B7-DC, and PDCD6); members of the TNF superfamily (e.g., 4-1BB/TNFSF9/CD137, 4-1BB Ligand/TNFSF9, BAFF/BLyS/TNFSF13B, BAFF R/TNFRSF13C, CD27/TNFRSF7, CD27 Ligand/TNFSF7, CD30/TNFRSF8, CD30 Ligand/TNFSF8, CD40/TNFRSF5, CD40 Ligand/TNFSF5, DR3/TNFRSF25, GITR/TNFRSF18, GITR Ligand/TNFSF18, HVEM/TNFRSF14, LIGHT/TNFSF14, Lymphotoxin-alpha/TNF-beta, OX40/TNFRSF4, OX40 Ligand/TNFSF4, RELT/TNFRSF19L, TACI/TNFRSF13B, TL1A/TNFSF15, TNF-alpha, and TNF RII/TNFRSF1B); members of the SLAM family (e.g., 2B4/CD244/SLAMF4, BLAME/SLAMF8, CD2, CD2F-10/SLAMF9, CD48/SLAMF2, CD58/LFA-3, CD84/SLAMF5, CD229/SLAMF3, CRACC/SLAMF7, NTB-A/SLAMF6, and SLAM/CD150); and any other co-stimulatory molecules, such as CD2, CD7, CD53, CD82/Kai-1, CD90/Thy1, CD96, CD160, Claudin18.20, CD300a/LMIR1, HLA Class I, HLA-DR, Ikaros, Integrin alpha 4/CD49d, Integrin alpha 4 beta 1, Integrin alpha 4 beta 7/LPAM-1, LAG-3, TCL1A, TCL1B, CRTAM, DAP12, Dectin-1/CLEC7A, DPPIV/CD26, EphB6, TIM-1/KIM-1/HAVCR, TIM-4, TSLP, TSLP R, lymphocyte function associated antigen-1 (LFA-1), and NKG2C.

**[0141]** In certain embodiments, in the CAR provided herein, the intracellular signaling domain further comprises a co-stimulatory signaling domain, and the co-stimulatory signaling domain is derived from a molecule selected from the group consisting of CD27, CD28, CD137, OX40, OX40L, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, ligands of CD83 and combinations thereof.

**[0142]** In some embodiments, the co-stimulatory signaling domain comprises an OX40L intracellular signaling domain, see, Matsumura et al., "Intracellular Signaling of gp34, the OX40 Ligand: Induction of c-jun and c-fos mRNA Expression Through gp34 upon Binding of Its Receptor, OX40," J. Immunol 163:3007–11 (1999), which is hereby incorporated by reference in its entirety. In some embodiments, the OX40L intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 56. In other embodiments, the OX40L intracellular signaling domain comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 56.

**[0143]** In some embodiments, the co-stimulatory signaling domain further comprises a CD28 intracellular signaling domain. In some embodiments, the CD28 intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 55. In some embodiments, the CD28 intracellular signaling domain comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 55.

**[0144]** More specifically, in some embodiments, the CAR provided herein comprises an OX40L intracellular signaling domain and a CD28 intracellular signaling domain in the co-stimulatory signaling domain, wherein the OX40L intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 56 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 56, and wherein the CD28 intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 55 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 55.

**[0145]** Also within the scope of the present disclosure are variants of any of the co-stimulatory signaling domains described herein, such that the co-stimulatory signaling domain is capable of modulating the immune response of the immune cell. In some embodiments, the co-stimulatory signaling domains comprises up to 10 amino acid residue variations (e.g., 1, 2, 3, 4, 5, or 8) as compared to a wild-type counterpart. Such co-stimulatory signaling domains comprising one or more amino acid variations may be referred to as variants. Mutation of amino acid residues of the co-stimulatory signaling domain may result in an increase in signaling

transduction and enhanced stimulation of immune responses relative to co-stimulatory signaling domains that do not comprise the mutation. Mutation of amino acid residues of the co-stimulatory signaling domain may result in a decrease in signaling transduction and reduced stimulation of immune responses relative to co-stimulatory signaling domains that do not comprise the mutation.

#### *4.2.5 Hinge region*

**[0146]** The CARs of the present disclosure may comprise a hinge domain that is located between the extracellular antigen binding domain and the transmembrane domain. A hinge domain is an amino acid segment that is generally found between two domains of a protein and may allow for flexibility of the protein and movement of one or both of the domains relative to one another. Any amino acid sequence that provides such flexibility and movement of the extracellular antigen binding domain relative to the transmembrane domain of the effector molecule can be used.

**[0147]** The hinge domain may contain about 10-100 amino acids, e.g., about any one of 15-75 amino acids, 20-50 amino acids, or 30-60 amino acids. In some embodiments, the hinge domain may be at least about any one of 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, or 75 amino acids in length.

**[0148]** In some embodiments, the hinge domain is a hinge domain of a naturally occurring protein. Hinge domains of any protein known in the art to comprise a hinge domain are compatible for use in the chimeric receptors described herein. In some embodiments, the hinge domain is at least a portion of a hinge domain of a naturally occurring protein and confers flexibility to the chimeric receptor.

**[0149]** Hinge domains of antibodies, such as an IgG, IgA, IgM, IgE, or IgD antibodies, are also compatible for use in the pH-dependent chimeric receptor systems described herein. In some embodiments, the hinge domain is the hinge domain that joins the constant domains CH1 and CH2 of an antibody. In some embodiments, the hinge domain is of an antibody and comprises the hinge domain of the antibody and one or more constant regions of the antibody. In some embodiments, the hinge domain comprises the hinge domain of an antibody and the CH3 constant region of the antibody. In some embodiments, the hinge domain comprises the hinge domain of an antibody and the CH2 and CH3 constant regions of the antibody. In some embodiments, the antibody is an IgG, IgA, IgM, IgE, or IgD antibody. In some embodiments, the antibody is an IgG antibody. In some embodiments, the antibody is an IgG1, IgG2, IgG3, or IgG4 antibody. In some embodiments, the hinge region comprises the hinge region and the CH2

and CH3 constant regions of an IgG1 antibody. In some embodiments, the hinge region comprises the hinge region and the CH3 constant region of an IgG1 antibody.

**[0150]** Non-naturally occurring peptides may also be used as hinge domains for the chimeric receptors described herein. In some embodiments, the hinge domain between the C-terminus of the extracellular ligand-binding domain of an Fc receptor and the N-terminus of the transmembrane domain is a peptide linker, such as a (GxS)<sub>n</sub> linker, wherein x and n, independently can be an integer between 3 and 12, including 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more (SEQ ID NO: 96).

**[0151]** In certain embodiments, the CAR provided herein further comprises a hinge domain located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain. In some embodiments, the hinge domain is derived from CD8 $\alpha$ .

**[0152]** In some embodiments, the hinge domain derived from CD8 $\alpha$  comprises the amino acid sequence of SEQ ID NO: 60. In some embodiments, the hinge domain derived from CD8 $\alpha$  comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 60.

**[0153]** In other embodiments, the hinge domain comprises at least a portion of an IgG1 hinge domain. In some embodiments, the IgG1 hinge domain comprises the amino acid sequence of SEQ ID NO: 53. In some embodiments, the IgG1 hinge domain comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 53.

**[0154]** According to particular embodiments, the CAR may further comprise a hinge region connecting the extracellular domain and the transmembrane domain. The hinge region functions to move the extracellular domain away from the surface of the engineered immune cell to enable proper cell/cell contact, binding to the target or antigen, and activation (Patel et al., Gene Therapy 6: 412-419 (1999)). Any suitable hinge region can be used in a CAR. It can be derived from a natural, synthetic, semi-synthetic, or recombinant source. Examples of a suitable hinge region for a CAR is a peptide comprising the amino acid sequence GS repeated up to six times, or a fragment thereof, or a hinge region from a CD8 protein, or a derivative thereof. A CD8 hinge comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 47, or the amino acid sequence of SEQ ID NO: 47.

#### *4.2.6 Signal peptide*

**[0155]** The CARs of the present disclosure may comprise a signal peptide (also known as a signal sequence) at the N-terminus of the polypeptide. In general, signal peptides are peptide

sequences that target a polypeptide to the desired site in a cell. In some embodiments, the signal peptide targets the effector molecule to the secretory pathway of the cell and will allow for integration and anchoring of the effector molecule into the lipid bilayer. Signal peptides including signal sequences of naturally occurring proteins or synthetic, non-naturally occurring signal sequences, which are compatible for use in the CARs described herein will be evident to one of skill in the art. In some embodiments, the signal peptide is derived from a molecule selected from the group consisting of CD8 $\alpha$ , GM-CSF receptor  $\alpha$ , and IgG1 heavy chain. In some embodiments, the signal peptide is derived from CD8 $\alpha$ .

**[0156]** In certain embodiments, the CAR provided herein further comprises a signal peptide located at the N-terminus of the polypeptide. In some embodiments, the signal peptide is derived from CD8 $\alpha$ . In some embodiments, the signal peptide derived from CD8 $\alpha$  comprises the amino acid sequence of SEQ ID NO: 51. In some embodiments, the signal peptide derived from CD8 $\alpha$  comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 51.

**[0157]** In some embodiments, in a nascent CAR, the extracellular domain is preceded by a signal or leader peptide at the N-terminus. Any suitable signal or leader peptide can be used in the disclosure. The signal peptide can be derived from a natural, synthetic, semi-synthetic or recombinant source. Examples of signal or leader peptides include those shown in SEQ ID NOs: 45 and 46.

#### *4.2.7 Exemplary CARs*

**[0158]** As illustrated in **FIG. 2**, an exemplary CAR may comprise (a) an extracellular domain comprising an scFv that specifically binds human MSLN; (b) a CD28 transmembrane domain; and (c) an intracellular signaling domain comprising a co-stimulatory domain comprising an OX40L intracellular signaling domain (e.g., the amino acid sequence set forth in SEQ ID NO: 56) and a CD28 intracellular signaling domain (e.g., the amino acid sequence set forth in SEQ ID NO: 55), and a primary signaling domain comprising the functional signaling domain of CD3 $\zeta$  (e.g., the amino acid sequence set forth in SEQ ID NO: 57), wherein the CAR optionally further comprises a hinge region connecting the extracellular domain and the transmembrane domain and wherein the CAR and IL-15 are linked with each other via a peptide linker.

**[0159]** Another exemplary CAR comprises (a) an extracellular domain comprising an scFv that specifically binds human MSLN; (b) a IgG1 hinge region; (c) a CD28 transmembrane domain; and (d) an intracellular signaling domain comprising a co-stimulatory domain comprising an OX40L intracellular signaling domain (e.g., the amino acid sequence set forth in

SEQ ID NO: 56) and a CD28 intracellular signaling domain (e.g., the amino acid sequence set forth in SEQ ID NO: 55), and a primary signaling domain derived from CD3 $\zeta$ .

**[0160]** Yet another exemplary CAR comprises (a) an extracellular domain comprising an scFv that specifically binds human MSLN; (b) a hinge domain derived from CD8 $\alpha$ ; (c) a CD28 transmembrane domain; and (d) an intracellular signaling domain comprising a co-stimulatory domain comprising an OX40L intracellular signaling domain (e.g., the amino acid sequence set forth in SEQ ID NO: 56) and a CD28 intracellular signaling domain (e.g., the amino acid sequence set forth in SEQ ID NO: 55), and a primary signaling domain comprising derived from CD3 $\zeta$ . Exemplary CARs are also described in Table 11.

**[0161]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 75 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 75.

**[0162]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 76 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 76.

**[0163]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 77 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 77.

**[0164]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 78 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 78.

**[0165]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 79 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 79.

**[0166]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 80 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 80.

**[0167]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 81 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 81.

**[0168]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 82 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 82.

**[0169]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 77 wherein the 1st amino acid residue of SEQ ID NO: 77 is pyroglutamate. In some specific embodiments, the CAR provided herein comprises an amino acid sequence having the 2nd amino acid residue to the 674th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 674th amino acid residue of SEQ ID NO: 77 and the 1st amino acid residue of the amino acid sequence being pyroglutamate.

**[0170]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 78 wherein the 1st amino acid residue of SEQ ID NO: 78 is pyroglutamate. In some specific embodiments, the CAR provided herein comprises an amino acid sequence having the 2nd amino acid residue to the 491st amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 491st amino acid residue of SEQ ID NO: 78 and the 1st amino acid residue of the amino acid sequence being pyroglutamate.

**[0171]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 79 wherein the 1st amino acid residue of SEQ ID NO: 79 is pyroglutamate. In some specific embodiments, the CAR provided herein comprises an amino acid sequence having the 2nd amino acid residue to the 671st amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 671st amino acid residue of SEQ ID NO: 79 and the 1st amino acid residue of the amino acid sequence being pyroglutamate.

**[0172]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 80 wherein the 1st amino acid residue of SEQ ID NO: 80 is pyroglutamate. In some specific embodiments, the CAR provided herein comprises an amino acid sequence having the 2nd amino acid residue to the 488th amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 488th amino acid residue of SEQ ID NO: 80 and the 1st amino acid residue of the amino acid sequence being pyroglutamate.

**[0173]** In some specific embodiments, the CAR provided herein comprises an amino acid sequence of SEQ ID NO: 81 wherein the 1st amino acid residue of SEQ ID NO: 81 is pyroglutamate. In some specific embodiments, the CAR provided herein comprises an amino acid sequence having the 2nd amino acid residue to the 671st amino acid residue of the amino acid sequence ranging from the 2nd amino acid residue to the 671st amino acid residue of SEQ ID NO: 81 and the 1st amino acid residue of the amino acid sequence being pyroglutamate.

**[0174]** In some specific embodiments, the CAR provided herein comprises the amino acid sequence of SEQ ID NO: 82 wherein the 1st amino acid residue of SEQ ID NO: 82 is pyroglutamate. In some specific embodiments, the CAR provided herein comprises an amino acid sequence having the 2nd amino acid residue to the 488th amino acid residue of the amino

acid sequence ranging from the 2nd amino acid residue to the 488th amino acid residue of SEQ ID NO: 82 and the 1st amino acid residue of the amino acid sequence being pyroglutamate.

**[0175]** Also provided herein are polyproteins comprising the CAR provided herein and an IL-15, wherein the CAR and the IL-15 are linked by a self-cleaving peptide, wherein optionally:

- (i) the IL-15 comprises the amino acid sequence of SEQ ID NO: 59; and/or
- (ii) the self-cleaving peptide comprises the amino acid sequence of SEQ ID NO: 58, wherein optionally the polyprotein comprises:
  - (a) the amino acid sequence of SEQ ID NO: 61 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 61,
  - (b) the amino acid sequence of SEQ ID NO: 62 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 62,
  - (c) the amino acid sequence of SEQ ID NO: 63 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 63,
  - (d) the amino acid sequence of SEQ ID NO: 64 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 64,
  - (e) the amino acid sequence of SEQ ID NO: 65 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 65,
  - (f) the amino acid sequence of SEQ ID NO: 66 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 66,
  - (g) the amino acid sequence of SEQ ID NO: 67 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 67, or
  - (h) the amino acid sequence of SEQ ID NO: 68 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 68.

#### 4.2.8 IL-15

**[0176]** In some embodiments, the engineered cells provided herein (e.g., NK cells) are engineered to express IL-15, e.g., human IL-15 (UniProtKB # P40933; NCBI Gene ID #3600),

e.g., soluble human IL-15 or an ortholog thereof, or a variant of any of the foregoing. In some embodiments, the IL-15 is expressed as part of a fusion protein further comprising a cleavage site. In some embodiments, the IL-15 is expressed as part of a polyprotein comprising a self-cleaving peptide such as a T2A ribosomal skip sequence site. *See, e.g., Radcliffe & Mitrophanous, "Multiple Gene Products from a Single Vector: 'Self-Cleaving' 2A Peptides," Gene Therapy 11:1673–4 (2004); see also Liu et al., "Systematic Comparison of 2A Peptides for Cloning Multi-Genes in a Polycistronic Vector," Scientific Reports 7(1):2193 (2017).*

**[0177]** In some embodiments, the IL-15 comprises or consists of SEQ ID NO: 59. In some embodiments, the IL-15 comprises an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 80 and retains essentially the functions of the IL-15 consisting of SEQ ID NO: 59.

**[0178]** In some embodiments of the various fusion proteins provided herein, the CAR and IL-15 are linked with each other via a peptide linker. In some embodiments, the peptide linker is a self-cleaving peptide such as 2A self-cleaving peptide, so that the CAR and the IL-15 become separate polypeptides upon cleavage in cells.

**[0179]** The members of 2A peptides are named after the virus in which they have been first described. For example, F2A, the first described 2A peptide, is derived from foot-and-mouth disease virus. The self-cleaving 18-22 amino acids long 2A peptides mediate 'ribosomal skipping' between the proline and glycine residues and inhibit peptide bond formation without affecting downstream translation. These peptides allow multiple proteins to be encoded as polyproteins, which dissociate into component proteins upon translation. Self-cleaving peptides are found in members of the picornaviridae virus family, including aphthoviruses such as foot-and-mouth disease virus (FMDV), equine rhinitis A virus (ERAV), thosa asigna virus (TaV) and porcine teschovirus-1 (PTV-1) (see Donnelly et al., J. Gen. Virol., 82: 1027-101 (2001); Ryan et al., J. Gen. Virol., 72: 2727-2732 (2001)) and cardioviruses such as theilovirus (e.g., Theiler's murine encephalomyelitis) and encephalomyocarditis viruses. The 2A peptides derived from FMDV, ERAV, PTV-1, and TaV are sometimes referred to as "F2A," "E2A," "P2A," and "T2A," respectively, and are included in the present disclosure, e.g., as described in Donnelly et al., J. Gen. Virol., 78: 13-21 (1997); Ryan and Drew, EMBO J., 13: 928-933 (1994); Szymczak et al., Nature Biotech., 5: 589-594 (2004); Hasegawa et al., Stem Cells, 25(7): 1707-12 (2007). In yet other embodiments, intein mediated protein splicing system is used herein, e.g., as described in Shah and Muir, Chem Sci., 5(1): 446–461 (2014) and Topilina and Mills, Mobile DNA, 5 (5) (2014). Other methods known in the art can also be used in the present constructs.

**[0180]** In some embodiments, the 2A self-cleaving peptide is selected from a group consisting of F2A, E2A, P2A, T2A, or variants thereof. In a specific embodiment, the self-cleaving peptide is T2A fragment comprising the amino acid sequence of SEQ ID NO: 58.

**[0181]** In some embodiments, the IL-15 is expressed as part of a fusion protein comprising a CAR, e.g., a CAR described herein.

**[0182]** In some embodiments, the fusion protein comprises (oriented from N-terminally to C-terminally): a CAR comprising, a cleavage site, and IL-15.

**[0183]** In some specific embodiments, provided herein is a polypeptide comprising the amino acid sequence of SEQ ID NO: 61 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 61.

**[0184]** In some specific embodiments, provided herein is a polypeptide comprising the amino acid sequence of SEQ ID NO: 62 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 62.

**[0185]** In some specific embodiments, provided herein is a polypeptide comprising the amino acid sequence of SEQ ID NO: 63 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 63.

**[0186]** In some specific embodiments, provided herein is a polypeptide comprising the amino acid sequence of SEQ ID NO: 64 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 64.

**[0187]** In some specific embodiments, provided herein is a polypeptide comprising the amino acid sequence of SEQ ID NO: 65 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 65.

**[0188]** In some specific embodiments, provided herein is a polypeptide comprising the amino acid sequence of SEQ ID NO: 66 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 66.

**[0189]** In some specific embodiments, provided herein is a polypeptide comprising the amino acid sequence of SEQ ID NO: 67 or an amino acid sequence having at least 85%, 86%, 87%,

88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 67.

**[0190]** In some specific embodiments, provided herein is a polypeptide comprising the amino acid sequence of SEQ ID NO: 68 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 68.

**[0191]** In some embodiments, the fusion protein(s) or components thereof described herein, are at least 80%, e.g., at least 85%, 90%, 95%, 98%, or 100% identical to the amino acid sequence of an exemplary sequence (e.g., as provided herein), e.g., have differences at up to 1%, 2%, 5%, 10%, 15%, or 20% of the residues of the exemplary sequence replaced, e.g., with conservative mutations, e.g., including or in addition to the mutations described herein. In preferred embodiments, the variant retains desired activity of the parent.

**[0192]** Alternatively, or additionally, in another embodiment of the present disclosure, the human MSLN binders, CARs, and other peptides provided herein may undergo post-translational modifications as known in the art. Examples of post-translational modifications include, but are not limited to, chemical modifications, such as disulfide bonds, oligosaccharides, N-terminal pyroglutamate formation, C-terminal lysine processing (such that lysine is removed), deamidation, isomerization, oxidation, glycation, peptide bond cleavage, non-reducible cross-linking, truncation and others known in the art. See, Liu, et. al., Heterogeneity of Monoclonal Antibodies, J. Pharma. Sci. vol. 97, no. 7, pp. 2426-2447 (July 2008). Other types of modifications include noncovalent interaction, conformational heterogeneity, and aggregation. *Id.*

**[0193]** In some embodiments, an N-terminal E or Q of a human MSLN binder provided herein is substituted with pyroglutamate. In some embodiments, a C-terminal K of a human MSLN binder provided herein is removed. In other embodiments, an N-terminal E or Q of a human MSLN binder provided herein is substituted with pyroglutamate and a C-terminal K of the human MSLN binder (e.g., heavy chain C terminal amino acid) is removed. The present disclosure includes any of the above described post-translational modifications of any of the human MSLN binders, CARs, and polypeptides provided herein. For example, in some embodiment, provided herein is a human MSLN binder that comprises the same sequences as 47D7, except that the first N-terminal amino acid of the VH region is substituted with pyroglutamate; and/or the C-terminal amino acid of the heavy chain is removed. In some embodiment, provided herein is a human MSLN binder that comprises the same sequences as 20B12, except that the first N-terminal amino acid of the VH region is substituted with

pyroglutamate; the first N-terminal amino acid of the VL region is substituted with pyroglutamate; and/or the C-terminal amino acid of the heavy chain is removed. In some embodiment, provided herein is a human MSLN binder that comprises the same sequences as 45B6, except that the first N-terminal amino acid of the VH region is substituted with pyroglutamate; the first N-terminal amino acid of the VL region is substituted with pyroglutamate; and/or the C-terminal amino acid of the heavy chain is removed.

**[0194]** In some specific embodiments, provided herein is an antibody or fragment thereof (e.g., scFv), a CAR, or a polyprotein comprising an amino acid sequence of any one of SEQ ID NOs: 3, 11, 12, 19, 20, 27 to 29, 77 to 82, except that the first amino acid at the N-terminus is substituted with pyroglutamate. In other specific embodiments, provided herein is a polypeptide (e.g., a CAR or a polyprotein) comprising an amino acid sequence of any one of SEQ ID NOs: 39 to 41, except that the first amino acid at the N-terminus is substituted with pyroglutamate.

#### **4.3 Nucleic Acid Molecules Encoding the CAR**

**[0195]** In another aspect, provided herein are nucleic acid molecules encoding the CAR provided here. In certain embodiments, provided herein is a polynucleotide comprising a nucleic acid encoding the CAR provided herein.

**[0196]** In certain embodiments, the polynucleotide provided herein further comprising a nucleic acid encoding an IL-15. In certain embodiments, the IL-15 comprises the amino acid sequence of SEQ ID NO: 59. In certain embodiments, the nucleic acid encoding the IL-15 comprises the nucleic acid sequence of SEQ ID NO: 93.

**[0197]** In certain embodiments, the polynucleotide encodes a polyprotein comprising the CAR and the IL-15. In certain embodiments, the polynucleotide provided herein further comprises a nucleic acid encoding a self-cleaving peptide, optionally a T2A self-cleaving peptide. In certain embodiments, the CAR is joined to the IL-15 by the self-cleaving peptide. In certain embodiments, the self-cleaving peptide is capable of inducing ribosomal skipping between the CAR and the IL-15. In certain embodiments, the self-cleaving peptide comprises the sequence of SEQ ID NO: 58. In certain embodiments, the nucleic acid encoding the self-cleaving peptide comprises the nucleic acid sequence of SEQ ID NO: 94.

**[0198]** In certain embodiments, the polynucleotide encodes a polyprotein comprising the amino acid sequence of SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, or SEQ ID NO: 68. In certain embodiments, the polynucleotide comprises the nucleic acid sequence of SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, or SEQ ID NO: 90.

**[0199]** In another aspect, provided herein are vectors comprising the polynucleotide provided herein. In certain embodiments, the vector is a viral vector, and wherein optionally the viral vector is a retroviral vector or a lentiviral vector.

**[0200]** The nucleic acid sequences encoding the CAR disclosed herein may be obtained by back-translating the amino acid sequence of the CAR into a nucleic acid sequence that encodes the CAR. The codons of the nucleic acid molecule so obtained may be further modified to correspond to codons commonly or more efficiently used when translated in a particular cell type. Methods and computer programs for back-translating and/or optimizing a nucleic acid molecule for enhancing expression in a particular host cell are well known in the art, e.g., the IDT Codon Optimization Tool commercially available from Integrated DNA Technologies, Inc. 1710 Commercial Park, Coralville, Iowa 52241, USA.; U.S. Pat. No. 8,326,547; WO2020024917A1.

**[0201]** In particular embodiments, the N-terminus of the scFv fusion protein is fused at the N-terminus to a leader or signal peptide to facilitate the transport of the scFv through the secretory pathway. Examples of leader/signal peptides that may be used those comprising the amino acid sequence set forth in SEQ ID NO: 45 or SEQ ID NO: 46. Thus, in particular embodiments, the aforementioned nucleic acid molecules may comprise a polynucleotide encoding a leader peptide linked to the 5' end of the nucleic acid molecule encoding the MSLN binder.

**[0202]** The nucleic acid molecules disclosed herein may include one or more substitutions that optimize one or more of the codons for enhancing the expression of the nucleic acid molecule in a particular host cell, e.g., yeast or fungal host cell, non-human mammalian host cell, human host cell, insect host cell, or prokaryote host cell.

#### **4.4 Engineered Cells**

**[0203]** In yet another aspect, provided herein are cells (such as immune effector cells, e.g., NK cells) comprising and/or expressing any one of the CARs described herein. In some embodiments, the engineered cell is an NK cell, a T cell, a peripheral blood mononuclear cell (PBMC), a hematopoietic stem cell, a pluripotent stem cell, or an embryonic stem cell. In some embodiments, the engineered cell is autologous. In some embodiments, the engineered cell is allogenic. Provided herein in some embodiments are engineered cells, e.g., engineered natural killer cells, e.g., CAR-NK cells, e.g., anti-MSLN CAR-NK cells. In some embodiments, the CAR-NK cells are engineered to express IL-15.

**[0204]** In some embodiments, the engineered cell is a lymphocyte. In some embodiments, the lymphocyte is a natural killer (NK) cell. In some embodiments, the lymphocyte is a T cell. In

some embodiments, the engineered cell is derived from a human cell. In some embodiments, the engineered cell is from a primary cell obtained from a subject. In some embodiments, the engineered cell is from a primary cell obtained from cord blood. In some embodiments, the engineered cell comprises a KIR-B haplotype. In some embodiments, the engineered cell expresses CD16 having the V/V polymorphism at F158.

**[0205]** In some embodiments, the engineered cells provided herein comprises the polynucleotide provided herein or the vector provided herein. In some embodiments, the engineered cells express the chimeric antigen receptor encoded by the polynucleotide provided herein or the vector provided herein. In some embodiments, the engineered cells express the chimeric antigen receptor and the IL-15 encoded by the polynucleotide provided herein or the vector provided herein.

**[0206]** In some embodiments, the NK cells are engineered, e.g., transduced, during expansion and stimulation, e.g., expansion and stimulation described herein. In some embodiments, the natural killer cells are engineered during expansion and stimulation, e.g., during production of a master cell bank (MCB), as described herein. In some embodiments, the natural killer cells are engineered during expansion and stimulation, e.g., during production of NK cells suitable for use in an injection-ready drug product and/or during production of a MCB, as described above. Thus, in some embodiments, the NK cells are host cells and provided herein are NK host cells expressing a heterogeneous protein, e.g., as described herein.

**[0207]** In some embodiments, the NK cells are engineered prior to expansion and stimulation. In some embodiments, the NK cells are engineered after expansion and stimulation.

**[0208]** In some embodiments, the NK cells are engineered by transducing with a vector. Suitable vectors are described herein, e.g., lentiviral vectors, e.g., lentiviral vectors comprising a heterologous protein, e.g., as described herein. In some embodiments, the NK cells are transduced during production of a first MCB, as described herein.

**[0209]** In some embodiments, the NK cells are transduced at a multiplicity of infection of from or from about 1 to or to about 40 viral particles per cell. In some embodiments, the NK cells are transduced at a multiplicity of infection of about 1, or of about 5, or of about 10, or of about 15, or of about 20, or of about 25, or of about 30, or of about 35, or of about 40 viral particles per cell.

**[0210]** In some embodiments, the NK cell is engineered to alter, e.g., reduce, expression of one or more inhibitor receptor genes.

[0211] In some embodiments, the inhibitory receptor gene is an HLA-specific inhibitory receptor. In some embodiments, the inhibitory receptor gene is a non-HLA-specific inhibitory receptor.

[0212] In some embodiments, the inhibitor receptor gene is selected from the group consisting of KIR, CD94/NKG2A, LILRB1, PD-1, IRp60, Siglec-7, LAIR-1, and combinations thereof.

[0213] In yet another aspect, provided herein are populations of cells comprising a plurality of the engineered cells provided herein. In some embodiments, at least 10% of the cells in the population of cells comprise the CAR provided herein, the polynucleotide provided herein, or the vector provided herein. In some embodiments, at least 10% of the cells express the polyprotein provided herein, the polynucleotide provided herein, or the vector provided herein.

#### **4.5 Expansion and Stimulation of NK Cells**

[0214] In some embodiments, natural killer cells are expanded and stimulated, e.g., by culturing and stimulation with feeder cells. NK cells can be expanded and stimulated as described, for example, in US 2020/0108096 or WO 2020/101361, both of which are incorporated herein by reference in their entirety. Briefly, the source cells can be cultured on modified HuT-78 (ATCC® TIB-161™) cells that have been engineered to express 4-1BBL, membrane bound IL-21, and a mutant TNF $\alpha$  as described in US 2020/0108096. Suitable NK cells can also be expanded and stimulated as described herein.

[0215] In some embodiments, NK cells are expanded and stimulated by a method comprising: (a) providing NK cells, e.g., a composition comprising NK cells, e.g., CD3(-) cells; and (b) culturing in a medium comprising feeder cells and/or stimulation factors, thereby producing a population of expanded and stimulated NK cells.

##### *4.5.1 Natural Killer Cell Sources*

[0216] In some embodiments, the NK cell source is selected from the group consisting of peripheral blood, peripheral blood lymphocytes (PBLs), peripheral blood mononuclear cells (PBMCs), bone marrow, umbilical cord blood (cord blood), isolated NK cells, NK cells derived from induced pluripotent stem cells, NK cells derived from embryonic stem cells, and combinations thereof.

[0217] In some embodiments, the NK cell source is a single unit of cord blood.

[0218] In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises from or from about  $1 \times 10^7$  to or to about  $1 \times 10^9$  total nucleated cells. In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises from or from about  $1 \times 10^8$  to or to about  $1.5 \times 10^8$  total nucleated cells. In some embodiments, the

natural killer cell source, e.g., single unit of cord blood, comprises  $1 \times 10^8$  total nucleated cells. In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises about  $1 \times 10^8$  total nucleated cells. In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises  $1 \times 10^9$  total nucleated cells. In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises about  $1 \times 10^9$  total nucleated cells.

**[0219]** In some embodiments, the NK cell source, e.g., the cord blood unit, comprises from about 20% to about 80% CD16+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises from or from about 20% to or to about 80%, from about 20% to or to about 70%, from about 20% to or to about 60%, from about 20% to or to about 50%, from about 20% to or to about 40%, from about 20% to or to about 30%, from about 30% to or to about 80%, from about 30% to or to about 70%, from about 30% to or to about 60%, from about 30% to or to about 50%, from about 30% to or to about 40%, from about 40% to or to about 80%, from about 40% to or to about 70%, from about 40% to or to about 60%, from about 40% to or to about 50%, from about 50% to or to about 80%, from about 50% to or to about 70%, from about 50% to or to about 60%, from about 60% to or to about 80%, from about 60% to or to about 70%, or from about 70% to or to about 80% CD16+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 80% CD16+ cells. Alternately, some NK cell sources may comprise CD16+ cells at a concentration of greater than 80%.

**[0220]** In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% MLG2A+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% NKG2C+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% NKG2D+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% NKp46+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% NKp30+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to

10%, e.g., less than or equal to 5% DNAM-1+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% NKp44+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% CD25+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% CD62L+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% CD69+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% CXCR3+ cells. In some embodiments, the NK cell source, e.g., the cord blood unit, comprises less than or equal to 40%, e.g., less than or equal to 30%, e.g., less than or equal to 20%, e.g., less than or equal to 10%, e.g., less than or equal to 5% CD57+ cells.

**[0221]** In some embodiments, NK cells in the NK cell source comprise a KIR B haplotype of the KIR receptor family. *See, e.g.,* Hsu et al., “The Killer Cell Immunoglobulin-Like Receptor (KIR) Genomic Region: Gene-Order, Haplotypes and Allelic Polymorphism,” *Immunological Review* 190:40–52 (2002); and Pyo et al., “Different Patterns of Evolution in the Centromeric and Telomeric Regions of Group A and B Haplotypes of the Human Killer Cell Ig-like Receptor Locus,” *PLoS One* 5:e15115 (2010).

**[0222]** In some embodiments, NK cells in the NK cell source comprise the 158 V/V variant of CD16 (i.e., homozygous CD16 158V polymorphism). *See, e.g.,* Koene et al., “FcγRIIIa-158V/F Polymorphism Influences the Binding of IgG by Natural Killer Cell FcγgammaRIIIa, Independently of the FcγgammaRIIIa-48L/R/H Phenotype,” *Blood* 90:1109–14 (1997).

**[0223]** In some embodiments, NK cells in the cell source comprises both the KIR B haplotype of the KIR receptor family and the 158 V/V variant of CD16. In some embodiments, the NK cells in the cell source are not genetically engineered. In some embodiments, the NK cells in the cell source do not comprise a CD16 transgene. In some embodiments, the NK cells in the cell source do not express an exogenous CD16 protein.

**[0224]** In some embodiments, the NK cell source is CD3(+) depleted. In some embodiments, the method comprises depleting the NK cell source of CD3(+) cells. In some embodiments, depleting the NK cell source of CD3(+) cells comprises contacting the NK cell source with a

CD3 binding antibody or antigen binding fragment thereof. In some embodiments, the CD3 binding antibody or antigen binding fragment thereof is selected from the group consisting of OKT3, UCHT1, HIT3a, SK7, and fragments thereof. In some embodiments, the CD3 binding antibody or antigen binding fragment thereof is OKT3 or an antigen binding fragment thereof. In some embodiments, the antibody or antigen binding fragment thereof is attached to a bead, e.g., a magnetic bead. In some embodiments, the depleting the composition of CD3(+) cells comprises contacting the composition with a CD3 targeting antibody or antigen binding fragment thereof attached to a bead and removing the bead-bound CD3(+) cells from the composition. The composition can be depleted of CD3 cells by immunomagnetic selection, for example, using a CliniMACS T cell depletion set ((LS Depletion set (162-01) Miltenyi Biotec).

**[0225]** In some embodiments, the NK cell source is CD56+ enriched, e.g., by gating on CD56 expression. In some embodiments, the NK cell source is both CD56+ enriched and CD3(+) depleted, e.g., by selecting for cells with CD56+CD3- expression. In some embodiments, the NK cell source comprises both the KIR B haplotype of the KIR receptor family and the 158 V/V variant of CD16 and is CD56+ enriched and CD3(+) depleted, e.g., by selecting for cells with CD56+CD3- expression.

#### *4.5.2 Feeder Cells*

**[0226]** Disclosed herein are feeder cells for the expansion of NK cells. These feeder cells advantageously allow NK cells to expand to numbers suitable for the preparation of a pharmaceutical composition as discussed herein. In some cases, the feeder cells allow the expansion of NK cells without the loss of CD16 expression, which often accompanies cell expansion on other types of feeder cells or using other methods. In some cases, the feeder cells make the expanded NK cells more permissive to freezing such that a higher proportion of NK cells remain viable after a freeze/thaw cycle or such that the cells remain viable for longer periods of time while frozen. In some cases, the feeder cells allow the NK cells to retain high levels of cytotoxicity, including ADCC, extend survival, increase persistence, and enhance or retain high levels of CD16. In some cases, the feeder cells allow the NK cells to expand without causing significant levels of exhaustion or senescence.

**[0227]** Feeder cells can be used to stimulate the NK cells and help them to expand more quickly, e.g., by providing substrate, growth factors, and/or cytokines. NK cells can be stimulated using various types of feeder cells, including, but not limited to peripheral blood mononuclear cells (PBMC), Epstein-Barr virus-transformed B-lymphoblastoid cells (e.g., EBV-LCL), myelogenous leukemia cells (e.g., K562), and CD4(+) T cells (e.g., HuT), and derivatives thereof. In some embodiments, the feeder cells are inactivated, e.g., by  $\gamma$ -irradiation or

mitomycin-c treatment. Suitable feeder cells for use in the methods described herein are described, for example, in US 2020/0108096, which is hereby incorporated by reference in its entirety.

#### *4.5.3 Stimulating Factors*

**[0228]** NK cells can also be stimulated using one or more stimulation factors other than feeder cells, e.g., signaling factors, in addition to or in place of feeder cells.

**[0229]** In some embodiments, the stimulating factor, e.g., signaling factor, is a component of the culture medium, as described herein. In some embodiments, the stimulating factor, e.g., signaling factor, is a supplement to the culture medium, as described herein.

**[0230]** In some embodiments, the stimulation factor(s) are cytokine(s). In some embodiments, the cytokine(s) are selected from the group consisting of IL-2, IL-12, IL-15, IL-18, IL-21, IL-23, IL-27, IFN- $\alpha$ , IFN $\beta$ , and combinations thereof. In some embodiments, the cytokine is IL-2. In some embodiments, the cytokines are a combination of IL-2 and IL-15. In some embodiments, the cytokines are a combination of IL-2, IL-15, and IL-18. In some embodiments, the cytokines are a combination of IL-2, IL-18, and IL-21.

#### *4.5.4 Culturing*

**[0231]** The NK cells can be expanded and stimulated by co-culturing an NK cell source and feeder cells and/or other stimulation factors. Suitable NK cell sources, feeder cells, and stimulation factors are described herein.

**[0232]** In some cases, the resulting population of expanded natural killer cells is enriched and/or sorted after expansion. In some cases, the resulting population of expanded natural killer cells is not enriched and/or sorted after expansion

**[0233]** Also described herein are compositions comprising the various culture compositions described herein, e.g., comprising NK cells. For example, a composition comprising a population of expanded cord blood-derived natural killer cells comprising a KIR-B haplotype and homozygous for a CD16 158V polymorphism and a plurality of engineered HuT78 cells.

**[0234]** Also described herein are vessels, e.g., vials, cryobags, and the like, comprising the resulting populations of expanded natural killer cells. In some cases, a plurality of vessels comprising portions of the resulting populations of expanded natural killer cells, e.g., at least 10, e.g., 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, or 1200 vessels.

**[0235]** Also described herein are bioreactors comprising the various culture compositions described herein, e.g., comprising NK cells. For example, a culture comprising natural killer cells from a natural killer cell source, e.g., as described herein, and feeder cells, e.g., as described

herein. Also described herein are bioreactors comprising the resulting populations of expanded natural killer cells.

#### *4.5.4.1 Culture Medium*

**[0236]** Disclosed herein are culture media for the expansion of NK cells. These culture media advantageously allow NK cells to expand to numbers suitable for the preparation of a pharmaceutical composition as discussed herein. In some cases, the culture media allows NK cells to expand without the loss of CD16 expression that often accompanies cell expansion on other helper cells or in other media.

**[0237]** In some embodiments, the culture medium is a basal culture medium, optionally supplemented with additional components, e.g., as described herein.

**[0238]** In some embodiments, the culture medium, e.g., the basal culture medium, is a serum-free culture medium. In some embodiments, the culture medium, e.g., the basal culture medium, is a serum-free culture medium supplemented with human plasma and/or serum.

**[0239]** Suitable basal culture media include, but are not limited to, DMEM, RPMI 1640, MEM, DMEM/F12, Ham's F12, SCGM (CellGenix®, 20802-0500 or 20806-0500), LGM-3™ (Lonza, CC-3211), TexMACS™ (Miltenyi Biotec, 130-097-196), NK MACS™ (Miltenyi Biotec, 130-114-429), ALyS™ 505NK-AC (Cell Science and Technology Institute, Inc., 01600P02), ALyS™ 505NK-EX (Cell Science and Technology Institute, Inc., 01400P10), CTS™ AIM-V™ SFM (ThermoFisher Scientific, A3830801), CTS™ OpTmizer™ (ThermoFisher Scientific, A1048501, ABS-001, StemXxVivo, SCGM, B0, HPLM, ExCellerate, X-VIVO 15, CTS expander, and combinations thereof.

**[0240]** The culture medium may comprise additional components, or be supplemented with additional components, such as growth factors, signaling factors, nutrients, antigen binders, and the like. Supplementation of the culture medium may occur by adding each of the additional component or components to the culture vessel either before, concurrently with, or after the medium is added to the culture vessel. The additional component or components may be added together or separately. When added separately, the additional components need not be added at the same time.

**[0241]** In some embodiments, the culture medium comprises plasma, e.g., human plasma. In some embodiments, the culture medium is supplemented with plasma, e.g., human plasma. In some embodiments, the plasma, e.g., human plasma, comprises an anticoagulant, e.g., trisodium citrate.

**[0242]** In some embodiments, the medium comprises and/or is supplemented with from or from about 0.5 % to or to about 10 % v/v plasma, e.g., human plasma. In some embodiments, the

medium is supplemented with from or from about 0.5% to or to about 9%, from or from about 0.5% to or to about 8%, from or from about 0.5% to or to about 7%, from or from about 0.5% to or to about 6%, from or from about 0.5% to or to about 5%, from or from about 0.5% to or to about 4%, from or from about 0.5% to or to about 3%, from or from about 0.5% to or to about 2%, from or from about 0.5% to or to about 1%, from or from about 1% to or to about 10%, from or from about 1% to or to about 9%, from or from about 1% to or to about 8%, from or from about 1% to or to about 7%, from or from about 1% to or to about 6%, from or from about 1% to or to about 5%, from or from about 1% to or to about 4%, from or from about 1% to or to about 3%, from or from about 1% to or to about 2%, from or from about 2% to or to about 10%, from or from about 2% to or to about 9%, from or from about 2% to or to about 8%, from or from about 2% to or to about 7%, from or from about 2% to or to about 6%, from or from about 2% to or to about 5%, from or from about 2% to or to about 4%, from or from about 2% to or to about 3%, from or from about 3% to or to about 10%, from or from about 3% to or to about 9%, from or from about 3% to or to about 8%, from or from about 3% to or to about 7%, from or from about 3% to or to about 6%, from or from about 3% to or to about 5%, from or from about 3% to or to about 4%, from or from about 4% to or to about 10%, from or from about 4% to or to about 9%, from or from about 4% to or to about 8%, from or from about 4% to or to about 7%, from or from about 4% to or to about 6%, from or from about 4% to or to about 5%, from or from about 5% to or to about 10%, from or from about 5% to or to about 9%, from or from about 4% to or to about 8%, from or from about 5% to or to about 7%, from or from about 5% to or to about 6%, from or from about 6% to or to about 10%, from or from about 6% to or to about 9%, from or from about 6% to or to about 8%, from or from about 6% to or to about 7%, from or from about 7% to or to about 10%, from or from about 7% to or to about 9%, from or from about 7% to or to about 8%, from or from about 8% to or to about 10%, from or from about 8% to or to about 9%, or from or from about 9% to or to about 10% v/v plasma, e.g., human plasma. In some embodiments, the culture medium comprises and/or is supplemented with from 0.8% to 1.2% v/v human plasma. In some embodiments, the culture medium comprises and/or is supplemented with 1.0 % v/v human plasma. In some embodiments, the culture medium comprises and/or is supplemented with about 1.0 % v/v human plasma.

**[0243]** In some embodiments, the culture medium comprises serum, e.g., human serum. In some embodiments, the culture medium is supplemented with serum, e.g., human serum. In some embodiments, the serum is inactivated, e.g., heat inactivated. In some embodiments, the serum is filtered, e.g., sterile-filtered.

**[0244]** In some embodiments, the medium comprises and/or is supplemented with from or from about 0.5 % to or to about 10 % v/v serum, e.g., human serum. In some embodiments, the medium is supplemented with from or from about 0.5% to or to about 9%, from or from about 0.5% to or to about 8%, from or from about 0.5% to or to about 7%, from or from about 0.5% to or to about 6%, from or from about 0.5% to or to about 5%, from or from about 0.5% to or to about 4%, from or from about 0.5% to or to about 3%, from or from about 0.5% to or to about 2%, from or from about 0.5% to or to about 1%, from or from about 1% to or to about 10%, from or from about 1% to or to about 9%, from or from about 1% to or to about 8%, from or from about 1% to or to about 7%, from or from about 1% to or to about 6%, from or from about 1% to or to about 5%, from or from about 1% to or to about 4%, from or from about 1% to or to about 3%, from or from about 1% to or to about 2%, from or from about 2% to or to about 10%, from or from about 2% to or to about 9%, from or from about 2% to or to about 8%, from or from about 2% to or to about 7%, from or from about 2% to or to about 6%, from or from about 2% to or to about 5%, from or from about 2% to or to about 4%, from or from about 2% to or to about 3%, from or from about 3% to or to about 10%, from or from about 3% to or to about 9%, from or from about 3% to or to about 8%, from or from about 3% to or to about 7%, from or from about 3% to or to about 6%, from or from about 3% to or to about 5%, from or from about 3% to or to about 4%, from or from about 4% to or to about 10%, from or from about 4% to or to about 9%, from or from about 4% to or to about 8%, from or from about 4% to or to about 7%, from or from about 4% to or to about 6%, from or from about 4% to or to about 5%, from or from about 5% to or to about 10%, from or from about 5% to or to about 9%, from or from about 4% to or to about 8%, from or from about 5% to or to about 7%, from or from about 5% to or to about 6%, from or from about 6% to or to about 10%, from or from about 6% to or to about 9%, from or from about 6% to or to about 8%, from or from about 6% to or to about 7%, from or from about 7% to or to about 10%, from or from about 7% to or to about 9%, from or from about 7% to or to about 8%, from or from about 8% to or to about 10%, from or from about 8% to or to about 9%, or from or from about 9% to or to about 10% v/v plasma, e.g., human serum. In some embodiments, the culture medium comprises and/or is supplemented with from 0.8% to 1.2% v/v human serum. In some embodiments, the culture medium comprises and/or is supplemented with 1.0 % v/v human serum. In some embodiments, the culture medium comprises and/or is supplemented with about 1.0 % v/v human serum.

**[0245]** In some embodiments, the culture medium comprises glutamine. In some embodiments, the culture medium is supplemented with glutamine. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 2.0 to or to about 6.0

mM glutamine. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 2.0 to or to about 5.5, from or from about 2.0 to or to about 5.0, from or from about 2.0 to or to about 4.5, from or from about 2.0 to or to about 4.0, from or from about 2.0 to or to about 3.5, from or from about 2.0 to or to about 3.0, from or from about 2.0 to or to about 2.5, from or from about 2.5 to or to about 6.0, from or from about 2.5 to or to about 5.5, from or from about 2.5 to or to about 5.0, from or from about 2.5 to or to about 4.5, from or from about 2.5 to or to about 4.0, from or from about 2.5 to or to about 3.5, from or from about 2.5 to or to about 3.0, from or from about 3.0 to or to about 6.0, from or from about 3.0 to or to about 5.5, from or from about 3.0 to or to about 5.0, from or from about 3.0 to or to about 4.5, from or from about 3.0 to or to about 4.0, from or from about 3.0 to or to about 3.5, from or from about 3.5 to or to about 6.0, from or from about 3.5 to or to about 5.5, from or from about 3.5 to or to about 5.0, from or from about 3.5 to or to about 4.5, from or from about 3.5 to or to about 4.0, from or from about 4.0 to or to about 6.0, from or from about 4.0 to or to about 5.5, from or from about 4.0 to or to about 5.0, from or from about 4.0 to or to about 4.5, from or from about 4.5 to or to about 6.0, from or from about 4.5 to or to about 5.5, from or from about 4.5 to or to about 5.0, from or from about 5.0 to or to about 6.0, from or from about 5.0 to or to about 5.5, or from or from about 5.5 to or to about 6.0 mM glutamine. In some embodiments, the culture medium comprises and/or is supplemented with from 3.2 mM glutamine to 4.8 mM glutamine. In some embodiments, the culture medium comprises and/or is supplemented with 4.0 mM glutamine. In some embodiments, the culture medium comprises and/or is supplemented with about 4.0 mM glutamine.

**[0246]** In some embodiments, the culture medium comprises one or more cytokines. In some embodiments, the culture medium is supplemented with one or more cytokines. In some embodiments, the cytokine is selected from IL-2, IL-12, IL-15, IL-18, IL-21, and combinations thereof.

**[0247]** In some embodiments, the culture medium comprises and/or is supplemented with IL-2. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 150 to or to about 2,500 IU/mL IL-2. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 200 to or to about 2,250, from or from about 200 to or to about 2,000, from or from about 200 to or to about 1,750, from or from about 200 to or to about 1,500, from or from about 200 to or to about 1,250, from or from 200 to or to about 1,000, from or from about 200 to or to about 750, from or from about 200 to or to about 500, from or from about 200 to or to about 250, from or from about 250 to or to about 2,500, from or from about 250 to or to about 2,250, from or from about 250 to or to about 2,000,

from or from about 250 to or to about 1,750, from or from about 250 to or to about 1,500, from or from about 250 to or to about 1,250, from or from about 250 to or to about 1,000, from or from about 250 to or to about 750, from or from about 250 to or to about 500, from or from about 500 to or to about 2,500, from or from about 500 to or to about 2,250, from or from about 500 to or to about 2,000, from or from about 500 to or to about 1,750, from or from about 500 to or to about 1,500, from or from about 500 to or to about 1,250, from or from about 500 to or to about 1,000, from or from about 500 to or to about 750, from or from about 750 to or to about 2,250, from or from about 750 to or to about 2,000, from or from about 750 to or to about 1,750, from or from about 750 to or to about 1,500, from or from about 750 to or to about 1,250, from or from about 750 to or to about 1,000, from or from about 1,000 to or to about 2,500, from or from about 1,000 to or to about 2,250, from or from about 1,000 to or to about 2,000, from or from about 1,000 to or to about 1,750, from or from about 1,000 to or to about 1,500, from or from about 1,000 to or to about 1,250, from or from about 1,250 to or to about 2,500, from or from about 1,250 to or to about 2,250, from or from about 1,250 to or to about 2,000, from or from about 1,250 to or to about 1,750, from or from about 1,250 to or to about 1,500, from or from about 1,500 to or to about 2,500, from or from about 1,500 to or to about 2,250, from or from about 1,500 to or to about 2,000, from or from about 1,500 to or to about 1,750, from or from about 1,750 to or to about 2,500, from or from about 1,750 to or to about 2,250, from or from about 1,750 to or to about 2,000, from or from about 2,000 to or to about 2,500, from or from about 2,000 to or to about 2,250, or from or from about 2,250 to or to about 2,500 IU/mL IL-2.

**[0248]** In some embodiments, the culture medium comprises and/or is supplemented with from 64 µg/L to 96 µg/L IL-2. In some embodiments, the culture medium comprises and/or is supplemented with 80 µg/L IL-2 (approximately 1,333 IU/mL). In some embodiments, the culture medium comprises and/or is supplemented with about 80 µg/L IL-2. In some embodiments, the culture medium comprises and/or is supplemented with from 10 µg/L to 100 µg/L IL-21. In some embodiments, the culture medium comprises and/or is supplemented with 20 µg/L IL-21. In some embodiments, the culture medium comprises and/or is supplemented with about 20 µg/L. In some embodiments, the culture medium comprises and/or is supplemented with a combination of IL-2 and IL-15. In some embodiments, the culture medium comprises and/or is supplemented with a combination of IL-2, IL-15, and IL-18. In some embodiments, the culture medium comprises and/or is supplemented with a combination of IL-2, IL-18, and IL-21.

**[0249]** In some embodiments, the culture medium comprises and/or is supplemented with glucose. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 0.5 to or to about 3.5 g/L glucose. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 0.5 to or to about 3.0, from or from about 0.5 to or to about 2.5, from or from about 0.5 to or to about 2.0, from or from about 0.5 to or to about 1.5, from or from about 0.5 to or to about 1.0, from or from about 1.0 to or to about 3.0, from or from about 1.0 to or to about 2.5, from or from about 1.0 to or to about 2.0, from or from about 1.0 to or to about 1.5, from or from about 1.5 to or to about 3.0, from or from about 1.5 to or to about 2.5, from or from about 1.5 to or to about 2.0, from or from about 2.0 to or to about 3.0, from or from about 2.0 to or to about 2.5, or from or from about 2.5 to or to about 3.0 g/L glucose. In some embodiments, the culture medium comprises and/or is supplemented with from 1.6 to 2.4 g/L glucose. In some embodiments, the culture medium comprises and/or is supplemented with 2.0 g/L glucose. In some embodiments, the culture medium comprises about 2.0 g/L glucose.

**[0250]** In some embodiments, the culture medium comprises and/or is supplemented with sodium pyruvate. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 0.1 to or to about 2.0 mM sodium pyruvate. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 0.1 to or to about 1.8, from or from about 0.1 to or to about 1.6, from or from about 0.1 to or to about 1.4, from or from about 0.1 to or to about 1.2, from or from about 0.1 to or to about 1.0, from or from about 0.1 to or to about 0.8, from or from about 0.1 to or to about 0.6, from or from about 0.1 to or to about 0.4, from or from about 0.1 to or to about 0.2, from or from about 0.2 to or to about 2.0, from or from about 0.2 to or to about 1.8, from or from about 0.2 to or to about 1.6, from or from about 0.2 to or to about 1.4, from or from about 0.2 to or to about 1.2, from or from about 0.2 to or to about 1.0, from or from about 0.2 to or to about 0.8, from or from about 0.2 to or to about 0.6, from or from about 0.2 to or to about 0.4, from or from about 0.4 to or to about 2.0, from or from about 0.4 to or to about 1.8, from or from about 0.4 to or to about 1.6, from or from about 0.4 to or to about 1.4, from or from about 0.4 to or to about 1.2, from or from about 0.4 to or to about 1.0, from or from about 0.4 to or to about 0.8, from or from about 0.4 to or to about 0.6, from or from about 0.6 to or to about 2.0, from or from about 0.6 to or to about 1.8, from or from about 0.6 to or to about 1.6, from or from about 0.6 to or to about 1.4, from or from about 0.6 to or to about 1.2, from or from about 0.6 to or to about 1.0, from or from about 0.6 to or to about 0.8, from or from about 0.8 to or to about 2.0, from or from about 0.8 to or to about 1.8, from or from about 0.8 to or to about 1.6, from or from about 0.8 to or to about 1.4, from or from about 0.8 to

or to about 1.4, from or from about 0.8 to or to about 1.2, from or from about 0.8 to or to about 1.0, from or from about 1.0 to or to about 2.0, from or from about 1.0 to or to about 1.8, from or from about 1.0 to or to about 1.6, from or from about 1.0 to or to about 1.4, from or from about 1.0 to or to about 1.2, from or from about 1.2 to or to about 2.0, from or from about 1.2 to or to about 1.8, from or from about 1.2 to or to about 1.6, from or from about 1.2 to or to about 1.4, from or from about 1.4 to or to about 2.0, from or from about 1.4 to or to about 1.8, from or from about 1.4 to or to about 1.6, from or from about 1.6 to or to about 2.0, from or from about 1.6 to or to about 1.8, or from or from about 1.8 to or to about 2.0 mM sodium pyruvate. In some embodiments, the culture medium comprises from 0.8 to 1.2 mM sodium pyruvate. In some embodiments, the culture medium comprises 1.0 mM sodium pyruvate. In some embodiments, the culture medium comprises about 1.0 mM sodium pyruvate.

**[0251]** In some embodiments, the culture medium comprises and/or is supplemented with sodium hydrogen carbonate. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 0.5 to or to about 3.5 g/L sodium hydrogen carbonate. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 0.5 to or to about 3.0, from or from about 0.5 to or to about 2.5, from or from about 0.5 to or to about 2.0, from or from about 0.5 to or to about 1.5, from or from about 0.5 to or to about 1.0, from or from about 1.0 to or to about 3.0, from or from about 1.0 to or to about 2.5, from or from about 1.0 to or to about 2.0, from or from about 1.0 to or to about 1.5, from or from about 1.5 to or to about 3.0, from or from about 1.5 to or to about 2.5, from or from about 1.5 to or to about 2.0, from or from about 2.0 to or to about 3.0, from or from about 2.0 to or to about 2.5, or from or from about 2.5 to or to about 3.0 g/L sodium hydrogen carbonate. In some embodiments, the culture medium comprises and/or is supplemented with from 1.6 to 2.4 g/L sodium hydrogen carbonate. In some embodiments, the culture medium comprises and/or is supplemented with 2.0 g/L sodium hydrogen carbonate. In some embodiments, the culture medium comprises about 2.0 g/L sodium hydrogen carbonate.

**[0252]** In some embodiments, the culture medium comprises and/or is supplemented with albumin, e.g., human albumin, e.g., a human albumin solution described herein. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 0.5% to or to about 3.5% v/v of a 20% albumin solution, e.g., a 20% human albumin solution. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 0.5% to or to about 3.0%, from or from about 0.5% to or to about 2.5%, from or from about 0.5% to or to about 2.0%, from or from about 0.5% to or to about 1.5%, from or from about 0.5% to or to about 1.0%, from or from about 1.0% to or to about 3.0%, from or from

about 1.0% to or to about 2.5%, from or from about 1.0% to or to about 2.0%, from or from about 1.0% to or to about 1.5%, from or from about 1.5% to or to about 3.0%, from or from about 1.5% to or to about 2.5%, from or from about 1.5% to or to about 2.0%, from or from about 2.0% to or to about 3.0%, from or from about 2.0% to or to about 2.5%, or from or from about 2.5% to or to about 3.0% v/v of a 20% albumin solution, e.g., a 20% human albumin solution. In some embodiments, the culture medium comprises and/or is supplemented with from 1.6% to 2.4% v/v of a 20% albumin solution, e.g., a 20% human albumin solution. In some embodiments, the culture medium comprises and/or is supplemented with 2.0% v/v of a 20% albumin solution, e.g., a 20% human albumin solution. In some embodiments, the culture medium comprises about 2.0% v/v of a 20% albumin solution, e.g., a 20% human albumin solution.

**[0253]** In some embodiments, the culture medium comprises and/or is supplemented with from or from about 2 to or to about 6 g/L albumin, e.g., human albumin. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 2 to or to about 5.5, from or from about 2 to or to about 5.0, from or from about 2 to or to about 4.5, from or from about 2 to or to about 4, from or from about 2 to or to about 3.5, from or from about 2 to or to about 3, from or from about 2 to or to about 2.5, from or from about 2.5 to or to about 6, from or from about 2.5 to or to about 5.5, from or from about 2.5 to or to about 5.5, from or from about 2.5 to or to about 5.0, from or from about 2.5 to or to about 4.5, from or from about 2.5 to or to about 4.0, from or from about 2.5 to or to about 3.5, from or from about 2.5 to or to about 3.0, from or from about 3 to or to about 6, from or from about 3 to or to about 5.5, from or from about 3 to or to about 5, from or from about 3 to or to about 4.5, from or from about 3 to or to about 4, from or from about 3 to or to about 3.5, from or from about 3.5 to or to about 6, from or from about 3.5 to or to about 5.5, from or from about 3.5 to or to about 5, from or from about 3.5 to or to about 4.5, from or from about 3.5 to or to about 4, from or from about 4 to or to about 6, from or from about 4 to or to about 5.5, from or from about 4 to or to about 5, from or from about 4 to or to about 4.5, from or from about 4.5 to or to about 6, from or from about 4.5 to or to about 5.5, from or from about 4.5 to or to about 5, from or from about 5 to or to about 6, from or from about 5 to or to about 5.5, or from or from about 5.5 to or to about 6 g/L albumin, e.g., human albumin. In some embodiments, the culture medium comprises and/or is supplemented with from 3.2 to 4.8 g/L albumin, e.g., human albumin. In some embodiments, the culture medium comprises 4 g/L albumin, e.g., human albumin. In some embodiments, the culture medium comprises about 4 g/L albumin, e.g., human albumin.

**[0254]** In some embodiments, the culture medium is supplemented with Poloxamer 188. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 0.1 to or to about 2.0 g/L Poloxamer 188. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 0.1 to or to about 1.8, from or from about 0.1 to or to about 1.6, from or from about 0.1 to or to about 1.4, from or from about 0.1 to or to about 1.2, from or from about 0.1 to or to about 1.0, from or from about 0.1 to or to about 0.8, from or from about 0.1 to or to about 0.6, from or from about 0.1 to or to about 0.4, from or from about 0.1 to or to about 0.2, from or from about 0.2 to or to about 2.0, from or from about 0.2 to or to about 1.8, from or from about 0.2 to or to about 1.6, from or from about 0.2 to or to about 1.4, from or from about 0.2 to or to about 1.2, from or from about 0.2 to or to about 1.0, from or from about 0.2 to or to about 0.8, from or from about 0.2 to or to about 0.6, from or from about 0.2 to or to about 0.4, from or from about 0.4 to or to about 2.0, from or from about 0.4 to or to about 1.8, from or from about 0.4 to or to about 1.6, from or from about 0.4 to or to about 1.4, from or from about 0.4 to or to about 1.2, from or from about 0.4 to or to about 1.0, from or from about 0.4 to or to about 0.8, from or from about 0.4 to or to about 0.6, from or from about 0.6 to or to about 2.0, from or from about 0.6 to or to about 1.8, from or from about 0.6 to or to about 1.6, from or from about 0.6 to or to about 1.4, from or from about 0.6 to or to about 1.2, from or from about 0.6 to or to about 1.0, from or from about 0.6 to or to about 0.8, from or from about 0.8 to or to about 2.0, from or from about 0.8 to or to about 1.8, from or from about 0.8 to or to about 1.6, from or from about 0.8 to or to about 1.4, from or from about 0.8 to or to about 1.2, from or from about 0.8 to or to about 1.0, from or from about 1.0 to or to about 2.0, from or from about 1.0 to or to about 1.8, from or from about 1.0 to or to about 1.6, from or from about 1.0 to or to about 1.4, from or from about 1.0 to or to about 1.2, from or from about 1.2 to or to about 2.0, from or from about 1.2 to or to about 1.8, from or from about 1.2 to or to about 1.6, from or from about 1.2 to or to about 1.4, from or from about 1.4 to or to about 2.0, from or from about 1.4 to or to about 1.8, from or from about 1.4 to or to about 1.6, from or from about 1.6 to or to about 2.0, from or from about 1.6 to or to about 1.8, or from or from about 1.8 to or to about 2.0 g/L Poloxamer 188. In some embodiments, the culture medium comprises from 0.8 to 1.2 g/L Poloxamer 188. In some embodiments, the culture medium comprises 1.0 g/L Poloxamer 188. In some embodiments, the culture medium comprises about 1.0 g/L Poloxamer 188.

**[0255]** In some embodiments, the culture medium comprises and/or is supplemented with one or more antibiotics.

**[0256]** A first exemplary culture medium is set forth in **Table 6**.

**Table 6. Exemplary culture medium #1**

| Component                 | Exemplary Concentration Range | Exemplary Concentration |
|---------------------------|-------------------------------|-------------------------|
| CellgroSCGM liquid medium | undiluted                     | undiluted               |
| Human Plasma              | 0.8 – 1.2 % (v/v)             | 1.0 % v/v               |
| Glutamine                 | 3.2 – 4.8 mM                  | 4.0 mM                  |
| IL-2                      | 64 - 96 µg/L                  | 80 µg/L                 |

**[0257]** A second exemplary culture medium is set forth in **Table 7**.

**Table 7. Exemplary culture medium #2**

| Component                 | Exemplary Concentration Range       | Exemplary Concentration |
|---------------------------|-------------------------------------|-------------------------|
| RPMI1640                  | 7.6 – 13.2 g/L                      | 10.4 g/L                |
| Human Plasma              | 0.8 – 1.2 % (v/v)                   | 1.0 % v/v               |
| Glucose                   | 1.6 – 2.4 g/L                       | 2.0 g/L                 |
| Glutamine                 | 3.2 – 4.8 mM                        | 4.0 mM                  |
| Sodium Pyruvate           | 0.8 – 1.2 mM                        | 1.0 mM                  |
| Sodium Hydrogen Carbonate | 1.6 – 2.4 g/L                       | 2.0 g/L                 |
| IL-2                      | 64 - 96 µg/L                        | 80 µg/L                 |
| Albumin 20% solution      | 1.6 – 2.5 % v/v<br>(3.2 to 4.8 g/L) | 2.0 % v/v<br>(4.0 g/L)  |
| Poloxamer 188             | 0.8 – 1.2 g/L                       | 1.0 g/L                 |

#### 4.5.4.2 CD3 Binding Antibodies

**[0258]** In some embodiments, the culture medium comprises and/or is supplemented with a CD3 binding antibody or antigen binding fragment thereof. In some embodiments, the CD3 binding antibody or antigen binding fragment thereof is selected from the group consisting of OKT3, UCHT1, and HIT3a, or variants thereof. In some embodiments, the CD3 binding antibody or antigen binding fragment thereof is OKT3 or an antigen binding fragment thereof.

**[0259]** In some embodiments, the CD3 binding antibody or antigen binding fragment thereof and feeder cells are added to the culture vessel before addition of NK cells and/or culture medium.

**[0260]** In some embodiments, the culture medium comprises and/or is supplemented with from or from about 5 ng/mL to or to about 15 ng/mL OKT3. In some embodiments, the culture medium comprises and/or is supplemented with from or from about 5 to or to about 12.5, from or from about 5 to or to about 10, from or from about 5 to or to about 7.5, from or from about 7.5 to or to about 15, from or from about 7.5 to or to about 12.5, from or from about 7.5 to or to about

10, from or from about 10 to or to about 15, from or from about 10 to or to about 12.5, or from or from about 12.5 to or to about 15 ng/mL OKT3. In some embodiments, the culture medium comprises and/or is supplemented with 10 ng/mL OKT3. In some embodiments, the culture medium comprises and/or is supplemented with about 10 ng/mL OKT3.

#### *4.5.4.3 Culture Vessels*

**[0261]** A number of vessels are consistent with the disclosure herein. In some embodiments, the culture vessel is selected from the group consisting of a flask, a bottle, a dish, a multiwell plate, a roller bottle, a bag, and a bioreactor.

**[0262]** In some embodiments, the culture vessel is treated to render it hydrophilic. In some embodiments, the culture vessel is treated to promote attachment and/or proliferation. In some embodiments, the culture vessel surface is coated with serum, collagen, laminin, gelatin, poy-L-lysine, fibronectin, extracellular matrix proteins, and combinations thereof.

**[0263]** In some embodiments, different types of culture vessels are used for different stages of culturing.

**[0264]** In some embodiments, the culture vessel has a volume of from or from about 100 mL to or to about 1,000 L. In some embodiments, the culture vessel has a volume of or about 125 mL, of or about 250 mL, of or about 500 mL, of or about 1 L, of or about 5 L, of about 10 L, or of or about 20 L.

**[0265]** In some embodiments, the culture vessel is a bioreactor. In some embodiments, the bioreactor is a rocking bed (wave motion) bioreactor. In some embodiments, the bioreactor is a stirred tank bioreactor. In some embodiments, the bioreactor is a rotating wall vessel. In some embodiments, the bioreactor is a perfusion bioreactor. In some embodiments, the bioreactor is an isolation/expansion automated system. In some embodiments, the bioreactor is an automated or semi-automated bioreactor. In some embodiments, the bioreactor is a disposable bag bioreactor.

**[0266]** In some embodiments, the bioreactor has a volume of from about 100 mL to about 1,000 L. In some embodiments, the bioreactor has a volume of from about 10 L to about 1,000 L. In some embodiments, the bioreactor has a volume of from about 100 L to about 900 L. In some embodiments, the bioreactor has a volume of from about 10 L to about 800 L. In some embodiments, the bioreactor has a volume of from about 10 L to about 700 L, about 10 L to about 600 L, about 10 L to about 500 L, about 10 L to about 400 L, about 10 L to about 300 L, about 10 L to about 200 L, about 10 L to about 100 L, about 10 L to about 90 L, about 10 L to about 80 L, about 10 L to about 70 L, about 10 L to about 60 L, about 10 L to about 50 L, about 10 L to about 40 L, about 10 L to about 30 L, about 10 L to about 20 L, about 20 L to about 1,000 L, about 20 L to about 900 L, about 20 L to about 800 L, about 20 L to about 700 L, about

20 L to about 600 L, about 20 L to about 500 L, about 20 L to about 400 L, about 20 L to about 300 L, about 20 L to about 200 L, about 20 L to about 100 L, about 20 L to about 90 L, about 20 L to about 80 L, about 20 L to about 70 L, about 20 L to about 60 L, about 20 L to about 50 L, about 20 L to about 40 L, about 20 L to about 30 L, about 30 L to about 1,000 L, about 30 L to about 900 L, about 30 L to about 800 L, about 30 L to about 700 L, about 30 L to about 600 L, about 30 L to about 500 L, about 30 L to about 400 L, about 30 L to about 300 L, about 30 L to about 200 L, about 30 L to about 100 L, about 30 L to about 90 L, about 30 L to about 80 L, about 30 L to about 70 L, about 30 L to about 60 L, about 30 L to about 50 L, about 30 L to about 40 L, about 40 L to about 1,000 L, about 40 L to about 900 L, about 40 L to about 800 L, about 40 L to about 700 L, about 40 L to about 600 L, about 40 L to about 500 L, about 40 L to about 400 L, about 40 L to about 300 L, about 40 L to about 200 L, about 40 L to about 100 L, about 40 L to about 90 L, about 40 L to about 80 L, about 40 L to about 70 L, about 40 L to about 60 L, about 40 L to about 50 L, about 50 L to about 1,000 L, about 50 L to about 900 L, about 50 L to about 800 L, about 50 L to about 700 L, about 50 L to about 600 L, about 50 L to about 500 L, about 50 L to about 400 L, about 50 L to about 300 L, about 50 L to about 200 L, about 50 L to about 100 L, about 50 L to about 90 L, about 50 L to about 80 L, about 50 L to about 70 L, about 50 L to about 60 L, about 60 L to about 1,000 L, about 60 L to about 900 L, about 60 L to about 800 L, about 60 L to about 700 L, about 60 L to about 600 L, about 60 L to about 500 L, about 60 L to about 400 L, about 60 L to about 300 L, about 60 L to about 200 L, about 60 L to about 100L, about 60 L to about 90 L, about 60 L to about 80 L, about 60 L to about 70 L, about 70 L to about 1,000 L, about 70 L to about 900 L, about 70 L to about 800 L, about 70 L to about 700 L, about 70 L to about 600 L, about 70 L to about 500 L, about 70 L to about 400 L, about 70 L to about 300 L, about 70 L to about 200 L, about 70 L to about 100 L, about 70 L to about 90 L, about 70 L to about 80 L, about 80 L to about 1,000 L, about 80 L to about 900 L, about 80 L to about 800 L, about 80 L to about 700 L, about 80 L to about 600 L, about 80 L to about 500 L, about 80 L to about 400 L, about 80 L to about 300 L, about 80 L to about 200 L, about 80 L to about 100 L, about 80 L to about 90 L, about 90 L to about 1,000 L, about 90 L to about 900 L, about 90 L to about 800 L, about 90 L to about 700 L, about 90 L to about 600 L, about 90 L to about 500 L, about 90 L to about 400 L, about 90 L to about 300 L, about 90 L to about 200 L, about 90 L to about 100 L, about 100 L to about 1,000 L, about 100 L to about 900 L, about 100 L to about 800 L, about 100 L to about 700 L, about 100 L to about 600 L, about 100 L to about 500 L, about 100 L to about 400 L, about 100 L to about 300 L, about 100 L to about 200 L, about 200 L to about 1,000 L, about 200 L to about 900 L, about 200 L to about 800 L, about 200 L to about 700 L, about 200 L to about 600 L, about 200 L to

about 500 L, about 200 L to about 400 L, about 200 L to about 300 L, about 300 L to about 1,000 L, about 300 L to about 900 L, about 300 L to about 800 L, about 300 L to about 700 L, about 300 L to about 600 L, about 300 L to about 500 L, about 300 L to about 400 L, about 400 L to about 1,000 L, about 400 L to about 900 L, about 400 L to about 800 L, about 400 L to about 700 L, about 400 L to about 600 L, about 400 L to about 500 L, about 500 L to about 1,000 L, about 500 L to about 900 L, about 500 L to about 800 L, about 500 L to about 700 L, about 500 L to about 600 L, about 600 L to about 1,000 L, about 600 L to about 900 L, about 600 L to about 800 L, about 600 L to about 700 L, about 700 L to about 1,000 L, about 700 L to about 900 L, about 700 L to about 800 L, about 800 L to about 1,000 L, about 800 L to about 900 L, or about 900 L to about 1,000 L. In some embodiments, the bioreactor has a volume of about 50 L.

**[0267]** In some embodiments, the bioreactor has a volume of from 100 mL to 1,000 L. In some embodiments, the bioreactor has a volume of from 10 L to 1,000 L. In some embodiments, the bioreactor has a volume of from 100 L to 900 L. In some embodiments, the bioreactor has a volume of from 10 L to 800 L. In some embodiments, the bioreactor has a volume of from 10 L to 700 L, 10 L to 600 L, 10 L to 500 L, 10 L to 400 L, 10 L to 300 L, 10 L to 200 L, 10 L to 100 L, 10 L to 90 L, 10 L to 80 L, 10 L to 70 L, 10 L to 60 L, 10 L to 50 L, 10 L to 40 L, 10 L to 30 L, 10 L to 20 L, 20 L to 1,000 L, 20 L to 900 L, 20 L to 800 L, 20 L to 700 L, 20 L to 600 L, 20 L to 500 L, 20 L to 400 L, 20 L to 300 L, 20 L to 200 L, 20 L to 100 L, 20 L to 90 L, 20 L to 80 L, 20 L to 70 L, 20 L to 60 L, 20 L to 50 L, 20 L to 40 L, 20 L to 30 L, 30 L to 1,000 L, 30 L to 900 L, 30 L to 800 L, 30 L to 700 L, 30 L to 600 L, 30 L to 500 L, 30 L to 400 L, 30 L to 300 L, 30 L to 200 L, 30 L to 100 L, 30 L to 90 L, 30 L to 80 L, 30 L to 70 L, 30 L to 60 L, 30 L to 50 L, 30 L to 40 L, 40 L to 1,000 L, 40 L to 900 L, 40 L to 800 L, 40 L to 700 L, 40 L to 600 L, 40 L to 500 L, 40 L to 400 L, 40 L to 300 L, 40 L to 200 L, 40 L to 100 L, 40 L to 90 L, 40 L to 80 L, 40 L to 70 L, 40 L to 60 L, 40 L to 50 L, 50 L to 1,000 L, 50 L to 900 L, 50 L to 800 L, 50 L to 700 L, 50 L to 600 L, 50 L to 500 L, 50 L to 400 L, 50 L to 300 L, 50 L to 200 L, 50 L to 100 L, 50 L to 90 L, 50 L to 80 L, 50 L to 70 L, 50 L to 60 L, 60 L to 1,000 L, 60 L to 900 L, 60 L to 800 L, 60 L to 700 L, 60 L to 600 L, 60 L to 500 L, 60 L to 400 L, 60 L to 300 L, 60 L to 200 L, 60 L to 100 L, 60 L to 90 L, 60 L to 80 L, 60 L to 70 L, 70 L to 1,000 L, 70 L to 900 L, 70 L to 800 L, 70 L to 700 L, 70 L to 600 L, 70 L to 500 L, 70 L to 400 L, 70 L to 300 L, 70 L to 200 L, 70 L to 100 L, 70 L to 90 L, 70 L to 80 L, 80 L to 1,000 L, 80 L to 900 L, 80 L to 800 L, 80 L to 700 L, 80 L to 600 L, 80 L to 500 L, 80 L to 400 L, 80 L to 300 L, 80 L to 200 L, 80 L to 100 L, 80 L to 90 L, 90 L to 1,000 L, 90 L to 900 L, 90 L to 800 L, 90 L to 700 L, 90 L to 600 L, 90 L to 500 L, 90 L to 400 L, 90 L to 300 L, 90 L to 200 L, 90 L to 100 L, 100 L to 1,000 L, 100 L to

900 L, 100 L to 800 L, 100 L to 700 L, 100 L to 600 L, 100 L to 500 L, 100 L to 400 L, 100 L to 300 L, 100 L to 200 L, 200 L to 1,000 L, 200 L to 900 L, 200 L to 800 L, 200 L to 700 L, 200 L to 600 L, 200 L to 500 L, 200 L to 400 L, 200 L to 300 L, 300 L to 1,000 L, 300 L to 900 L, 300 L to 800 L, 300 L to 700 L, 300 L to 600 L, 300 L to 500 L, 300 L to 400 L, 400 L to 1,000 L, 400 L to 900 L, 400 L to 800 L, 400 L to 700 L, 400 L to 600 L, 400 L to 500 L, 500 L to 1,000 L, 500 L to 900 L, 500 L to 800 L, 500 L to 700 L, 500 L to 600 L, 600 L to 1,000 L, 600 L to 900 L, 600 L to 800 L, 600 L to 700 L, 700 L to 1,000 L, 700 L to 900 L, 700 L to 800 L, 800 L to 1,000 L, 800 L to 900 L, or 900 L to 1,000 L. In some embodiments, the bioreactor has a volume of 50 L.

#### *4.5.4.4 Cell Expansion and Stimulation*

**[0268]** In some embodiments, the natural killer cell source, e.g., single unit of cord blood, is co-cultured with feeder cells to produce expanded and stimulated NK cells.

**[0269]** In some embodiments, the co-culture is carried out in a culture medium described herein, e.g., exemplary culture medium #1 (**Table 6**) or exemplary culture medium #2 (**Table 7**).

**[0270]** In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises from or from about  $1 \times 10^7$  to or to about  $1 \times 10^9$  total nucleated cells prior to expansion. In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises from or from about  $1 \times 10^8$  to or to about  $1.5 \times 10^8$  total nucleated cells prior to expansion. In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises  $1 \times 10^8$  total nucleated cells prior to expansion. In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises about  $1 \times 10^8$  total nucleated cells prior to expansion. In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises  $1 \times 10^9$  total nucleated cells prior to expansion. In some embodiments, the natural killer cell source, e.g., single unit of cord blood, comprises about  $1 \times 10^9$  total nucleated cells prior to expansion.

**[0271]** In some embodiments, cells from the co-culture of the natural killer cell source, e.g., single unit of cord blood and feeder cells are harvested and frozen, e.g., in a cryopreservation composition described herein. In some embodiments, the frozen cells from the co-culture are an infusion-ready drug product. In some embodiments, the frozen cells from the co-culture are used as a master cell bank (MCB) from which to produce an infusion-ready drug product, e.g., through one or more additional co-culturing steps, as described herein. Thus, for example, a natural killer cell source can be expanded and stimulated as described herein to produce expanded and stimulated NK cells suitable for use in an infusion-ready drug product without generating any intermediate products. A natural killer cell source can also be expanded and

stimulated as described herein to produce an intermediate product, e.g., a first master cell bank (MCB). The first MCB can be used to produce expanded and stimulated NK cells suitable for use in an infusion-ready drug product, or, alternatively, be used to produce another intermediate product, e.g., a second MCB. The second MCB can be used to produce expanded and stimulated NK cells suitable for an infusion-ready drug product, or alternatively, be used to produce another intermediate product, e.g., a third MCB, and so on.

**[0272]** In some embodiments, the ratio of feeder cells to cells of the natural killer cell source or MCB cells inoculated into the co-culture is from or from about 1:1 to or to about 4:1. In some embodiments, the ratio of feeder cells to cells of the natural killer cell source or MCB cells is from or from about 1:1 to or to about 3.5:1, from or from about 1:1 to or to about 3:1, from or from about 1:1 to or to about 2.5:1, from or from about 1:1 to or to about 2:1, from or from about 1:1 to or to about 1.5:1, from or from about 1.5:1 to or to about 4:1, from or from about 1.5:1 to or to about 3.5:1, from or from about 1.5:1 to or to about 3:1, from or from about 1.5:1 to or to about 2.5:1, from or from about 1.5:1 to or to about 2:1, from or from about 2:1 to or to about 4:1, from or from about 2:1 to or to about 3.5:1, from or from about 2:1 to or to about 3:1, from or from about 2:1 to or to about 2.5:1, from or from about 2.5:1 to or to about 4:1, from or from about 2.5:1 to or to about 3.5:1, from or from about 2.5:1 to or to about 3:1, from or from about 3:1 to or to about 4:1, from or from about 3:1 to or to about 3.5:1, or from or from about 3.5:1 to or to about 4:1. In some embodiments, the ratio of feeder cells to cells of the natural killer cell source or MCB inoculated into the co-culture is 2.5:1. In some embodiments, the ratio of feeder cells to cells of the natural killer cell source or MCB inoculated into the co-culture is about 2.5:1.

**[0273]** In some embodiments, the co-culture is carried out in a disposable culture bag, e.g., a 1L disposable culture bag. In some embodiments, the co-culture is carried out in a bioreactor, e.g., a 50L bioreactor. In some embodiments, culture medium is added to the co-culture after the initial inoculation.

**[0274]** In some embodiments, the co-culture is carried out for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or more days. In some embodiments, the co-culture is carried out for a maximum of 16 days.

**[0275]** In some embodiments, the co-culture is carried out at 37 °C or about 37°C. In some embodiments, the co-culture is carried out at pH 7.9 or about pH 7.9. In some embodiments, the co-culture is carried out at a dissolved oxygen (DO) level of 50% or more.

**[0276]** In some embodiments, exemplary culture medium #1 (**Table 6**) is used to produce a MCB and exemplary culture medium #2 (**Table 7**) is used to produce cells suitable for an infusion-ready drug product.

**[0277]** In some embodiments, the co-culture of the natural killer cell source, e.g., single unit of cord blood, with feeder cells yields from or from about  $50 \times 10^8$  to or to about  $50 \times 10^{12}$  cells, e.g., MCB cells or infusion-ready drug product cells. In some embodiments, the expansion yields from or from about  $50 \times 10^8$  to or to about  $25 \times 10^{10}$ , from or from about  $10 \times 10^8$  to or to about  $1 \times 10^{10}$ , from or from about  $50 \times 10^8$  to or to about  $75 \times 10^9$ , from or from about  $50 \times 10^8$  to or to about  $50 \times 10^9$ , from or from about  $50 \times 10^8$  to or to about  $25 \times 10^9$ , from or from about  $50 \times 10^8$  to or to about  $1 \times 10^9$ , from or from about  $50 \times 10^8$  to or to about  $75 \times 10^8$ , from or from about  $75 \times 10^8$  to or to about  $50 \times 10^{10}$ , from or from about  $75 \times 10^8$  to or to about  $25 \times 10^{10}$ , from or from about  $75 \times 10^8$  to or to about  $1 \times 10^{10}$ , from or from about  $75 \times 10^8$  to or to about  $75 \times 10^9$ , from or from about  $75 \times 10^8$  to or to about  $50 \times 10^9$ , from or from about  $75 \times 10^8$  to or to about  $25 \times 10^9$ , from or from about  $75 \times 10^8$  to or to about  $1 \times 10^9$ , from or from about  $1 \times 10^9$  to or to about  $50 \times 10^{10}$ , from or from about  $1 \times 10^9$  to or to about  $25 \times 10^{10}$ , from or from about  $1 \times 10^9$  to or to about  $1 \times 10^{10}$ , from or from about  $1 \times 10^9$  to or to about  $75 \times 10^9$ , from or from about  $1 \times 10^9$  to or to about  $50 \times 10^9$ , from or from about  $1 \times 10^9$  to or to about  $25 \times 10^9$ , from or from about  $25 \times 10^9$  to or to about  $50 \times 10^{10}$ , from or from about  $25 \times 10^9$  to or to about  $25 \times 10^{10}$ , from or from about  $25 \times 10^9$  to or to about  $1 \times 10^{10}$ , from or from about  $25 \times 10^9$  to or to about  $75 \times 10^9$ , from or from about  $25 \times 10^9$  to or to about  $50 \times 10^9$ , from or from about  $50 \times 10^9$  to or to about  $50 \times 10^{10}$ , from or from about  $50 \times 10^9$  to or to about  $25 \times 10^{10}$ , from or from about  $50 \times 10^9$  to or to about  $1 \times 10^{10}$ , from or from about  $50 \times 10^9$  to or to about  $75 \times 10^9$ , from or from about  $75 \times 10^9$  to or to about  $50 \times 10^{10}$ , from or from about  $75 \times 10^9$  to or to about  $25 \times 10^{10}$ , from or from about  $75 \times 10^9$  to or to about  $1 \times 10^{10}$ , from or from about  $1 \times 10^{10}$  to or to about  $50 \times 10^{10}$ , from or from about  $1 \times 10^{10}$  to or to about  $25 \times 10^{10}$ , or from or from about  $25 \times 10^{10}$  to or to about  $50 \times 10^{10}$  cells, e.g., MCB cells or infusion-ready drug product cells.

**[0278]** In some embodiments, the expansion yields from or from about 60 to or to about 100 vials, each comprising from or from about 600 million to or to about 1 billion cells, e.g., MCB cells or infusion-ready drug product cells. In some embodiments, the expansion yields 80 or about 80 vials, each comprising or consisting of 800 million or about 800 million cells, e.g., MCB cells or infusion-ready drug product cells.

**[0279]** In some embodiments, the expansion yields from or from about a 100 to or to about a 500 fold increase in the number of cells, e.g., the number of MCB cells relative to the number of cells, e.g., NK cells, in the natural killer cell source. In some embodiments, the expansion yields from or from about a 100 to or to about a 500, from or from about a 100 to or to about a 400, from or from about a 100 to or to about a 300, from or from about a 100 to or to about a 200, from or from about a 200 to or to about a 450, from or from about a 200 to or to about a 400,

from or from about a 100 to or to about a 350, from or from about a 200 to or to about a 300, from or from about a 200 to or to about a 250, from or from about a 250 to or to about a 500, from or from about a 250 to or to about a 450, from or from about a 200 to or to about a 400, from or from about a 250 to or to about a 350, from or from about a 250 to or to about a 300, from or from about a 300 to or to about a 500, from or from about a 300 to or to about a 450, from or from about a 300 to or to about a 400, from or from about a 300 to or to about a 350, from or from about a 350 to or to about a 500, from or from about a 350 to or to about a 450, from or from about a 350 to or to about a 400 fold increase in the number of cells, e.g., the number of MCB cells relative to the number of cells, e.g., NK cells, in the natural killer cell source.

**[0280]** In some embodiments, the expansion yields from or from about a 100 to or to about a 70,000 fold increase in the number of cells, e.g., the number of MCB cells relative to the number of cells, e.g., NK cells, in the natural killer cell source. In some embodiments, the expansion yields at least a 10,000 fold, e.g., 15,000 fold, 20,000 fold, 25,000 fold, 30,000 fold, 35,000 fold, 40,000 fold, 45,000 fold, 50,000 fold, 55,000 fold, 60,000 fold, 65,000 fold, or 70,000 fold increase in the number of cells, e.g., the number of MCB cells relative to the number of cells, e.g., NK cells, in the natural killer cell source.

**[0281]** In some embodiments, the co-culture of the MCB cells and feeder cells yields from or from about 500 million to or to about 1.5 billion cells, e.g., NK cells suitable for use in an MCB and/or in an infusion-ready drug product. In some embodiments, the co-culture of the MCB cells and feeder cells yields from or from about 500 million to or to about 1.5 billion, from or from about 500 million to or to about 1.25 billion, from or from about 500 million to or to about 1 billion, from or from about 500 million to or to about 750 million, from or from about 750 million to or to about 1.5 billion, from or from about 500 million to or to about 1.25 billion, from or from about 750 million to or to about 1 billion, from or from about 1 billion to or to about 1.5 billion, from or from about 1 billion to or to about 1.25 billion, or from or from about 1.25 billion to or to about 1.5 billion cells, e.g., NK cells suitable for use in an MCB and/or an infusion-ready drug product.

**[0282]** In some embodiments, the co-culture of the MCB cells and feeder cells yields from or from about 50 to or to about 150 vials of cells, e.g., infusion-ready drug product cells, each comprising from or from about 750 million to or to about 1.25 billion cells, e.g., NK cells suitable for use in an MCB and/or an infusion-ready drug product. In some embodiments, the co-culture of the MCB cells and feeder cells yields 100 or about 100 vials, each comprising or

consisting of 1 billion or about 1 billion cells, e.g., NK cells suitable for use in an MCB and/or an infusion-ready drug product.

**[0283]** In some embodiments, the expansion yields from or from about a 100 to or to about a 500 fold increase in the number of cells, e.g., the number of NK cells suitable for use in an MCB and/or an infusion-ready drug product relative to the number of starting MCB cells. In some embodiments, the expansion yields from or from about a 100 to or to about a 500, from or from about a 100 to or to about a 400, from or from about a 100 to or to about a 300, from or from about a 100 to or to about a 200, from or from about a 200 to or to about a 450, from or from about a 200 to or to about a 400, from or from about a 100 to or to about a 350, from or from about a 200 to or to about a 300, from or from about a 200 to or to about a 250, from or from about a 250 to or to about a 500, from or from about a 250 to or to about a 450, from or from about a 200 to or to about a 400, from or from about a 250 to or to about a 350, from or from about a 250 to or to about a 300, from or from about a 300 to or to about a 500, from or from about a 300 to or to about a 450, from or from about a 300 to or to about a 400, from or from about a 300 to or to about a 350, from or from about a 350 to or to about a 500, from or from about a 350 to or to about a 450, from or from about a 350 to or to about a 400 fold increase in the number of cells, e.g., the number of NK cells suitable for use in an MCB and/or an infusion-ready drug product relative to the number of starting MCB cells.

**[0284]** In some embodiments, the expansion yields from or from about a 100 to or to about a 70,000 fold increase in the number of cells, e.g., the number of NK cells suitable for use in an MCB and/or an infusion-ready drug product relative to the number of starting MCB cells. In some embodiments, the expansion yields at least a 10,000 fold, e.g., 15,000 fold, 20,000 fold, 25,000 fold, 30,000 fold, 35,000 fold, 40,000 fold, 45,000 fold, 50,000 fold, 55,000 fold, 60,000 fold, 65,000 fold, or 70,000 fold increase in the number of cells, e.g., the number of NK cells suitable for use in an MCB and/or an infusion-ready drug product relative to the number of starting MCB cells.

**[0285]** In embodiments where the cells are engineered during expansion and stimulation, as described herein, not all of the expanded and stimulated cells will necessarily be engineered successfully, e.g., transduced successfully, e.g., transduced successfully with a vector comprising a heterologous protein, e.g., a heterologous protein comprising a CAR and/or IL-15 as described herein. Thus, the methods described herein can further comprise sorting engineered cells, e.g., engineered cells described herein, away from non-engineered cells.

**[0286]** In some embodiments, the engineered cells, e.g., transduced cells, are sorted from the non-engineered cells, e.g., the non-transduced cells using a reagent specific to an antigen of the

engineered cells, e.g., an antibody that targets an antigen of the engineered cells but not the non-engineered cells. In some embodiments, the antigen of the engineered cells is a component of a CAR, e.g., a CAR described herein.

**[0287]** Systems for antigen-based cell separation of cells are available commercially, e.g., the CliniMACS® sorting system (Miltenyi Biotec).

**[0288]** In some embodiments, the engineered cells, e.g., transduced cells, are sorted from the non-engineered cells, e.g., the non-transduced cells using flow cytometry.

**[0289]** In some embodiments, the sorted engineered cells are used as an MCB. In some embodiments, the sorted engineered cells are used as a component in an infusion-ready drug product.

**[0290]** In some embodiments, the engineered cells, e.g., transduced cells, are sorted from the non-engineered cells, e.g., the non-transduced cells using a microfluidic cell sorting method. Microfluidic cell sorting methods are described, for example, in Dalili et al., “A Review of Sorting, Separation and Isolation of Cells and Microbeads for Biomedical Applications: Microfluidic Approaches,” *Analyst* 144:87 (2019).

**[0291]** In some embodiments, from or from about 1% to or to about 99% of the expanded and stimulated cells are engineered successfully, e.g., transduced successfully, e.g., transduced successfully with a vector comprising a heterologous protein, e.g., a heterologous protein comprising a CAR and/or IL-15 as described herein. In some embodiments, from or from about 1% to or to about 90%, from or from about 1% to or to about 80%, from or from about 1% to or to about 70%, from or from about 1% to or to about 60%, from or from about 1% to or to about 50%, from or from about 1% to or to about 40%, from or from about 1% to or to about 30%, from or from about 1% to or to about 20%, from or from about 1% to or to about 10%, from or from about 1% to or to about 5%, from or from about 5% to or to about 99%, from or from about 5% to or to about 90%, from or from about 5% to or to about 80%, from or from about 5% to or to about 70%, from or from about 5% to or to about 60%, from or from about 5% to or to about 50%, from or from about 5% to or to about 40%, from or from about 5% to or to about 30%, from or from about 5% to or to about 20%, from or from about 5% to or to about 10%, from or from about 10% to or to about 99%, from or from about 10% to or to about 90%, from or from about 10% to or to about 80%, from or from about 10% to or to about 70%, from or from about 10% to or to about 60%, from or from about 10% to or to about 50%, from or from about 10% to or to about 40%, from or from about 10% to or to about 30%, from or from about 10% to or to about 20%, from or from about 20% to or to about 99%, from or from about 20% to or to about 90%, from or from about 20% to or to about 80%, from or from about 20% to or to about 70%,

from or from about 20% to or to about 60%, from or from about 20% to or to about 50%, from or from about 20% to or to about 40%, from or from about 20% to or to about 30%, from or from about 30% to or to about 99%, from or from about 30% to or to about 90%, from or from about 30% to or to about 80%, from or from about 30% to or to about 70%, from or from about 30% to or to about 60%, from or from about 30% to or to about 50%, from or from about 30% to or to about 40%, from or from about 40% to or to about 99%, from or from about 40% to or to about 90%, from or from about 40% to or to about 80%, from or from about 40% to or to about 70%, from or from about 40% to or to about 60%, from or from about 40% to or to about 50%, from or from about 50% to or to about 99%, from or from about 50% to or to about 90%, from or from about 50% to or to about 80%, from or from about 50% to or to about 70%, from or from about 50% to or to about 60%, from or from about 60% to or to about 99%, from or from about 60% to or to about 90%, from or from about 60% to or to about 80%, from or from about 60% to or to about 70%, from or from about 70% to or to about 99%, from or from about 70% to or to about 90%, from or from about 70% to or to about 80%, from or from about 80% to or to about 99%, from or from about 80% to or to about 90%, or from or from about 90% to or to about 99% of the expanded and stimulated cells are engineered successfully, e.g., transduced successfully, e.g., transduced successfully with a vector comprising a heterologous protein, e.g., a heterologous protein comprising a CAR and/or IL-15 as described herein.

**[0292]** In some embodiments, frozen cells of a first or second MCB are thawed and cultured. In some embodiments, a single vial of frozen cells of the first or second MCB e.g., a single vial comprising 800 or about 800 million cells, e.g., first or second MCB cells, are thawed and cultured. In some embodiments, the frozen first or second MCB cells are cultured with additional feeder cells to produce cells suitable for use either as a second or third MCB or in an infusion-ready drug product. In some embodiments, the cells from the co-culture of the first or second MCB are harvested and frozen.

**[0293]** In some embodiments, the cells from the co-culture of the natural killer cell source, a first MCB, or a second MCB are harvested, and frozen in a cryopreservation composition, e.g., a cryopreservation composition described herein. In some embodiments, the cells are washed after harvesting. Thus, provided herein is a pharmaceutical composition comprising activated and stimulated NK cells, e.g., activated and stimulated NK cells produced by the methods described herein, e.g., harvested and washed activated and stimulated NK cells produced by the methods described herein and a cryopreservation composition, e.g., a cryopreservation composition described herein.

[0294] In some embodiments, the cells are mixed with a cryopreservation composition, e.g., as described herein, before freezing. In some embodiments, the cells are frozen in cryobags. In some embodiments, the cells are frozen in cryovials.

[0295] In some embodiments, the method further comprises isolating NK cells from the population of expanded and stimulated NK cells.

#### 4.5.5 Properties of Expanded and Stimulated NK Cells

[0296] After having been *ex vivo* expanded and stimulated, e.g., as described herein, the expanded and stimulated NK cell populations not only have a number/density (e.g., as described above) that could not occur naturally in the human body, but they also differ in their phenotypic characteristics, (e.g., gene expression and/or surface protein expression) with the starting source material or other naturally occurring populations of NK cells.

[0297] In some cases, the starting NK cell source is a sample derived from a single individual, e.g., a single cord blood unit that has not been *ex vivo* expanded. Therefore, in some cases, the expanded and stimulated NK cells share a common lineage, i.e., they all result from expansion of the starting NK cell source, and, therefore, share a genotype via clonal expansion of a population of cells that are, themselves, from a single organism. Yet, they could not occur naturally at the density achieved with *ex vivo* expansion and also differ in phenotypic characteristics from the starting NK cell source.

[0298] In some cases, the population of expanded and stimulated NK cells comprises at least 100 million expanded natural killer cells, e.g., 200 million, 250 million, 300 million, 400 million, 500 million, 600 million, 700 million, 750 million, 800 million, 900 million, 1 billion, 2 billion, 3 billion, 4 billion, 5 billion, 6 billion, 7 billion, 8 billion, 9 billion, 10 billion, 15 billion, 20 billion, 25 billion, 50 billion, 75 billion, 80 billion, 9- billion, 100 billion, 200 billion, 250 billion, 300 billion, 400 billion, 500 billion, 600 billion, 700 billion, 800 billion, 900 billion, 1 trillion, 2 trillion, 3 trillion, 4 trillion, 5 trillion, 6 trillion, 7 trillion, 8 trillion, 9 trillion, or 10 trillion expanded natural killer cells.

[0299] In some embodiments, the expanded and stimulated NK cells comprise at least 80%, e.g., at least 90%, at least 95%, at least 99%, or 100% CD56+CD3- cells. In some embodiments, the expanded and stimulated NK cells are not genetically engineered. In some embodiments, the expanded and stimulated NK cells do not comprise a CD16 transgene. In some embodiments, the expanded and stimulated NK cells do not express an exogenous CD16 protein.

[0300] The expanded and stimulated NK cells can be characterized, for example, by surface expression, e.g., of one or more of CD16, CD56, CD3, CD38, CD14, CD19, NKG2D, NKp46, NKp30, DNAM-1, and NKp44.

**[0301]** The surface protein expression levels stated herein, in some cases are achieved without positive selection on the particular surface protein referenced. For example, in some cases, the NK cell source, e.g., a single cord unit, comprises both the KIR B haplotype of the KIR receptor family and the 158 V/V variant of CD16 and is + enriched and CD3(+) depleted, e.g., by gating on CD56+CD3- expression, but no other surface protein expression selection is carried out during expansion and stimulation.

**[0302]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprise at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% NKG2D+ cells.

**[0303]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprise at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% NKp46+ cells.

**[0304]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprise at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% NKp30+ cells.

**[0305]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprise at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% DNAM-1+ cells.

**[0306]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprise at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% NKp44+ cells.

**[0307]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprise at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% CD94+ (KLRD1) cells.

**[0308]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprises less than or equal to 20%, e.g., less than or equal to 10%, less than or equal to 5%, less than or equal to 1% or 0% CD3+ cells.

**[0309]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprises less than or equal to 20%, e.g., less than or equal to 10%, less than or equal to 5%, less than or equal to 1% or 0% CD14+ cells.

**[0310]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprises less than or equal to 20%, e.g., less than or equal to 10%, less than or equal to 5%, less than or equal to 1% or 0% CD19+ cells.

**[0311]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprises less than or equal to 20%, e.g., less than or equal to 10%, less than or equal to 5%, less than or equal to 1% or 0% CXCR+ cells.

**[0312]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprises less than or equal to 20%, e.g., less than or equal to 10%, less than or equal to 5%, less than or equal to 1% or 0% CD122+ (IL2RB) cells.

**[0313]** In some embodiments, the NK cells expanded and stimulated by the methods described herein express CD16 at high levels throughout the expansion and stimulation process, resulting in a cell population with high CD16 expression. The high expression of CD16 obviates the need for engineering the expanded cells to express CD16, which is important for initiating ADCC. Thus, in some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprise 50% or more, e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% CD16+ NK cells.

**[0314]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprises both the KIR B haplotype of the KIR receptor family and the 158 V/V variant of CD16 and comprise 50% or more, e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% CD16+ NK cells.

**[0315]** In some embodiments, the percentage of expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, expressing CD16 is the same or higher than the percentage of natural killer cells in the seed cells from umbilical cord blood.

**[0316]** In some embodiments, the percentage of expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, expressing NKG2D is the same or higher than the percentage of natural killer cells in the seed cells from umbilical cord blood.

**[0317]** In some embodiments, the percentage of expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, expressing

NKp30 is the same or higher than the percentage of natural killer cells in the seed cells from umbilical cord blood.

**[0318]** In some embodiments, the percentage of expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, expressing DNAM-1 is the same or higher than the percentage of natural killer cells in the seed cells from umbilical cord blood.

**[0319]** In some embodiments, the percentage of expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, expressing NKp44 is the same or higher than the percentage of natural killer cells in the seed cells from umbilical cord blood.

**[0320]** In some embodiments, the percentage of expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, expressing NKp46 is the same or higher than the percentage of natural killer cells in the seed cells from umbilical cord blood.

**[0321]** In some embodiments, the NK cells expanded and stimulated by the methods described herein express CD38 at low levels. CD38 is an effective target for certain cancer therapies (e.g., multiple myeloma and acute myeloid leukemia). *See, e.g.,* Jiao et al., “CD38: Targeted Therapy in Multiple Myeloma and Therapeutic Potential for Solid Cancers,” *Expert Opinion on Investigational Drugs* 29(11):1295–1308 (2020). Thus, in some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprise less than or equal to 80% CD38+ cells, e.g., less than or equal to 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, or 20% CD38+ cells.

**[0322]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprises both the KIR B haplotype of the KIR receptor family and the 158 V/V variant of CD16 and comprise less than or equal to 80% CD38+ cells, e.g., less than or equal to 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, or 20% CD38+ cells.

**[0323]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprises both the KIR B haplotype of the KIR receptor family and the 158 V/V variant of CD16 and comprise less than or equal to 80% CD38+ cells, e.g., less than or equal to 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, or 20% CD38+ cells, and 50% or more, e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% CD16+ NK cells.

**[0324]** In some embodiments, the expanded and stimulated NK cells, e.g., from expansion and stimulation of a single cord blood unit, e.g., as described above, comprises both the KIR B haplotype of the KIR receptor family and the 158 V/V variant of CD16 and comprise: i) 50% or more, e.g., 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% CD16<sup>+</sup> NK cells; and/or ii) less than or equal to 80% CD38<sup>+</sup> cells, e.g., less than or equal to 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, or 20% CD38<sup>+</sup> cells; and/or iii) at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% NKG2D<sup>+</sup> cells; and/or iv) at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% NKp46<sup>+</sup> cells; and/or v) at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% NKp30<sup>+</sup> cells; and/or vi) at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% DNAM-1<sup>+</sup> cells; and/or vii) at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% NKp44<sup>+</sup> cells; and/or viii) at least 60%, e.g., at least 70%, at least 80%, at least 90% at least 95%, at least 99%, or 100% CD94<sup>+</sup> (KLRD1) cells; and/or ix) less than or equal to 20%, e.g., less than or equal to 10%, less than or equal to 5%, less than or equal to 1% or 0% CD3<sup>+</sup> cells; and/or x) less than or equal to 20%, e.g., less than or equal to 10%, less than or equal to 5%, less than or equal to 1% or 0% CD14<sup>+</sup> cells; and/or xi) less than or equal to 20%, e.g., less than or equal to 10%, less than or equal to 5%, less than or equal to 1% or 0% CD19<sup>+</sup> cells; and/or xii) less than or equal to 20%, e.g., less than or equal to 10%, less than or equal to 5%, less than or equal to 1% or 0% CXCR<sup>+</sup> cells; and/or xiii) less than or equal to 20%, e.g., less than or equal to 10%, less than or equal to 5%, less than or equal to 1% or 0% CD122<sup>+</sup> (IL2RB) cells.

**[0325]** In some embodiments, feeder cells do not persist in the expanded and stimulated NK cells, though, residual signature of the feeder cells may be detected, for example, by the presence of residual cells (e.g., by detecting cells with a particular surface protein expression) or residual nucleic acid and/or proteins that are expressed by the feeder cells.

**[0326]** For example, in some cases, the methods described herein include expanding and stimulating natural killer cells using engineered feeder cells, e.g., eHuT-78 feeder cells described above, which are engineered to express sequences that are not expressed by cells in the natural killer cell source, including the natural killer cells.

**[0327]** While these feeder cells may not persist in the expanded and stimulated NK cells, the expanded and stimulated NK cells may retain detectable residual amounts of cells, proteins, and/or nucleic acids from the feeder cells. Thus, their residual presence in the expanded and stimulated NK cells may be detected, for example, by detecting the cells themselves (e.g., by flow cytometry), proteins that they express, and/or nucleic acids that they express.

[0328] Thus, also described herein is a population of expanded and stimulated NK cells comprising residual feeder cells (live cells or dead cells) or residual feeder cell cellular impurities (e.g., residual feeder cell proteins or portions thereof, and/or genetic material such as a nucleic acid or portion thereof). In some cases, the expanded and stimulated NK cells comprise more than 0% and, but 0.3% or less residual feeder cells, e.g., eHuT-78 feeder cells.

[0329] A wide variety of different methods can be used to analyze and detect the presence of nucleic acids or protein gene products in a biological sample. As used herein, “detecting” can refer to a method used to discover, determine, or confirm the existence or presence of a compound and/or substance (e.g., a cell, a protein and/or a nucleic acid). In some embodiments, a detecting method can be used to detect a protein. In some embodiments, detecting can include chemiluminescence or fluorescence techniques. In some embodiments, detecting can include immunological-based methods (e.g., quantitative enzyme-linked immunosorbent assays (ELISA), Western blotting, or dot blotting) wherein antibodies are used to react specifically with entire proteins or specific epitopes of a protein. In some embodiments, detecting can include immunoprecipitation of the protein (Jungblut et al., *J Biotechnol.* 31;41(2-3):111-20 (1995); Franco et al., *Eur J Morphol.* 39(1):3-25 (2001)). In some embodiments, a detecting method can be used to detect a nucleic acid (e.g., DNA and/or RNA). In some embodiments, detecting can include Northern blot analysis, nuclease protection assays (NPA), in situ hybridization, or reverse transcription-polymerase chain reaction (RT-PCR) (Raj et al., *Nat. Methods* 5, 877–879 (2008); Jin et al., *J Clin Lab Anal.* 11(1):2-9 (1997); Ahmed, *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev.* 20(2):77-116 (2002)).

[0330] Thus, also described herein, are methods for detecting a population of expanded and stimulated NK cells, e.g., expanded and stimulated using the methods described herein, that have been co-cultured with engineered feeder cells, e.g., eHuT-78 feeder cells described herein.

#### 4.6 Engineering of NK Cells

[0331] In some embodiments, the method further comprises engineering NK cells, e.g., to express a heterologous protein, e.g., a heterologous protein described herein, e.g., a heterologous protein comprising a CAR and/or IL-15.

[0332] In some embodiments, engineering the NK cells to express a heterologous protein described herein comprises transforming or transducing, e.g., stably transforming or transducing the NK cells with a vector comprising a polynucleic acid encoding a heterologous protein described herein. Suitable vectors are described herein.

[0333] In some embodiments, engineering the NK cells to express a heterologous protein described herein comprises introducing the heterologous protein via gene editing (e.g., zinc

finger nuclease (ZFN) gene editing, ARCUS gene editing, CRISPR-Cas9 gene editing, or megaTAL gene editing) combined with adeno-associated virus (AAV) technology.

**[0334]** In some embodiments, the NK cells are engineered to express a heterologous protein described herein, e.g., during or after culturing the composition in a medium comprising feeder cells. For example, in some cases, engineering (e.g., transduction) occurs during the expansion and stimulation process described herein, e.g., during co-culturing NK cell source(s) and feeder cells as described herein, e.g., at day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 of co-culturing.

**[0335]** In some embodiments, the method further comprises engineering NK cells, e.g., to express, over-express, knock-out, or knock-down gene(s) or gene product(s).

**[0336]** In some embodiments, the natural killer cells are not genetically engineered.

**[0337]** In some embodiments, the NK cells are engineered (e.g., transduced) in a culture medium supplemented with a stimulating factor (e.g., as described herein). Such cytokines can be used to provide growth or survival signals to the NK cells during the engineering process or to increase transduction efficiency. In some embodiments, the stimulation factor(s) are cytokine(s). In some embodiments, the cytokine(s) are selected from the group consisting of IL-2, IL-12, IL-15, IL-18, IL-21, IL-23, IL-27, IFN- $\alpha$ , IFN $\beta$ , and combinations thereof.

**[0338]** In some embodiments, the cytokine is IL-21. IL-21 can be used, for example, at a final concentration of between 10 and 100 ng/mL, including, for example, at or at about 10, 15, 20, 25, 30, 34, 40, 45, 50, 55, 60, 70, 80, 90, or 100 ng/mL. In some embodiments, the cytokine is IL-2. In some embodiments, the cytokines are a combination of IL-2 and IL-21. In some embodiments, the cytokines are a combination of IL-2, IL-18, and IL-21.

**[0339]** In some embodiments, the stimulating factor is added to the culture medium at the time of engineering (e.g., transduction). In some embodiments, the stimulating factor is added to the culture medium after the time of engineered (e.g., transducing), e.g., from 1 to 48 hours after engineering, e.g., from 1 to 36, 1 to 24, 1 to 12, 12 to 28, 12 to 36, 12 to 24, 24 to 48, 24 to 36, or 36 to 48 hours after engineering. In some embodiments, the stimulating factor is added to the culture medium both at the time of transduction and after the time of engineering (e.g., from 1 to 48 hours after transduction).

**[0340]** In some embodiments, the culture is supplemented with the stimulating factor after culturing in a medium comprising feeder cells. Thus, in some cases, the culture medium will contain feeder cells at the time of engineering (e.g., transduction). In some cases, the feeder cells are removed from the culture prior to supplementation with the stimulating factor or engineering. In some cases, the feeder cells are not removed from the culture prior to supplementation with

the stimulating factor or engineering. In some cases, no additional feeder cells are added to the culture during engineering, whether or not any residual feeder cells are removed. In some cases, both additional feeder cells and a stimulating factor are added to the culture during engineering. In some cases, additional feeder cells are not added to the culture during engineering but stimulating factors are added to the culture during engineering.

## 4.7 Cryopreservation

### 4.7.1 Cryopreservation Compositions

**[0341]** Provided herein are cryopreservation compositions, e.g., cryopreservation compositions suitable for intravenous administration, e.g., intravenous administration of NK cells, e.g., the NK cells described herein. In some embodiments, a pharmaceutical composition comprises the cryopreservation composition and cells, e.g., the NK cells described herein.

#### 4.7.1.1 Albumin

**[0342]** In some embodiments, the cryopreservation composition comprises albumin protein, e.g., human albumin protein (UniProtKB Accession P0278) or variant thereof. In some embodiments, the cryopreservation composition comprises an ortholog of an albumin protein, e.g., human albumin protein, or variant thereof. In some embodiments, the cryopreservation composition comprises a biologically active portion of an albumin protein, e.g., human albumin, or variant thereof.

**[0343]** In some embodiments, the albumin, e.g., human albumin, is provided as a solution, also referred to herein as an albumin solution or a human albumin solution. Thus, in some embodiments, the cryopreservation composition is or comprises an albumin solution, e.g., a human albumin solution. In some embodiments, the albumin solution is a serum-free albumin solution.

**[0344]** In some embodiments, the albumin solution is suitable for intravenous use.

**[0345]** In some embodiments, the albumin solution comprises from or from about 40 to or to about 200 g/L albumin. In some embodiments, the albumin solution comprises from or from about 40 to or to about 50 g/L albumin, e.g., human albumin. In some embodiments, the albumin solution comprises about 200 g/L albumin, e.g., human albumin. In some embodiments, the albumin solution comprises 200 g/L albumin, e.g., human albumin.

**[0346]** In some embodiments, the albumin solution comprises a protein composition, of which 95% or more is albumin protein, e.g., human albumin protein. In some embodiments, 96%, 97%, 98%, or 99% or more of the protein is albumin, e.g., human albumin.

**[0347]** In some embodiments, the albumin solution further comprises sodium. In some embodiments, the albumin solution comprises from or from about 100 to or to about 200 mmol

sodium. In some embodiments, the albumin solution comprises from or from about 130 to or to about 160 mmol sodium.

**[0348]** In some embodiments, the albumin solution further comprises potassium. In some embodiments, the albumin solution comprises 3 mmol or less potassium. In some embodiments, the albumin solution further comprises 2 mmol or less potassium.

**[0349]** In some embodiments, the albumin solution further comprises one or more stabilizers. In some embodiments, the stabilizer(s) are selected from the group consisting of sodium caprylate, caprylic acid, (2*S*)-2-acetamido-3-(1*H*-indol-3-yl)propanoic acid (also referred to as acetyl tryptophan, N-Acetyl-L-tryptophan and Acetyl-L-tryptophan), 2-acetamido-3-(1*H*-indol-3-yl)propanoic acid (also referred to as N-acetyltryptophan, DL-Acetyltryptophan and N-Acetyl-DL-tryptophan). In some embodiments, the solution comprises less than .1 mmol of each of the one or more stabilizers per gram of protein in the solution. In some embodiments, the solution comprises from or from about 0.05 to or to about 0.1, e.g., from or from about 0.064 to or to about 0.096 mmol of each of the stabilizers per gram of protein in the solution. In some embodiments, the solution comprises less than 0.1 mmol of total stabilizer per gram of protein in the solution. In some embodiments, the solution comprises from or from about 0.05 to or to about 0.1, e.g., from or from about 0.064 to or to about 0.096 mmol of total stabilizer per gram of protein in the solution.

**[0350]** In some embodiments, the albumin solution consists of a protein composition, of which 95% or more is albumin protein, sodium, potassium, and one or more stabilizers selected from the group consisting of sodium caprylate, caprylic acid, (2*S*)-2-acetamido-3-(1*H*-indol-3-yl)propanoic acid (also referred to as acetyl tryptophan, N-Acetyl-L-tryptophan and Acetyl-L-tryptophan), 2-acetamido-3-(1*H*-indol-3-yl)propanoic acid (also referred to as N-acetyltryptophan, DL-Acetyltryptophan and N-Acetyl-DL-tryptophan) in water.

**[0351]** In some embodiments, the cryopreservation composition comprises from or from about 10% v/v to or to about 50% v/v of an albumin solution, e.g., an albumin solution described herein. In some embodiments, the cryopreservation composition comprises from or from about 10% to or to about 50%, from or from about 10% to or to about 45%, from or from about 10% to or to about 40%, from or from about 10% to or to about 35%, from or from about 10% to or to about 30%, from or from about 10% to or to about 25%, from or from about 10% to or to about 20%, from or from about 10% to or to about 15%, from or from about 15% to or to about 50%, from or from about 15% to or to about 45%, from or from about 15% to or to about 40%, from or from about 15% to or to about 35%, from or from about 15% to or to about 30%, from or from about 15% to or to about 25%, from or from about 15% to or to about 20%, from or from about

20% to or to about 50%, from or from about 20% to or to about 45%, from or from about 20% to or to about 40%, from or from about 20% to or to about 35%, from or from about 20% to or to about 30%, from or from about 20% to or to about 25%, from or from about 25% to or to about 50%, from or from about 25% to or to about 45%, from or from about 25% to or to about 40%, from or from about 25% to or to about 35%, from or from about 25% to or to about 30%, from or from about 30% to or to about 50%, from or from about 30% to or to about 45%, from or from about 30% to or to about 40%, from or from about 30% to or to about 35%, from or from about 35% to or to about 50%, from or from about 35% to or to about 45%, from or from about 35% to or to about 40%, from or from about 40% to or to about 50%, from or from about 40% to or to about 45%, or from or from about 45% to or to about 50% v/v of an albumin solution described herein. In some embodiments, the cryopreservation composition comprises about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, or about 50% v/v of an albumin solution described herein. In some embodiments, the cryopreservation composition comprises 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% v/v of an albumin solution described herein.

**[0352]** In some embodiments, the cryopreservation composition comprises from or from about 20 to or to about 100 g/L albumin, e.g., human albumin. In some embodiments, the cryopreservation composition comprises from or from about 20 to or to about 100, from or from about 20 to or to about 90, from or from about 20 to or to about 80, from or from about 20 to or to about 70, from or from about 20 to or to about 60, from or from about 20 to or to about 50, from or from about 20 to or to about 40, from or from about 20 to or to about 30, from or from about 30 to or to about 100, from or from about 30 to or to about 90, from or from about 30 to or to about 80, from or from about 30 to or to about 70, from or from about 30 to or to about 60, from or from about 30 to or to about 50, from or from about 30 to or to about 40, from or from about 40 to or to about 100, from or from about 40 to or to about 90, from or from about 40 to or to about 80, from or from about 40 to or to about 70, from or from about 40 to or to about 60, from or from about 40 to or to about 50, from or from about 50 to or to about 100, from or from about 50 to or to about 90, from or from about 50 to or to about 80, from or from about 50 to or to about 70, from or from about 50 to or to about 60, from or from about 60 to or to about 100, from or from about 60 to or to about 90, from or from about 60 to or to about 80, from or from about 60 to or to about 70, from or from about 70 to or to about 100, from or from about 70 to or to about 90, from or from about 70 to or to about 80, from or from about 80 to or to about 100, from or from about 80 to or to about 90, or from or from about 90 to or to about 100 g/L albumin, e.g., human albumin.

**[0353]** In some embodiments, the cryopreservation composition comprises 20 g/L albumin, e.g., human albumin. In some embodiments, the cryopreservation composition comprises 40 g/L albumin, e.g., human albumin. In some embodiments, the cryopreservation composition comprises 70 g/L albumin, e.g., human albumin. In some embodiments, the cryopreservation composition comprises 100 g/L albumin, e.g., human albumin.

**[0354]** In some embodiments, the cryopreservation composition comprises about 20 g/L albumin, e.g., human albumin. In some embodiments, the cryopreservation composition comprises about 40 g/L albumin, e.g., human albumin. In some embodiments, the cryopreservation composition comprises about 70 g/L albumin, e.g., human albumin. In some embodiments, the cryopreservation composition comprises about 100 g/L albumin, e.g., human albumin.

**[0355]** In some embodiments, the cryopreservation composition further comprises a stabilizer, e.g., an albumin stabilizer. In some embodiments, the stabilizer(s) are selected from the group consisting of sodium caprylate, caprylic acid, (2*S*)-2-acetamido-3-(1*H*-indol-3-yl)propanoic acid (also referred to as acetyl tryptophan, N-Acetyl-L-tryptophan and Acetyl-L-tryptophan), 2-acetamido-3-(1*H*-indol-3-yl)propanoic acid (also referred to as N-acetyltryptophan, DL-Acetyltryptophan and N-Acetyl-DL-tryptophan). In some embodiments, the cryopreservation composition comprises less than .1 mmol of each of the one or more stabilizers per gram of protein, e.g., per gram of albumin protein, in the composition. In some embodiments, the cryopreservation composition comprises from or from about 0.05 to or to about 0.1, e.g., from or from about 0.064 to or to about 0.096 mmol of each of the stabilizers per gram of protein, e.g., per gram of albumin protein in the composition. In some embodiments, the cryopreservation composition comprises less than 0.1 mmol of total stabilizer per gram of protein, e.g., per gram of albumin protein in the cryopreservation composition. In some embodiments, the cryopreservation composition comprises from or from about 0.05 to or to about 0.1, e.g., from or from about 0.064 to or to about 0.096 mmol of total stabilizer per gram of protein, e.g., per gram of albumin protein, in the cryopreservation composition.

#### *4.7.1.2 Dextran*

**[0356]** In some embodiments, the cryopreservation composition comprises Dextran, or a derivative thereof.

**[0357]** Dextran is a polymer of anhydroglucose composed of approximately 95%  $\alpha$ -D-(1-6) linkages (designated  $(C_6H_{10}O_5)_n$ ). Dextran fractions are supplied in molecular weights of from about 1,000 Daltons to about 2,000,000 Daltons. They are designated by number (Dextran X), e.g., Dextran 1, Dextran 10, Dextran 40, Dextran 70, and so on, where X corresponds to the

mean molecular weight divided by 1,000 Daltons. So, for example, Dextran 40 has an average molecular weight of or about 40,000 Daltons.

**[0358]** In some embodiments, the average molecular weight of the dextran is from or from about 1,000 Daltons to or to about 2,000,000 Daltons. In some embodiments, the average molecular weight of the dextran is or is about 40,000 Daltons. In some embodiments, the average molecular weight of the dextran is or is about 70,000 Daltons.

**[0359]** In some embodiments, the dextran is selected from the group consisting of Dextran 40, Dextran 70, and combinations thereof. In some embodiments, the dextran is Dextran 40.

**[0360]** In some embodiments, the dextran, e.g., Dextran 40, is provided as a solution, also referred to herein as a dextran solution or a Dextran 40 solution. Thus, in some embodiments, the composition comprises a dextran solution, e.g., a Dextran 40 solution.

**[0361]** In some embodiments, the dextran solution is suitable for intravenous use.

**[0362]** In some embodiments, the dextran solution comprises about 5% to about 50% w/w dextran, e.g., Dextran 40. In some embodiments, the dextran solution comprises from or from about 5% to or to about 50%, from or from about 5% to or to about 45%, from or from about 5% to or to about 40%, from or from about 5% to or to about 35%, from or from about 5% to or to about 30%, from or from about 5% to or to about 25%, from or from about 5% to or to about 20%, from or from about 5% to or to about 15%, from or from about 5% to or to about 10%, from or from about 10% to or to about 50%, from or from about 10% to or to about 45%, from or from about 10% to or to about 40%, from or from about 10% to or to about 35%, from or from about 10% to or to about 30%, from or from about 10% to or to about 25%, from or from about 10% to or to about 20%, from or from about 10% to or to about 15%, from or from about 15% to or to about 50%, from or from about 15% to or to about 45%, from or from about 15% to or to about 40%, from or from about 15% to or to about 35%, from or from about 15% to or to about 30%, from or from about 15% to or to about 25%, from or from about 15% to or to about 20%, from or from about 20% to or to about 50%, from or from about 20% to or to about 45%, from or from about 20% to or to about 40%, from or from about 20% to or to about 35%, from or from about 20% to or to about 30%, from or from about 20% to or to about 25%, from or from about 25% to or to about 50%, from or from about 25% to or to about 45%, from or from about 25% to or to about 40%, from or from about 25% to or to about 35%, from or from about 25% to or to about 30%, from or from about 30% to or to about 50%, from or from about 30% to or to about 45%, from or from about 30% to or to about 40%, from or from about 30% to or to about 35%, from or from about 35% to or to about 50%, from or from about 35% to or to about 45%, from or from about 35% to or to about 40%, from or from about 40% to or to about 50%, from or from

about 40% to or to about 45%, or from or from about 45% to or to about 50% w/w dextran, e.g., Dextran 40. In some embodiments, the dextran solution comprises 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% w/w dextran, e.g., Dextran 40. In some embodiments, the dextran solution comprises about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, or about 50% w/w dextran, e.g., Dextran 40.

**[0363]** In some embodiments, the dextran solution comprises from or from about 25 g/L to or to about 200 g/L dextran, e.g., Dextran 40. In some embodiments, the dextran solution comprises from or from about 35 to or to about 200, from or from about 25 to or to about 175, from or from about 25 to or to about 150, from or from about 25 to or to about 125, from or from about 25 to or to about 100, from or from about 25 to or to about 75, from or from about 25 to or to about 50, from or from about 50 to or to about 200, from or from about 50 to or to about 175, from or from about 50 to or to about 150, from or from about 50 to or to about 125, from or from about 50 to or to about 100, from or from about 50 to or to about 75, from or from about 75 to or to about 200, from or from about 75 to or to about 175, from or from about 75 to or to about 150, from or from about 75 to or to about 125, from or from about 75 to or to about 100, from or from about 100 to or to about 200, from or from about 100 to or to about 175, from or from about 100 to or to about 150, from or from about 100 to or to about 125, from or from about 125 to or to about 200, from or from about 125 to or to about 175, from or from about 125 to or to about 150, from or from about 150 to or to about 200, from or from about 150 to or to about 175, or from or from about 175 to or to about 200 g/L dextran e.g., Dextran 40. In some embodiments, the dextran solution comprises 25, 50, 75, 100, 125, 150, 175, or 200 g/L dextran, e.g., Dextran 40. In some embodiments, the dextran solution comprises 100 g/L dextran, e.g., Dextran 40. In some embodiments, the dextran solution comprises about 25, about 50, about 75, about 100, about 125, about 150, about 175, or about 200 g/L dextran, e.g., Dextran 40. In some embodiments, the dextran solution comprises about 100 g/L dextran, e.g., Dextran 40.

**[0364]** In some embodiments, the dextran solution further comprises glucose (also referred to as dextrose). In some embodiments, the dextran solution comprises from or from about 10 g/L to or to about 100 g/L glucose. In some embodiments, the dextran solution comprises from or from about 10 to or to about 100, from or from about 10 to or to about 90, from or from about 10 to or to about 80, from or from about 10 to or to about 70, from or from about 10 to or to about 60, from or from about 10 to or to about 50, from or from about 10 to or to about 40, from or from about 10 to or to about 30, from or from about 10 to or to about 20, from or from about 20 to or to about 100, from or from about 20 to or to about 90, from or from about 20 to or to about 80, from or from about 20 to or to about 70, from or from about 20 to or to about 60, from or from

about 20 to or to about 50, from or from about 20 to or to about 40, from or from about 20 to or to about 30, from or from about 30 to or to about 100, from or from about 30 to or to about 90, from or from about 30 to or to about 80, from or from about 30 to or to about 70, from or from about 30 to or to about 60, from or from about 30 to or to about 50, from or from about 30 to or to about 40, from or from about 40 to or to about 100, from or from about 40 to or to about 90, from or from about 40 to or to about 80, from or from about 40 to or to about 70, from or from about 40 to or to about 60, from or from about 40 to or to about 50, from or from about 50 to or to about 100, from or from about 50 to or to about 90, from or from about 50 to or to about 80, from or from about 50 to or to about 70, from or from about 50 to or to about 60, from or from about 60 to or to about 100, from or from about 60 to or to about 90, from or from about 60 to or to about 80, from or from about 60 to or to about 70, from or from about 70 to or to about 100, from or from about 70 to or to about 90, from or from about 70 to or to about 80, from or from about 80 to or to about 90, from or from about 80 to or to about 100, from or from about 80 to or to about 90, or from or from about 90 to or to about 100 g/L glucose. In some embodiments, the dextran solution comprises 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 g/L glucose. In some embodiments, the dextran solution comprises 50 g/L glucose. In some embodiments, the dextran solution comprises about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, or about 100 g/L glucose. In some embodiments, the dextran solution comprises 50 g/L glucose.

**[0365]** In some embodiments, the dextran solution consists of dextran, e.g., Dextran 40, and glucose in water.

**[0366]** In some embodiments, the cryopreservation composition comprises from or from about 10% v/v to or to about 50% v/v of a dextran solution described herein. In some embodiments, the cryopreservation composition comprises from or from about 10% to 50%, from or from about 10% to or to about 45%, from or from about 10% to or to about 40%, from or from about 10% to or to about 35%, from or from about 10% to or to about 30%, from or from about 10% to or to about 25%, from or from about 10% to or to about 20%, from or from about 10% to or to about 15%, from or from about 15% to or to about 50%, from or from about 15% to or to about 45%, from or from about 15% to or to about 40%, from or from about 15% to or to about 35%, from or from about 15% to or to about 30%, from or from about 15% to or to about 25%, from or from about 15% to or to about 20%, from or from about 20% to or to about 50%, from or from about 20% to or to about 45%, from or from about 20% to or to about 40%, from or from about 20% to or to about 35%, from or from about 20% to or to about 30%, from or from about 20% to or to about 25%, from or from about 25% to or to about 50%, from or from about

25% to or to about 45%, from or from about 25% to or to about 40%, from or from about 25% to or to about 35%, from or from about 25% to or to about 30%, from or from about 30% to or to about 50%, from or from about 30% to or to about 45%, from or from about 30% to or to about 40%, from or from about 30% to or to about 35%, from or from about 35% to or to about 50%, from or from about 35% to or to about 45%, from or from about 35% to or to about 40%, from or from about 40% to or to about 50%, from or from about 40% to or to about 45%, or from or from about 45% to or to about 50% v/v of a dextran solution, e.g., a dextran solution described herein. In some embodiments, the cryopreservation composition comprises 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% v/v of a dextran solution, e.g., a dextran solution described herein. In some embodiments, the cryopreservation composition comprises about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, or about 50% v/v of a dextran solution, e.g., a dextran solution described herein.

**[0367]** In some embodiments, the cryopreservation composition comprises from or from about 10 to or to about 50 g/L dextran, e.g., Dextran 40. In some embodiments, the cryopreservation composition comprises from or from about 10 to or to about 50, from or from about 10 to or to about 45, from or from about 10 to or to about 40, from or from about 10 to or to about 35, from or from about 10 to or to about 30, from or from about 10 to or to about 25, from or from about 10 to or to about 20, from or from about 10 to or to about 15, from or from about 15 to or to about 50, from or from about 15 to or to about 45, from or from about 15 to or to about 40, from or from about 15 to or to about 35, from or from about 15 to or to about 30, from or from about 15 to or to about 25, from or from about 15 to or to about 20, from or from about 20 to or to about 50, from or from about 20 to or to about 45, from or from about 20 to or to about 40, from or from about 20 to or to about 30, from or from about 20 to or to about 25, from or from about 25 to or to about 50, from or from about 25 to or to about 45, from or from about 25 to or to about 40, from or from about 25 to or to about 35, from or from about 25 to or to about 30, from or from about 30 to or to about 50, from or from about 30 to or to about 45, from or from about 30 to or to about 40, from or from about 30 to or to about 35, from or from about 35 to or to about 50, from or from about 35 to or to about 45, from or from about 35 to or to about 40, from or from about 40 to or to about 50, from or from about 40 to or to about 45, or from or from about 45 to or to about 50 g/L dextran, e.g., Dextran 40. In some embodiments, the cryopreservation composition comprises 10, 15, 20, 25, 30, 30, 35, 40, 45, or 50 g/L dextran, e.g., Dextran 40. In some embodiments, the cryopreservation composition comprises about 10, about 15, about 20, about 25, about 30, about 30, about 35, about 40, about 45, or about 50 g/L dextran, e.g., Dextran 40.

#### 4.7.1.3 Glucose

[0368] In some embodiments, the cryopreservation composition comprises glucose.

[0369] In some embodiments, as described above, the cryopreservation composition comprises a Dextran solution comprising glucose.

[0370] In some embodiments, the cryopreservation composition comprises a Dextran solution that does not comprise glucose. In some embodiments, e.g., when the Dextran solution does not comprise glucose, glucose is added separately to the cryopreservation composition.

[0371] In some embodiments, the cryopreservation composition comprises from or from about 5 to or to about 25 g/L glucose. In some embodiments, the cryopreservation composition comprises from or from about 5 to or to about 25, from or from about 5 to or to about 20, from or from about 5 to or to about 15, from or from about 5 to or to about 10, from or from about 10 to or to about 25, from or from about 10 to or to about 20, from or from about 10 to or to about 15, from or from about 15 to or to about 25, from or from about 15 to or to about 20, or from or from about 20 to or to about 25 g/L glucose. In some embodiments, the cryopreservation composition comprises 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, or 25 g/L glucose. In some embodiments, the cryopreservation composition comprises 12.5 g/L glucose. In some embodiments, the cryopreservation composition comprises about 5, about 7.5, about 10, about 12.5, about 15, about 17.5, about 20, about 22.5, or about 25 g/L glucose. In some embodiments, the cryopreservation composition comprises about 12.5 g/L glucose.

[0372] In some embodiments, the cryopreservation composition comprises less than 2.75% w/v glucose. In some embodiments, the cryopreservation composition comprises less than 27.5 g/L glucose. In some embodiments, the cryopreservation composition comprises less than 2% w/v glucose. In some embodiments, the cryopreservation composition comprises less than 1.5% w/v glucose. In some embodiments, the cryopreservation composition comprises about 1.25% w/v or less glucose.

#### 4.7.1.4 Dimethyl Sulfoxide

[0373] In some embodiments, the cryopreservation composition comprises dimethyl sulfoxide (DMSO, also referred to as methyl sulfoxide and methylsulfinylmethane).

[0374] In some embodiments, the DMSO is provided as a solution, also referred to herein as a DMSO solution. Thus, in some embodiments, the cryopreservation composition comprises a DMSO solution.

[0375] In some embodiments, the DMSO solution is suitable for intravenous use.

[0376] In some embodiments, the DMSO solution comprises 1.1 g/mL DMSO. In some embodiments, the DMSO solution comprises about 1.1 g/mL DMSO.

**[0377]** In some embodiments, the cryopreservation composition comprises from or from about 1% to or to about 10% v/v of the DMSO solution. In some embodiments, the cryopreservation composition comprises from or from about 1% to or to about 10%, from or from about 1% to or to about 9%, from or from about 1% to or to about 8%, from or from about 1% to or to about 7%, from or from about 1% to or to about 6%, from or from about 1% to or to about 5%, from or from about 1% to or to about 4%, from or from about 1% to or to about 3%, from or from about 1% to or to about 2%, from or from about 2% to or to about 10%, from or from about 2% to or to about 9%, from or from about 8%, from or from about 2% to or to about 7%, from or from about 2% to or to about 6%, from or from about 2% to or to about 5%, from or from about 2% to or to about 4%, from or from about 2% to or to about 3%, from or from about 3% to or to about 10%, from or from about 3% to or to about 9%, from or from about 3% to or to about 8%, from or from about 3% to or to about 7%, from or from about 3% to or to about 6%, from or from about 3% to or to about 5%, from or from about 3% to or to about 4%, from or from about 4% to or to about 10%, from or from about 4% to or to about 9%, from or from about 4% to or to about 8%, from or from about 4% to or to about 7%, from or from about 4% to or to about 6%, from or from about 4% to or to about 5%, from or from about 5% to or to about 10%, from or from about 5% to or to about 9%, from or from about 5% to or to about 8%, from or from about 5% to or to about 7%, from or from about 5% to or to about 6%, from or from about 6% to or to about 10%, from or from about 6% to or to about 9%, from or from about 6% to or to about 8%, from or from about 6% to or to about 7%, from or from about 7% to or to about 10%, from or from about 7% to or to about 9%, from or from about 7% to or to about 8%, from or from about 8% to or to about 10%, from or from about 8% to or to about 9%, or from or from about 9% to or to about 10% v/v of the DMSO solution. In some embodiments, the cryopreservation composition comprises 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% v/v of the DMSO solution. In some embodiments, the cryopreservation composition comprises 5% of the DMSO solution. In some embodiments, the cryopreservation composition comprises about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, or about 10% v/v of the DMSO solution. In some embodiments, the cryopreservation composition comprises about 5% of the DMSO solution.

**[0378]** In some embodiments, the cryopreservation composition comprises from or from about 11 to or to about 110 g/L DMSO. In some embodiments, from or from about the cryopreservation composition comprises from or from about 11 to or to about 110, from or from about 11 to or to about 99, from or from about 11 to or to about 88, from or from about 11 to or to about 77, from or from about 11 to or to about 66, from or from about 11 to or to about 55,

from or from about 11 to or to about 44, from or from about 11 to or to about 33, from or from about 11 to or to about 22, from or from about 22 to or to about 110, from or from about 22 to or to about 99, from or from about 22 to or to about 88, from or from about 22 to or to about 77, from or from about 22 to or to about 77, from or from about 22 to or to about 66, from or from about 22 to or to about 55, from or from about 22 to or to about 44, from or from about 22 to or to about 33, from or from about 33 to or to about 110, from or from about 33 to or to about 99, from or from about 33 to or to about 88, from or from about 33 to or to about 77, from or from about 33 to or to about 66, from or from about 33 to or to about 55, from or from about 33 to or to about 44, from or from about 44 to or to about 110, from or from about 44 to or to about 99, from or from about 44 to or to about 88, from or from about 44 to or to about 77, from or from about 44 to or to about 66, from or from about 44 to or to about 55, from or from about 55 to or to about 110, from or from about 55 to or to about 99, from or from about 55 to or to about 88, from or from about 55 to or to about 77, from or from about 55 to or to about 66, from or from about 66 to or to about 110, from or from about 66 to or to about 99, from or from about 66 to or to about 88, from or from about 66 to or to about 77, from or from about 77 to or to about 119, from or from about 77 to or to about 88, from or from about 88 to or to about 110, from or from about 88 to or to about 99, or from or from about 99 to or to about 110 g/L DMSO. In some embodiments, the cryopreservation composition comprises 11, 22, 33, 44, 55, 66, 77, 88, 99, or 110 g/L DMSO. In some embodiments, the cryopreservation composition comprises 55 g/L DMSO. In some embodiments, the cryopreservation composition comprises about 11, about 22, about 33, about 44, about 55, about 66, about 77, about 88, about 99, or about 110 g/L DMSO. In some embodiments, the cryopreservation composition comprises about 55 g/L DMSO.

#### *4.7.1.5 Buffers*

**[0379]** In some embodiments, the cryopreservation composition comprises a buffer solution, e.g., a buffer solution suitable for intravenous administration.

**[0380]** Buffer solutions include, but are not limited to, phosphate buffered saline (PBS), Ringer's Solution, Tyrode's buffer, Hank's balanced salt solution, Earle's Balanced Salt Solution, saline, and Tris.

**[0381]** In some embodiments, the buffer solution is phosphate buffered saline (PBS).

#### *4.7.1.6 Exemplary Cryopreservation Compositions*

**[0382]** In some embodiments, the cryopreservation composition comprises or consists of: 1) albumin, e.g., human albumin, 2) dextran, e.g., Dextran 40, 3) DMSO, and 4) a buffer solution. In some embodiments, the cryopreservation composition further comprises glucose. In some

embodiments, the cryopreservation composition consists of 1) albumin, e.g., human albumin, 2) dextran, e.g., Dextran 40, 3) glucose, 4) DMSO, and 5) a buffer solution.

**[0383]** In some embodiments, the cryopreservation composition comprises: 1) an albumin solution described herein, 2) a dextran solution described herein, 3) a DMSO solution described herein, and 4) a buffer solution.

**[0384]** In some embodiments, the cryopreservation composition consists of: 1) an albumin solution described herein, 2) a dextran solution described herein, 3) a DMSO solution described herein, and 4) a buffer solution.

**[0385]** In some embodiments, the cryopreservation composition does not comprise a cell culture medium.

**[0386]** In one embodiment, the cryopreservation composition comprises or comprises about 40 mg/mL human albumin, 25 mg/mL Dextran 40, 12.5 mg/mL glucose, and 55 mg/mL DMSO.

**[0387]** In one embodiment, the cryopreservation composition comprises or comprises about or consists of or consists of about 40 mg/mL human albumin, 25 mg/mL Dextran 40, 12.5 mg/mL glucose, 55 mg/mL DMSO, and 0.5 mL/mL 100% phosphate buffered saline (PBS) in water.

**[0388]** In one embodiment, the cryopreservation composition comprises or comprises about 32 mg/mL human albumin, 25 mg/mL Dextran 40, 12.5 mg/mL glucose, and 55 mg/mL DMSO.

**[0389]** In one embodiment, the cryopreservation composition comprises or comprises about or consists of or consists of about 32 mg/mL human albumin, 25 mg/mL Dextran 40, 12.5 mg/mL glucose, 55 mg/mL DMSO, and 0.54 mL/mL 100% phosphate buffered saline (PBS) in water.

**[0390]** Exemplary Cryopreservation Compositions are shown in **Tables 8-11**.

**Table 8. Exemplary cryopreservation compositions**

| Excipient Solution  | Concentration Range of Solution                        | Exemplary Solution Concentration   | Exemplary Range v/v% in Cryopreservation Composition |
|---------------------|--------------------------------------------------------|------------------------------------|------------------------------------------------------|
| Albumin Solution    | 40–200 g/L albumin in water                            | 200 g/L albumin                    | 10%–50%                                              |
| Dextran 40 Solution | 25–200 g/L Dextran 40; and 0–100 g/L glucose; in water | 100 g/L Dextran 40; 50 g/L glucose | 10%–50%                                              |
| DMSO                | 11–110 g/L DMSO in water                               | 1,100 g/L DMSO                     | 1%–10%                                               |
| Buffer              | to volume                                              | to volume                          | to volume                                            |

**Table 9. Exemplary cryopreservation composition #1**

| Excipient Solution  | Solution Composition                             | Exemplary v/v% in Cryopreservation Composition #1 | Final Concentration in Cryopreservation Composition #1 |
|---------------------|--------------------------------------------------|---------------------------------------------------|--------------------------------------------------------|
| Albumin Solution    | 200 g/L albumin in water                         | 20%                                               | 40 mg/mL albumin                                       |
| Dextran 40 Solution | 100 g/L Dextran 40; and 50 g/L glucose; in water | 25%                                               | 25 mg/mL Dextran 40; 12.5 mg/mL glucose                |
| DMSO                | 100% DMSO (1,100 g/L)                            | 5%                                                | 55 mg/mL                                               |
| Buffer              | 100% Phosphate Buffered Saline (PBS)             | 50%                                               | 0.5 mL/mL                                              |

**Table 10. Exemplary cryopreservation composition #2**

| Excipient Solution  | Solution Composition                             | Exemplary v/v% in Cryopreservation Composition #2 | Final Concentration in Cryopreservation Composition #2 |
|---------------------|--------------------------------------------------|---------------------------------------------------|--------------------------------------------------------|
| Albumin Solution    | 200 g/L albumin in water                         | 16%                                               | 32 mg/mL albumin                                       |
| Dextran 40 Solution | 100 g/L Dextran 40; and 50 g/L glucose; in water | 25%                                               | 25 mg/mL Dextran 40; 12.5 mg/mL glucose                |
| DMSO                | 100% DMSO (1,100 g/L)                            | 5%                                                | 55 mg/mL                                               |
| Buffer              | 100% Phosphate Buffered Saline (PBS)             | 54%                                               | 0.54 mL/mL                                             |

**Table 11. Exemplary cryopreservation composition #3**

| Excipient Solution  | Solution Composition                             | Exemplary v/v% in Cryopreservation Composition #3 | Final Concentration in Cryopreservation Composition #3 |
|---------------------|--------------------------------------------------|---------------------------------------------------|--------------------------------------------------------|
| Albumin Solution    | 250 g/L albumin in water                         | 16%                                               | 40 mg/mL albumin                                       |
| Dextran 40 Solution | 100 g/L Dextran 40; and 50 g/L glucose; in water | 25%                                               | 25 mg/mL Dextran 40; 12.5 mg/mL glucose                |
| DMSO                | 100% DMSO (1,100 g/L)                            | 5%                                                | 55 mg/mL                                               |
| Buffer              | 100% Phosphate Buffered Saline (PBS)             | 54%                                               | 0.54 mL/mL                                             |

#### 4.7.2 Methods of Cryopreserving

[0391] The cryopreservation compositions described herein can be used for cryopreserving cells, e.g., therapeutic cells, e.g., natural killer (NK) cells, e.g., the NK cells described herein. In some embodiments, the cells are animal cells. In some embodiments, the cells are human cells. In some embodiments, the cells are immune cells. In some embodiments, the immune cells are selected from basophils, eosinophils, neutrophils, mast cells, monocytes, macrophages, neutrophils, dendritic cells, natural killer cells, B cells, T cells, and combinations thereof. In some embodiments, the immune cells are natural killer (NK) cells. In some embodiments, the natural killer cells are expanded and stimulated by a method described herein. In some embodiments, the NK cells are CAR-NK cells, for example CAR-NK cells described herein.

**[0392]** In some embodiments, cryopreserving the cells comprises: mixing the cells with a cryopreservation composition or components thereof described herein to produce a composition, e.g., a pharmaceutical composition; and freezing the mixture.

**[0393]** In some embodiments, cryopreserving the cells comprises: mixing a composition comprising the cells with a cryopreservation composition or components thereof described herein to produce a composition, e.g., a pharmaceutical composition; and freezing the mixture. In some embodiments, the composition comprising the cells comprises: the cells and a buffer. Suitable buffers are described herein.

**[0394]** In some embodiments, cryopreserving the cells comprises: mixing a composition comprising the cells and a buffer, e.g., PBS, with a composition comprising albumin, Dextran, and DMSO, e.g., as described herein; and freezing the mixture.

**[0395]** In some embodiments, cryopreserving the cells comprises: mixing a composition comprising the cells and a buffer, e.g., PBS 1:1 with a composition comprising 40 mg/mL albumin, e.g., human albumin, 25 mg/mL Dextran, e.g., Dextran 40, 12.5 mg/mL glucose and 55 mg/mL DMSO.

**[0396]** In some embodiments, the composition comprising the cells and the buffer, e.g., PBS, comprises from or from about  $2 \times 10^7$  to or to about  $2 \times 10^9$  cells/mL. In some embodiments, the composition comprising the cells and the buffer, e.g., PBS, comprises  $2 \times 10^8$  cells/mL. In some embodiments, the composition comprising the cells and the buffer, e.g., PBS, comprising about  $2 \times 10^8$  cells/mL.

**[0397]** In some embodiments, cryopreserving the cells comprising mixing: the cells, a buffer, e.g., PBS, albumin, e.g., human albumin, Dextran, e.g., Dextran 40, and DMSO; and freezing the mixture.

**[0398]** In some embodiments, the mixture comprises from or from about  $1 \times 10^7$  to or to about  $1 \times 10^9$  cells/mL. In some embodiments, the mixture comprises  $1 \times 10^8$  cells/mL. In some embodiments, the mixture comprises about  $1 \times 10^8$  cells/mL.

**[0399]** Suitable ranges for albumin, Dextran, and DMSO are set forth above.

**[0400]** In some embodiments, the composition is frozen at or below  $-135^\circ\text{C}$ .

**[0401]** In some embodiments, the composition is frozen at a controlled rate.

#### **4.8 Pharmaceutical Compositions**

**[0402]** In yet another aspect, provided herein are pharmaceutical compositions. In certain embodiments, provided herein is a pharmaceutical composition comprising (i) the population of cells provided herein, CAR provided herein, the engineered immune cell provided herein, the

polypeptide provided herein, the polynucleotide provided herein, or the vector provided herein; and (ii) a pharmaceutically acceptable excipient.

**[0403]** In certain embodiments, provided herein is a pharmaceutical composition comprising the population of cells provided herein and a pharmaceutically acceptable excipient. In certain embodiments, provided herein is a pharmaceutical composition comprising the CAR provided herein and a pharmaceutically acceptable excipient. In certain embodiments, provided herein is a pharmaceutical composition comprising the engineered immune cell provided herein and a pharmaceutically acceptable excipient. In certain embodiments, provided herein is a pharmaceutical composition comprising the polypeptide provided herein and a pharmaceutically acceptable excipient. In certain embodiments, provided herein is a pharmaceutical composition comprising the polynucleotide provided herein and a pharmaceutically acceptable excipient.

**[0404]** In certain embodiments, provided herein is a pharmaceutical composition comprising the vector provided herein and a pharmaceutically acceptable excipient.

**[0405]** In certain embodiments, provided herein is a pharmaceutical composition comprising the population of cells provided herein, the CAR provided herein, the engineered immune cell provided herein, the polypeptide provided herein, the polynucleotide provided herein, or the vector provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising the population of cells provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising the CAR provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising the engineered immune cell provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising the polypeptide provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising the polynucleotide provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising the vector provided herein.

**[0406]** In certain embodiments, provided herein is a pharmaceutical composition comprising an effective amount of the population of cells provided herein, an effective amount of the CAR provided herein, an effective amount of the engineered immune cell provided herein, an effective amount of the polypeptide provided herein, an effective amount of the polynucleotide provided herein, or an effective amount of the vector provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising an effective amount of the population of cells. In certain embodiments, provided herein is a pharmaceutical composition comprising an effective amount of the CAR provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising an effective amount of the engineered immune cell

provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising an effective amount of the polypeptide provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising an effective amount of the polynucleotide provided herein. In certain embodiments, provided herein is a pharmaceutical composition comprising an effective amount of the vector provided herein.

#### **4.9 CAR-NK Cellular Therapies**

**[0407]** In another aspect, the engineered cells provided herein can be used for treating cancer or other proliferative disorders, for example, the NK cells described herein, e.g., the NK cells expressing the anti-MSLN CARs described herein, can be used for treating cancer or other proliferative disorders. Thus, also provided herein are methods of treating a patient suffering from a disorder, e.g., a disorder associated with a cancer, e.g., a MSLN+ cancer, comprising administering the cells, e.g., the NK cells described herein, e.g., the CAR-NK cells described herein.

**[0408]** In yet another aspect, provided herein is a method of treatment comprising administering the engineered cell, the population of cells, or the pharmaceutical composition provided herein to a subject having a disease or condition associated with MSLN. In yet another aspect, provided herein is use of the engineered cell, the population of cells, or the pharmaceutical composition provided herein in the manufacture of a medicament for treating a disease or condition associated with MSLN. In yet another aspect, provide herein is use of the engineered cell, the population of cells, or the pharmaceutical composition provide herein in the manufacture of a medicament for treating a disease or condition associated with MSLN. In some embodiments, the disease or condition associated with MSLN is cancer.

**[0409]** In certain embodiments, provided herein are methods of preventing, reducing and/or inhibiting the recurrence, growth, proliferation, migration and/or metastasis of a cancer cell or population of cancer cells in a subject in need thereof, comprising administering the cells, e.g., the NK cells described herein, e.g., the CAR-NK cells described herein.

**[0410]** Also provided herein are methods of enhancing, improving, and/or increasing the response to an anticancer therapy in a subject in need thereof, comprising administering the cells, e.g., the NK cells described herein, e.g., the CAR-NK cells described herein.

**[0411]** Also provided herein are methods for inducing the immune system in a subject in need thereof comprising administering the cells, e.g., the NK cells described herein, e.g., the CAR-NK cells described herein.

**[0412]** The methods described herein include methods for the treatment of disorders associated with abnormal apoptotic or differentiative processes, e.g., cellular proliferative

disorders or cellular differentiative disorders, e.g., cancer, including both solid tumors and hematopoietic cancers. Generally, the methods include administering a therapeutically effective amount of a treatment as described herein, to a subject who is in need of, or who has been determined to be in need of, such treatment. In some embodiments, the methods include administering a therapeutically effective amount of a treatment comprising NK cells, e.g., CAR-NK cells described herein.

**[0413]** In some embodiments, the cells, e.g., the NK cells described herein, e.g., the CAR-NK cells described herein are administered as part of a pharmaceutical composition, e.g., a pharmaceutical composition described herein. In some embodiments, the cells, e.g., the NK cells described herein, e.g., the CAR-NK cells described herein, are administered at or at about  $1 \times 10^6$  to or to about  $1 \times 10^9$  NK cells per dose.

#### **4.10 Combination Therapies**

**[0414]** The combination therapy of the present disclosure comprises the cells, e.g., the NK cells described herein, e.g., the CAR-NK cells described herein, and another therapeutic agent (small molecule or antibody) may be used for the treatment any proliferative disease, in particular, treatment of cancer.

**[0415]** The combination therapy of the present disclosure may be administered to an individual having a cancer in combination with chemotherapy. The individual may undergo the chemotherapy at the same time the individual is undergoing the combination therapy of the present disclosure. The individual may undergo the combination therapy of the present disclosure after the individual has completed chemotherapy. The individual may be administered the chemotherapy after completion of the combination therapy. The combination therapy of the present disclosure may also be administered to an individual having recurrent or metastatic cancer with disease progression or relapse cancer and who is undergoing chemotherapy or who has completed chemotherapy.

**[0416]** The cells, e.g., the NK cells described herein, e.g., the CAR-NK cells described herein, may be administered in combination with one or more therapeutic agent, which is an antibody, for treatment of cancer or proliferative disease. The individual may undergo treatment with the therapeutic antibody at the same time the individual is undergoing the combination therapy of the present disclosure. The individual may undergo the combination therapy of the present disclosure after the individual has completed treatment with the therapeutic antibody. The individual may be administered the treatment with the therapeutic antibody after completion of the combination therapy. The combination therapy of the present disclosure may also be

administered to an individual having recurrent or metastatic cancer with disease progression or relapse cancer and who is undergoing chemotherapy or who has completed chemotherapy.

[0417] The following examples are intended to promote a further understanding of the present disclosure.

## 5. EXAMPLES

### 5.1 Example 1

[0418] Three MSLN antibody sequences were selected for CAR-NK generation based on binding affinity and binding to different regions of MSLN. The three anti-MSLN antibodies are shown to bind to distinct MSLN epitopes. The regions of human MSLN protein are illustrated in **FIG. 1A**. As shown in **FIGs. 1A-1B**, anti-MSLN antibody clone 45B6 binds to region 1 in the human MSLN protein with relatively low affinity; anti-MSLN antibody clone 47D7 binds to region 3 (membrane proximal) in the human MSLN protein with relatively high affinity; anti-MSLN antibody clone 20B12 binds to region 1-2 junction (tentative) in the human MSLN protein with relatively lower affinity. The anti-MSLN antibodies above are described in PCT/US22/01253, which is incorporated by reference herein in its entirety.

[0419] The three antibodies were evaluated by enzyme-linked immunosorbent assay (ELISA) for binding to human MSLN (huMSLN, 87BGR), human MSLN expressed by Chinese hamster ovary K1 (CHOK1) cells genetically modified to express human MSLN as membrane-bound protein, or human MSLN expressed by the OVCAR3 cell line (a human ovarian cancer cell line that expresses high levels of human MSLN). The  $K_D$  values of the tested clones were measured using SPR at pH 7.4. ELISA results are shown in **FIG. 1B**.

### 5.2 Example 2

[0420] Amino acid sequences for the anti-MSLN scFvs were converted to DNA sequences through reverse translation. The scFv sequences were codon optimized at Lentigen, for expression in human cells and synthesized in conjuncture with the hinge and endodomain sequences. IL-15 was linked downstream to CAR using a T2A element. The entire expression cassette was cloned into Lentigen's proprietary lentiviral expression vector driven by EF1a promoter. The expression cassette was subsequently packaged into a lentiviral vector.

[0421] Four iterations of CARs were designed for the MSLN antibodies and 15B6 clone (U.S. Pub. No. US 2020/0247901 A1, which is incorporated by reference herein in its entirety). Exemplary CAR designs included VL-VH – short hinge; VL-VH – long hinge; VH-VL – short hinge; and VH-VL – long hinge (**FIG. 2**). The heavy and light chains were connected by a (G4S)<sub>3</sub> linker (SEQ ID NO: 52). The constructs co-express sIL-15. For gene synthesis, lentiviral

vector production was completed for CARs expressing 47D7, 45B6, 20B12, and 15B6 in the VL/VH orientation.

**[0422]** With respect to the binding domain of 15B6, the epitope spans TACE cleavage sequence. MSLN cleavage interrupts the antigen recognized by 15B6. 15B6 does not bind to soluble MSLN. 15B6 may provide mitigation strategy for sMSLN sink effect on CAR.

### 5.3 Example 3

**[0423]** The binding of the MSLN mAb-converted scFv-Fc constructs to MSLN-expressing CHO cells and wild-type CHO cells was measured using cell ELISA. The EC<sub>50</sub> values are shown in **FIG. 3A** whereas the EC<sub>50</sub> curves are shown in **FIGs. 3B-3C**. MSLN mAb-converted scFv-Fc constructs showed good binding to MSLN-expressing CHO cells (**FIGs. 3A-3C**). The binding curves indicate that clones 45B6, 20B6, & 47D7 all have picomolar half maximal binding concentrations and bind to hMSLN on the cell surface, and do not show any binding in the negative cell line.

#### 5.3.1 Basic cell ELISA

**[0424]** CHO-WT and CHO-Human MSLN cells were seeded at a density of 20K cells per well and incubated overnight at 37°C, 5% CO<sub>2</sub>. Aspirate cell media, add 50 µL of 100 nM titrated at a 4-fold dilution of antibodies, and incubate for 1 hour. Then cells are washed 2X with 1X DPBST (150 µL/well). Next, a secondary detection antibody (Immuno Jackson 109-035-003: Peroxidase-conjugated AffiniPure Goat Anti-Human IgG (H+L)) is added at a 1:5000 dilution at 50 µL per well and incubated for 30 minutes. Then the cells are washed 2X with 1X DPBST, and 50 µL of TMB substrate (Thermo Scientific 34029) is added to each well for 7 minutes. Lastly, 50 µL of stop solution is added to each well to stop the reaction, and each plate is read at 450nm.

### 5.4 Example 4

#### 5.4.1 Affinity determination of 45B6 Fab, 45B6 ScFv-Fc, and 47D7 ScFv-Fc against human mesothelin

**[0425]** To determine the affinities of 45B6 Fab, 45B6 ScFv-Fc, and 47D7 ScFv-Fc against human mesothelin protein, the proteins were analyzed using surface plasmon resonance (SPR) assay on a Biacore T200 (Cytiva) instrument. Biacore T200 Evaluation Software was used to fit each titration series to a 1:1 binding model. The association ( $k_{on}$  M<sup>-1</sup> s<sup>-1</sup>) and dissociation ( $k_{off}$  s<sup>-1</sup>) rate constants were determined for each set of titrations and used to calculate the dissociation constant,  $K_D = k_{off}/k_{on}$ , of each molecule against the human mesothelin protein. To measure the affinity of the mesothelin molecules the mesothelin antigen was captured on an anti-his capture surface at low density followed by one of the 45B6 molecules or the 47D7 molecule as the

analyte. SPR plots for 45B6 Fab, 45B6 ScFv-Fc, and 47D7 ScFv-Fc are each shown in **FIGs 4A-4C**, respectively.

**[0426]** As shown in **FIG. 4D**, the affinity of both 45B6 molecules for human mesothelin protein is in the range of 82.8-640 pM, and the affinity of the 47D7 molecule was measured at 49.5 pM. Since the ScFv-Fc molecules were run in avidity format due to homodimerization of the ScFV portions of the molecule, the  $K_D$  value should be considered an apparent affinity.

## 5.5 Example 5

### *5.5.1 Epitope mapping of antibody 45B6 by hydrogen deuterium exchange mass spectrometry (HDX-MS)*

**[0427]** The contact areas between antibody 45B6 and human soluble mesothelin were determined by HDX-MS analysis. HDX-MS measures the exchange of deuterium with hydrogen into the amide backbone of the protein. One factor influencing the exchange rate is the hydrogen's exposure to solvent. Comparison of the exchange levels in the antigen when the antibody is bound can identify regions of the protein where the antibody is binding.

#### *5.5.1.1 Materials*

**[0428]** Human soluble mesothelin was produced: MSLN AviTag His6 (aa296-638) in phosphate buffered saline (PBS), pH 7.4 and 3% sucrose with a molecular weight of 38381 Da. Antibody 45B6 scFv-Fc was generated with a molecular weight of 102000 Da. Before HDX-MS analysis, the antibody was buffer exchanged from its storage buffer 20 mM sodium acetate, 9% Sucrose, pH 5.5 to PBS, pH 7.4.

#### *5.5.1.2 Pepsin/XIII digestion and Liquid Chromatography-Mass Spectrometry*

**[0429]** Four micrograms of the recombinant mesothelin in 100  $\mu$ L of control buffer (50 mM phosphate, 100 mM sodium chloride at pH 7.4) was denatured by adding 100  $\mu$ L of 8 M urea, 1.25 M TCEP buffer (final pH was 2.5), which was incubated for 3 minutes at 20 °C. Then, the mixture was subjected to on-column pepsin/XIII digestion using a pepsin/XIII column (2.1 x 30 mm, NovaBioAssays, Woburn, MA, NBA2014002). The resultant peptides were trapped and desalted on an ACQUITY UPLC BEH C18 VanGuard pre-column (130Å, 1.7  $\mu$ m, 2.1 mm X 5 mm, 186003975, Waters) for 3.5 min at 160  $\mu$ L/min. Peptides were then eluted from the trap using a 2-35% gradient of acetonitrile (with 0.3% formic acid) over 12.5 min at a flow rate of 150  $\mu$ L/min and are separated on a 50 x 1 mm C8 column (3  $\mu$ m, NBA2014015, NovaBioAssays). A UPLC-MS system comprised of a Waters Acquity UPLC coupled to an Orbitrap Exploris™ 480 Mass Spectrometer (ThermoFisher) was used. Solvent A was 0.3% formic acid in water. The injection valve, enzyme column and their related connecting tubing were stored inside a cooling box maintained at 20 °C. The second switching valve, C8 column

and their related connecting tubing were inside another chilled circulating box maintained at -6 °C. Peptide identification was done through searching MS/MS data against the mesothelin sequence with Byonic search algorithm from Protein Metrics. The mass tolerance for the precursor and product ions were 10 ppm and 0.02 Da, respectively.

*5.4.1.3 HDX-MS for the recombinant mesothelin with and without the presence of the antibody*

**[0430]** Buffer exchange was performed for the antibody, which changes the original buffer (20 mM sodium acetate, 9% Sucrose, pH 5.5) to PBS buffer, pH 7.4. The concentration of the antibody after buffer exchange was 4.59 mg/mL.

**[0431]** Ten microliters of the recombinant mesothelin (4 µg, 115 pmole) or 10 µL of recombinant mesothelin & antibody (4 µg (115 pmole): 19 µg (184 pmole), molar ratio is 1:1.6) was incubated with 90 µL deuterium oxide labeling buffer (50 mM sodium phosphate, 100 mM sodium chloride at pD 7.0) for 0 s, 15 s, 60 s, 600 s, or 3600 s at 20 °C. Hydrogen/deuterium exchange was quenched by adding 100 µL of 8 M urea, 1.25 M TCEP buffer (final pH was 2.5). Subsequently, the quenched samples were subjected to on column pepsin/XIII digestion and LC-MS analysis as described above. The mass spectra were recorded in MS only mode.

**[0432]** Raw MS data was processed using HDX WorkBench software for the analysis of H/D exchange MS data (J. Am. Soc. Mass Spectrom. 2012, 23 (9), 1512-1521). The deuterium levels were calculated using the average mass difference between the deuterated peptide and its undeuterated form (t0).

*5.4.1.4 Results*

**[0433]** Approximately 99.4% sequence coverage was achieved for the recombinant mesothelin. The recombinant mesothelin showed significant reduction in deuterium uptakes upon binding to the antibody at region I of the protein: sequences 296-315 (EVEKTACPSGKKAREIDSL) (SEQ ID NO: 97) and 326-337 (CVDAALLATQMD) (SEQ ID NO: 98), which should be assigned as the main binding site on mesothelin. In addition, modest reduction in deuterium uptakes was observed at sequence 338-349 (RVNAIPFTYEQL) (SEQ ID NO: 99), which could be the peripheral region of the main binding site.

**[0434]** **FIG. 5A** shows the differential heat map comparing hydrogen/deuterium exchange of recombinant mature mesothelin alone to that of recombinant mature mesothelin & antibody (protected sequence regions are shown; residue numbers for mesothelin that includes the precursor sequence would increase by 295, the length of the precursor sequence; see SEQ ID NO: 1).

### 5.5.2 Epitope mapping of antibody 20B12 and 47D7 by HDX-MS

**[0435]** The contact areas between antibody 20B12 or 47D7 and human soluble mesothelin were determined by hydrogen deuterium exchange mass spectrometry (HDX-MS) analysis. HDX-MS measures the exchange of deuterium with hydrogen into the amide backbone of the protein. One factor influencing the exchange rate is the hydrogen's exposure to solvent. Comparison of the exchange levels in the antigen when the antibody is bound can identify regions of the protein where the antibody is binding.

#### 5.4.2.1 Materials

**[0436]** Recombinant human soluble mesothelin was purchased from ACRO Biosystems (Catalogue#: MSN-H5223; aa296-580). The protein was reconstituted to 20  $\mu$ M in PBS, pH 7.4. Fabs 20B12 and 47D7 were produced, which were buffered exchanged from their storage buffer 20 mM sodium acetate, 9% Sucrose, pH 5.5 to PBS, pH 7.4.

#### 5.4.2.2 Liquid Chromatography-Mass Spectrometry

**[0437]** The mass spectrometer was a Waters Synapt G2Si Quadrupole Time-of-flight (TOF). For peptide identification and the measurement of deuterium labeled samples, the mass spectrometer was set to acquire one full scan MS data (low energy) and one MS(e) data (high energy) with TOF-only mode. The scan time was set to 0.4 second. Ramp trap collision energy was from 15 to 45 volts.

**[0438]** The liquid chromatography system was a Waters nanoAcquity binary pump for the analytical column gradient and auxiliary pump for the sample digestion and loading. For sample digestion and loading, the buffer used was 100% water and 0.1% formic acid at a flow rate of 100  $\mu$ L/minute. For the analytical gradient, the buffers were Buffer A) 0.1% formic acid in water and Buffer B) 0.1% formic acid in acetonitrile.

**[0439]** The gradient was at 40  $\mu$ L/minute from 5% B to 35% B in 9 minutes, followed by a ramp to 85% B in one minute, and a wash of 85% B for one minute and a re-equilibration at 5% B for one minute. The column was then washed by cycling the gradient between 5% and 95% B, four times with one minute at each step, followed by a final equilibration at 5% B for one minute. The trapping column was a Waters Vanguard BEH C18 1.7  $\mu$ m Guard Column and the analytical column was a Waters BEH C18, 1.7  $\mu$ m 1x50 mm column.

**[0440]** Sample handling for the deuterium labeling was done by a Waters HDX unit which consists of Leaptac H/D-X PAL system and a Waters HDX chamber for column cooling. The labeling sample tray was set to a temperature of 20°C; the quenching tray was set to 4°C and the trap and analytical column chamber was set to 0.5°C. Poroszyme™ immobilized pepsin cartridge (P/N: 2-3131-00) from Applied Biosystems was kept at 20°C in the enzyme column chamber.

#### 5.4.2.3 Deuterium Labeling

**[0441]** For 20B12 epitope mapping, human mesothelin was first deglycosylated with PNGaseF (NEW England Biolabs, catalogue# P0704L) overnight at 37°C using 1:10 enzyme to protein ratio (i.e., 1 µL of PNGaseF in 10 µg of protein). For 47D7, mesothelin protein was used without deglycosylation. Mesothelin protein was mixed with the Fab to final concentrations of 10 µM for mesothelin and 10 µM for the Fab. An unbound control was prepared by incubating mesothelin in PBS, pH 7.4. The Fab bound sample and the unbound control were incubated at room temperature for one hour before beginning the labeling experiment.

**[0442]** To deuterium label the samples, six microliters of sample were mixed with 54 µL of 10 mM sodium phosphate, 150 mM sodium chloride in deuterium oxide pD 7.4. Labeling time points for 20B12 were 0, 10, 60, 600, 6000 seconds and for 47D7 were 0, 10, 60, 600 seconds. After the time point, 50 µL of the labeling mixture was added to 50 µL of cold quench buffer (quench using 3 M urea, 250 mM TCEP in phosphate buffer, pH 2.5 at 4°C). After mixing one time, 90 µL was injected into the column cooling chamber where the sample was passed over the pepsin column and the resulting peptides were loaded onto the trapping column. After four minutes, a valve switch took the pepsin column out of line. The trap was then switched in-line with the analytical column. The analytical gradient and mass spectrometer data acquisition were then initiated.

#### 5.4.2.4 Data Analysis

**[0443]** LC-MS/MS data were acquired using unlabeled bound and unbound samples in triplicates, and database was searched to verify successful digestion of the proteins and to generate a list of peptides from the pepsin digestion. Database search was done using ProteinLynx Global Server 3.0 (Waters Corporation) using mesothelin protein sequence. The protein database used was the human mesothelin combined with a randomized human mesothelin sequence to reduce false identifications.

**[0444]** MS data from the deuterium labeling experiment was processed by DynamX (version 3.0.0, Waters Corporation) to generate relative deuterium uptake level in each peptide, which was used to generate deuterium uptake graphs and difference maps. For each peptide, the mass, retention time and charge state selected by the software were manually verified.

#### 5.4.2.5 Results

**[0445]** Approximately 97% sequence coverage was achieved using human mesothelin with or without deglycosylation. The peptides protected upon Fab 20B12 binding are illustrated in the H/D difference plot in **FIG. 5B** (top). Significant H/D protection observed covers part of region I and II of the protein: amino acid residues 314-325 (SLIFYKKWELEA, region I) (SEQ ID NO:

100), 337-349 (DRVNAIPFTYEQL, region I) (SEQ ID NO: 101), 369-376 (IQHLGYLF, region I) (SEQ ID NO: 102), 385-392 (RKWNVTSL, region II) (SEQ ID NO: 103) and 423-433 (IDRFVKGRGQL, region II) (SEQ ID NO: 104). The peptides protected upon Fab 47D7 binding are listed in the H/D difference plot in **FIG. 5B** (bottom). Significant H/D protection was detected in sequence region III: amino acid residues 546-558 (EVQKLLGPHVEGL) (SEQ ID NO: 105) and 573-588 (RQRQDDLDTLGLGLQG) (SEQ ID NO: 106).

**[0446]** **FIG. 5B** provides the H/D Difference Plot showing deuterium uptake protection upon antibody binding to human mesothelin protein: (top) 20B12 and (bottom) 47D7. Each gray bar represents the sum of the deuterium uptake difference across all time points between bound and unbound mesothelin protein for each peptic peptide.

#### *5.5.3 Epitope Mapped onto AlphaFold Model of Mesothelin*

**[0447]** The X-ray structure of full length mesothelin has been resolved (Cancer Research Communications, 2023, 3(2), 175-191). However, the electron densities were only sufficiently clear to allow modeling of residues from K299 to L543, missing about 50 C-terminal residues. To illustrate relative location of each epitope, a model of the full length mesothelin was generated using AlphaFold 2.0. HD protections were mapped onto this model as shown in Figure 3. Based on the model, 45B6 is clearly a region I binder and 47D7 a region III binder. 20B12 binds to mesothelin with a conformational epitope spanning part of region I and II.

**[0448]** In **FIG. 5C**, H/D protection from all three antibodies were mapped onto mesothelin AlphaFold model. Epitopes detected for 45B6 (black) 20B12 (light grey), and 47D7 (dark grey) are illustrated.

### **5.6 Example 6**

**[0449]** Sandwich ELISA was performed to detect amount of soluble mesothelin released by Human Mesothelin cells. Cells were plated on Day 0 and antibodies were added on Day 1. Antibodies were titrated at an 8-fold dilution with a starting concentration of 50 µg/mL (333 nM). Samples were collected and tested on Day 3 for soluble MSLN. 15B6 mAb decreased soluble MSLN levels in the CHO supernatant, suggesting the ability to block MSLN from shedding (**FIG. 6**).

### **5.7 Example 7**

**[0450]** The long-term cytotoxicity data at the end point of 5-day study was used to generate a heat map comparing the various CARs for anti-tumor cytotoxicity against MSLN +/- cell lines in the presence or absence of soluble mesothelin. The data is normalized to unmodified NK cells (CB-NK cells/ NK cells) and represented as a percent increase in lytic function of CAR-NK cells against tumor lines expressing surface mesothelin. C AR clones 45B6 and 20B12 with CD8a

hinge showed consistent tumor lysis in the presence or absence of 500ng/ml of soluble mesothelin. In case of CAR clone 20B12 containing IgG mutant hinge addition of soluble mesothelin resulted in reduction of CAR mediated cytotoxicity in one out of three MSLN+ cell lines tested; whereas for CAR clone 47D7 with CD8a and IgG mutant hinges, soluble mesothelin resulted in reduction of CAR mediated anti-tumor function against two out of the three cell lines tested.

**[0451]** As illustrated in **FIG. 7**, CAR-NK demonstrated robust cytotoxic activity in long-term co-culture assays, as shown in the percent kill at 5 days. Untransduced NK cells showed variable basal activity across tumor cell lines. CAR-NK cells showed minimal activity on MSLN negative cell line (A431). Multiple CAR constructs demonstrated robust activity in long-term killing assays. Antibodies with lower-affinity (measure by Biacore) showed robust killing in the presence of sMSLN. All CAR constructs drove CAR-dependent IFN $\gamma$  and IL-15 production. The promising initial long-term cytotoxicity data prioritized 5 clones for *in vivo* analysis.

## 5.8 Example 8

### 5.8.1 Long-term cytotoxicity assay

**[0452]** NucLight-red stable cell lines A-431, KLM-1, NCI-N87 and OVCAR-3 were harvested in assay medium. Cell count was measured and cells were resuspended at  $1 \times 10^5$ /mL for A-431, NCI-N87 and OVCAR-3; and  $3 \times 10^4$  cells/mL for KLM-1. 100 $\mu$ L of tumor cells were plated in a 96 well flat-bottom plate one day prior to co-culture with CBNK and MSLN CAR-NK. The plate was placed into the IncuCyte device in a CO $_2$  incubator at 37°C, and the cells were cultured for 16-18 hours. The protocol for the IncuCyte software (v2019B) was set as follows: Scan type: Standard; Image Channels: Phase, Red (Acquisition time: 400 ms); Objective: 10X; Scan pattern: 2 images per well; Scan schedule: Scan every 2 hours and infinitely. The following day, the NK cells were prepared in the assay medium at appropriate cell density to create E:T ratios of 3:1, 1:1 or 0.3:1 and 100  $\mu$ L of the NK cells were added to each well.

**[0453]** For wells where sMSLN was to be added, 50  $\mu$ L of NK cells (reconstituted at 2x cell density) and 50  $\mu$ L of soluble MSLN (or 50  $\mu$ L of assay medium as control) were added to the well containing target cells to obtain E:T ratios of 3:1 and 1:1. The plate was returned to the IncuCyte S3 device, and the image collection was continued. Following incubation, the result was analyzed and quantified as follows. Cell count was determined by number of Nuclight red<sup>+</sup> cells. Cell count of each time point was divided by cell count of the initial time point.

$$\text{Normalized percentage increase in cell number} = \frac{\text{Cell count of indicated time point}}{\text{Cell count of initial time point}} \times 100$$

### 5.8.2 Result

**[0454]** In the long-term cytotoxicity assay, CBNK demonstrated anti-tumor activity against NCI-N87 (vs tumor only group) and MSLN CAR-NK showed higher lysis of MSLN+ tumor cells. In the presence of soluble MSLN, 20B12 and 45B6 CAR-NK cells retained their lytic activity, while the activity of 47D7 CAR-NK cells was suppressed. 47D7 CAR with IgGm hinge was more inhibited by soluble MSLN compared to MSLN CAR with CD8a hinge. As illustrated in **FIGs. 8A-8J**, MSLN CAR-NK demonstrated robust cytotoxicity activity in long-term co-culture assays.

## 5.9 Example 9

### 5.9.1 Phenotypic characterization assay

**[0455]** The antibody cocktails were prepared in amber microcentrifuge tubes to assess NK cell purity using CD3, CD56 and CD16; CD3, CD19 and CD14. NK phenotypic markers were combined in three-antibody cocktail panels containing CD3, CD56 and either NKG2A, NKG2C, NKG2D, NKp30, NKp44, NKp46, DNAM-1, CD25, CD62L, CD69, CXCR3, CD57, TIM-3, TIGIT, PD-1 or LAG-3. Viability was assessed by 7-AAD. The cell suspension was prepared at a cell density of  $2.0 \times 10^6$  cells/mL with FACS buffer and aliquoted at  $0.2 \times 10^6$  cells/100  $\mu$ L in each well of a 96-well plate. 100  $\mu$ L of each antibody cocktail was added to cells and the cells were incubated for 30 minutes at 4 °C. Cells were centrifuged at 800  $\times$ g, 4 °C for 3 min. The cell pellet was resuspended with 200  $\mu$ L of FACS buffer for washing, and centrifuged at 800  $\times$ g, 4°C for 3min. The washing step was repeated. 200  $\mu$ L of cytofixation buffer was added to each well, and the sample was transferred to new 5 mL round bottom tubes. The sample was analyzed using flow cytometry within 72 hours of preparation. The expression of indicated surface proteins was analyzed using the FlowJo software as follows: Singlet gating (FSC-H / FSC-A); Lymphocyte gating (SSC-A / FSC-A); Live cell gating (SSC-A / 7AAD-); Purity and identity gating CD56/CD16 purity gating (CD56+CD16+) and CD56/CD3 purity gating (CD56-CD3+)-CD14/CD19 purity gating (CD19+ / CD14+). The expression of each protein was determined based on the FMO and appropriate isotype controls (mIgG1 $\kappa$ )

### 5.9.2 Result

**[0456]** The MSLN CAR-NK cells showed high expression of CD16 and major NK cell activating receptors such as NKG2D, NKp30, NKp46, and DNAM-1. The expression of NK cell markers in MSLN CAR-NK remained similar to that of CBNK.

**[0457]** The profile of NK cell activation and inhibitory receptors was determined. As illustrated in **FIGs. 9A-9B**, the percent expression of NK cell markers in MSLN CAR-NK

remained vastly like cord blood-derived natural killer cells (CB-NK or CBNK). All CAR NK constructs conferred the same overall NK cell phenotype.

### 5.10 Example 10

**[0458]** Cellular persistence and plasma IL-15 levels were evaluated in naïve NSG mice dosed with different MSLN CAR NK constructs from 4 donors. Female NSG mice (n= 3/group) were administered a single intravenous injection of MSLN CAR NK cells at a  $1 \times 10^7$  cell dose on day 0. Blood samples were collected on days 7, 14, 21, 28, and 35 post-injection.

**[0459]** *In vivo* persistence of MSLN CAR-NK in naïve NSG mice was evaluated in peripheral blood by flow cytometry. In brief, peripheral blood was collected into K2-EDTA microvette tubes, and 50  $\mu$ L was used for flow cytometry analyses. Samples were first incubated with mouse and human Fc block at 4 °C for 15 minutes followed by staining with viability dye, human NK cell surface antibodies, and PE-conjugated recombinant MSLN at room temperature for 30 minutes. Following staining, blood was lysed in 1mL of RBC Lysis/Fixation solution (1X) for 15 minutes at room temperature followed by centrifugation at 350 x g for 5 minutes. Cells were subsequently washed in 1mL of staining buffer (Cat#554657) and centrifuged at 350 x g for 5 minutes. Cells were resuspended in 80  $\mu$ L of stain buffer, and 50  $\mu$ L of each sample was acquired by using a Cytex Aurora flow cytometer and analyzed using FlowJo software.

**[0460]** The data was analyzed by using FlowJo software. The gating strategy and hierarchy are the followings: Lymphocyte gating (FSC-A / SSC-A); Singlets (FSC-A/FSC-H); Live cells (FVD780-/SSC-A); hCD45+ cells (hCD45+/SSC-A). Human CD45 positive cells were presented as frequency (%) and in cell counts per  $\mu$ L blood.

**[0461]** IL-15 levels in mouse plasma were evaluated in duplicates for each sample using MSD human IL-15 V-PLEX kit (Cat# K151RDD) according to the manufacturer's procedure. Linear regression analysis was performed using a standard curve by Prism software to obtain interpolated IL-15 levels in pg/mL.

**[0462]** **FIGs. 10A-10C** show the *in vivo* persistence of MSLN CAR-NK in naïve NSG mice. The percent hCD45, plasma sIL-15 and hCD45 count were measured. 315339P 45B6-CD8aH CAR-NK (repeat) showed substantially higher persistence compared to 45B6- and 20B12-CD8aH from the other donors. Plasma IL-15 showed relative correlation to the persistence of the CAR-NK (each sample was run in duplicates).

### 5.11 Example 11

**[0463]** MSLN CAR-NK-mediated efficacy against MSLN+ gastric tumors was evaluated in a subcutaneous NCI-N87 xenograft tumor model in NSG mice. In this study, female NSG mice (n = 5/group) were irradiated with 1.5 Gy dose on Day -1 and inoculated with  $1 \times 10^7$  NCI-N87

cells subcutaneously on Day 0. On Day 5, animals were randomized into seven groups based on tumor burden. Mice bearing subcutaneous NCI-N87 tumors received intravenous administration of CBNK and different MSLN CAR-NK constructs at  $1 \times 10^7$  cell dose. Tumor cells alone served as the untreated group. Tumor growth was monitored by tumor volumes measured twice a week by caliper.

[0464] *In vivo* persistence of MSLN CAR-NK in peripheral blood in the NCI-N87 efficacy study was assessed on Day 8, 29, and 49 by flow cytometry. In brief, 50  $\mu$ L of blood from each mouse was stained for human CD45 and NK cell markers prior to RBC lysis and analysis. Persistence data was presented in hCD45+ cell counts per  $\mu$ L blood.

[0465] MSLN CAR-NK efficacy was measured in NCI-N87 tumor model. Results were shown in tumor volume, hCD45+ count and immunohistochemistry. As illustrated in **FIGs. 11A-11C**, surprisingly, only 45B6-CD8aH MSLN-CAR NK cells promoted tumor regression and demonstrated significant anti-tumor efficacy against N87 subcutaneous (SC) tumors when compared to untreated and CBNK groups. 45B6-CD8aH CAR-NK showed superior persistence in the peripheral blood, which correlated with efficacy. No efficacy observed for 47D7- and 20B12-CD8aH and IgGmH MSLN CAR-NK candidates. NCI-N87 SC tumors expressed high level of MSLN. High level of NK cell infiltration was found in tumors treated with 45B6-CD8aH CAR-NK cells, correlating with anti-tumor efficacy.

[0466] While the present disclosure is described herein with reference to illustrated embodiments, it should be understood that the disclosure is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof. Therefore, the present disclosure is limited only by the claims attached herein.

6. SEQUENCES

Table 12. Sequences

| SEQ ID NO: | Description                                                                                                                                    | Sequence                                                                                                                                                                                                                                                                                                                                                                               |
|------------|------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1          | Human mesothelin (precursor)<br>Features:<br>Amino acids 1-295 (MPF; megakaryocyte potentiating factor);<br>Amino acids 296-622 mature protein | MALPTARPLLGSCGTPALGSLLFLLFSLGWVQPSRTLGETGQE<br>AAPLDGVLANPPNISSLSRQLLGFPCEVSGSLSTERVRELAVA<br>LAQKNVKLSTEQLRCLAHRLSEPPEDLDALPLDLLFLNPDAFS<br>GPQACTRFFFSRITKANVDLLPRGAPERQRLLPALACWVGRLSL<br>LSEADVRLGGLACDLPGRFVAESAEVLLPRLVSCPGPLDQDQQ<br>EAARAALQGGPPYGGPSTWSVSTMDALRGLLPVLGQPIIRSIP<br>QGIVAARQRSSRDPSWRQPRTLRLPRFRREVEKTACPSGKKA<br>REIDESLIIFYKKWELEACVDAALLATQMDRVNAIPFTYEQLDVL |

| SEQ ID NO: | Description                                                                                                                                   | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
|------------|-----------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|            |                                                                                                                                               | KHKLDELYPQGYPESVIQHLGYLFLKMSPEDIRKWNVTSLET<br>LKALLEVNKGHEMSPQVATLIDRFVKGRGQLDKDTLDTLTA<br>FYPGYLCSLSPEELSSVPPSSIWAVRPQDLDTCDPRQLD<br>VLYPKARLAFQNMNGSEYFVKIQSFLGGAPTE<br>DLKALSQQNVSMDLATFMKLRTDAVLPLTVAEVQKLL<br>GPHVEGLKAEERHRPVRDWILRQRQDDLD<br>TLGLGLQGGIPNGYLVLDSLMSQEALSGTPCLLGPGP<br>VLTVLALLLASTLA                                                                                                                                                                                                                                                                                                                                                                                                                 |
| 2          | Rhesus mesothelin precursor<br>Features:<br>Amino acids 1-294 (MPF; megakaryocyte potentiating factor);<br>Amino acids 295-623 mature protein | MALPMARPLSGSCGTPAVGSLLFLLFSLGWVQPSRVLAGET<br>RQEAAPLDGILTNAPDIASLSPRQLLGFTCVEVSGLSTELV<br>QELAVALLGQKNVKLSAEQLRCLAHRLSEPPEDLALPLD<br>LLFLNPDAFSGPQACTHFFSRVAKANVDLLPRGAPERQ<br>RLLPAALTCWGVGRGSLLSEADVRLGGLACDLPGCFVA<br>ESAEEVVLPRLVRCGLGPLDQDQQAARAALQRGGP<br>PYGPPSTWSISTLDDLQSLLPVLGQPVIIHSIPK<br>GILAAWRQRSSRDPSWQQPEQTVLRPRFRDVERTT<br>CPPEKEVHEIDESLIFYKKRELEACVDAALLAAQMD<br>RVDAIPTTYEQLDVLKHKLDELYPQGYPESVIRH<br>LGHFLFKMSPEDIRKWNVTSLET<br>LKAALLKVSKGHEMSAQVATLIDRVVVG<br>RGQLDKDTVDTLTAFCPGCLCSLSPERLSSVPPSV<br>IGAVRPQDLDTCGPRQLDVLYPKARLAFQ<br>NMSGSEYFVKIRPFLGGAPTE<br>DVKALSQQNVSMDLATFMKLRR<br>EAVLPLTVAEVQKLLGPHVEGLKVEEQHS<br>PVRDWILKQRQDDLDTLGLGLQGGIPNGYL<br>ILDLSVREALSGTPCLLGPGP<br>VLTVLALLLASTLA |
| 3          | SV017.47D7.1C2 VH                                                                                                                             | EVQLVESGGGLVQPGGSLRLS<br>CAATGFTTFSSYWMHWRQGP<br>GKGLVWVSRINSDGTSTVYAD<br>SVKGRFTISRDNKNTLYLQMN<br>SLRAEDTAVYYCARGRGIMTY<br>WGQGT<br>LTVTVSS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| 4          | SV017.47D7.1C2 VL                                                                                                                             | DIQMTQSPSSLSASVGRVTIT<br>CRASQDIGSWLTWYQRKPEKAP<br>KSLIYAASSLQSGVPSRFSG<br>SGSGTDFTLTISSLQPEDFAT<br>YYCQQYKSYPLTFGQAKLEIK                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| 5          | SV017.47D7.1C2 VH-<br>CDR1 Kabat                                                                                                              | SYWMH                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| 6          | SV017.47D7.1C2 VH-<br>CDR2 Kabat                                                                                                              | RINSDGTSTVYADSVKG                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| 7          | SV017.47D7.1C2 VH-<br>CDR3 Kabat                                                                                                              | GRGIMTY                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| 8          | SV017.47D7.1C2 VL-<br>CDR1 Kabat                                                                                                              | RASQDIGSWLT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| 9          | SV017.47D7.1C2 VL-<br>CDR2 Kabat                                                                                                              | AASSLQS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| 10         | SV017.47D7.1C2 VL-<br>CDR3 Kabat                                                                                                              | QQYKSYPLT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| 11         | SV018.20B12.1D1 VH                                                                                                                            | QVQLQESGPGLVKPSSETLSL<br>TCTVSGGSISSYYWSWIRQP<br>PGKGLIEWIGYIYYSGSTN<br>YNPSLKSRTISVDTSKNQFSL<br>KLSSVTAA<br>DTAVYYCARTGYIPMDVWGQ<br>GTTVT<br>TVSS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |

| SEQ ID NO: | Description                       | Sequence                                                                                                                       |
|------------|-----------------------------------|--------------------------------------------------------------------------------------------------------------------------------|
| 12         | SV018.20B12.1D1 VL                | EIVLTQSPDFQSVTPKEKVTITCRASQSIGSNLHWYQQKPDQSP<br>KLLIKYVSQSFSGVPSRFSGSGSGTDFTLTINSLEAEDAATYYC<br>HQSRLPYTFGQGTKLEIK             |
| 13         | SV018.20B12.1D1 VH-<br>CDR1 Kabat | SYYS                                                                                                                           |
| 14         | SV018.20B12.1D1 VH-<br>CDR2 Kabat | YIYYSGSTNYNPSLKS                                                                                                               |
| 15         | SV018.20B12.1D1 VH-<br>CDR3 Kabat | TGYIPMDV                                                                                                                       |
| 16         | SV018.20B12.1D1 VL-<br>CDR1 Kabat | RASQSIGSNLH                                                                                                                    |
| 17         | SV018.20B12.1D1 VL-<br>CDR2 Kabat | YVSQSFS                                                                                                                        |
| 18         | SV018.20B12.1D1 VL-<br>CDR3 Kabat | HQSRLPYT                                                                                                                       |
| 19         | SV018.45B6.1G1 VH                 | QVQLLQSGAEVKKPGASVKVSCKASGYTFTTYGISWVRQAPGQG<br>LEWLGWITAYNRNTNYAQKLQGRVTMTTDTSTSTSFMVLRSLRS<br>DDTAVYYCARVGDSDAFDIWGQGTMTVTSS |
| 20         | SV018.45B6.1G1 VL                 | EIVMTQSPATLSLSPGERATLSCRTSQSVFNSFLSWYQQKPGQA<br>PRLLLYGTSTRATGIPARFSGSGSGTDFTLTISLQPEDFAVYY<br>CQQDYKLPLTFGGGTKVEIK            |
| 21         | SV018.45B6.1G1 VH-<br>CDR1 Kabat  | TYGIS                                                                                                                          |
| 22         | SV018.45B6.1G1 VH-<br>CDR2 Kabat  | WITAYNRNTNYAQKLQG                                                                                                              |
| 23         | SV018.45B6.1G1 VH-<br>CDR3 Kabat  | VGDSDAFDI                                                                                                                      |
| 24         | SV018.45B6.1G1 VL-<br>CDR1 Kabat  | RTSQSVFNSFLS                                                                                                                   |
| 25         | SV018.45B6.1G1 VL-<br>CDR2 Kabat  | GTSTRAT                                                                                                                        |
| 26         | SV018.45B6.1G1 VL-<br>CDR3 Kabat  | QQDYKLPLT                                                                                                                      |
| 27         | SV017.13B12.1E1 VH                | EVQLVESGGGLVQPGGSLRLSCAATGFTTFSSYWMHWVRQGPGKG<br>LVWVSRINDGTSTVYADSVKGRFTISRDNKNTLHLQMNSLRA<br>EDTAVYYCARGRGIMTYWGQGTTLVTVSS   |
| 28         | SV017.50G3.1B1 VH                 | EVQLVESGGGLVQPGESLRLSCAATGFTTFSSYWMHWVRQGPGKG<br>LVWVSRINDGTSTVYADSVKGRFTISRDNKNTLFLQMNSLRA<br>EDTAVYYCARGRGIMTYWGQGTTLVTVSS   |
| 29         | SV017.51F4.1A1 VH                 | EVQLVESGGGLVQPGGSLRLSCAATGFTTFSSYWMHWVRQGPGKG<br>LVWVSRINDGTSTVYADSVKGRFTISRDNKNTLYLQMNSLRA<br>EDTAVYYCARGRGIMTYWGQGTTLVTVSS   |

| SEQ ID NO: | Description        | Sequence                                                                                                                                                                                                                                                           |
|------------|--------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 30         | 15B6 VH            | EVQLQQSGPVLVKPGASVKISCKASGYSTGYMHVWRQSLVKRL<br>EWIGRINPYTGVPSYKHNFKDKASLTVDKSSSTAYMELHSLTSED<br>SAVYYCARELGGYWGQGTTLTVSS                                                                                                                                           |
| 31         | 15B6 VL            | QAVVTQESALTTSPGETVTLTCRSSTGAVTTGNYPNWWQEKPDHL<br>FTGLIAGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFC<br>ALWFSSHWVFGGGTKLTVL                                                                                                                                              |
| 32         | 15B6 VH-CDR1       | GYMH                                                                                                                                                                                                                                                               |
| 33         | 15B6 VH-CDR2       | RINPYTGVPSYKHNFKD                                                                                                                                                                                                                                                  |
| 34         | 15B6 VH-CDR3       | ELGGY                                                                                                                                                                                                                                                              |
| 35         | 15B6 VL-CDR1       | RSSTGAVTTGNYPN                                                                                                                                                                                                                                                     |
| 36         | 15B6 VL-CDR2       | GTNNRAP                                                                                                                                                                                                                                                            |
| 37         | 15B6 VL-CDR3       | ALWFSSHWV                                                                                                                                                                                                                                                          |
| 38         | 47D7 VL-(G4S)3-VH  | DIQMTQSPSSLSASVGDRVITTCRASQDIGSWLTWYQRKPEKAP<br>KSLIYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFATYYC<br>QQYKSYPLTFGQGALEIKGGGSGGGGSGGGGSEVQLVESGGG<br>LVQPGGSLRLSCAATGFTTFSSYWMHWVRQGPGLVWVSRINSD<br>GTSTVYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAR<br>GRGIMTYWGQGTTLTVSS      |
| 39         | 45B6 VL-(G4S)3-VH  | EIVMTQSPATLSLSPGERATLSCRTSQSVFNSFLSWYQQKPGQAP<br>RLLLYGTSTRATGIPARFSGSGSGTDFTLTISLQPEDFAVYYCQ<br>QDYKLPLTFGGGTKVEIKGGGSGGGGSGGGGSQVQLQSGAEVK<br>KPGASVKVSKASGYTFTTYGISWVRQAPGQGLEWLGWITAYNRN<br>TNYAQKLQGRVTMTTDTSTSTSFVLRSLRSDDTAVYYCARVGD<br>DAFDIWGQGTMTVTVSS   |
| 40         | 20B12 VL-(G4S)3-VH | EIVLTQSPDFQSVTPKEKVTITCRASQSIGSNLHWYQQKPDQSPK<br>LLIKYVSQSFSGVPSRFSGSGSGTDFTLTINSLEAEDAATYYCHQ<br>SRSLPYTFGQGTKLEIKGGGSGGGGSGGGGSQVQLQESGPGLVK<br>PSETLSLTCTVSGGSISSYYWSWIRQPPGKGLEWIGYIYSGSTN<br>YNPSLKSRTVISVDTSKNQFSLKLSSVTAADTAVYYCARTGYIPM<br>DVWGQGTTLTVTVSS |
| 41         | 15B6 VL-(G4S)3-VH  | QAVVTQESALTTSPGETVTLTCRSSTGAVTTGNYPNWWQEKPDHL<br>FTGLIAGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFC<br>ALWFSSHWVFGGGTKLTVLGGGSGGGGSGGGGSEVQLQQSGPVL<br>VKPGASVKISCKASGYSTGYMHVWRQSLVKRLEWIGRINPYTG<br>VPSYKHNFKDKASLTVDKSSSTAYMELHSLTSEDSAVYYCARELG<br>GYWGQGTTLTVSS    |
| 42         | 13B12 VL-(G4S)3-VH | DIQMTQSPSSLSASVGDRVITTCRASQDIGSWLTWYQRKPEKAP<br>KSLIYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFATYYC<br>QQYKSYPLTFGQGALEIKGGGSGGGGSGGGGSEVQLVESGGG<br>LVQPGGSLRLSCAATGFTTFSSYWMHWVRQGPGLVWVSRINSD<br>GTSTVYADSVKGRFTISRDNKNTLHLQMNSLRAEDTAVYYCAR<br>GRGIMTYWGQGTTLTVTVSS    |
| 43         | 50G3 VL-(G4S)3-VH  | DIQMTQSPSSLSASVGDRVITTCRASQDIGSWLTWYQRKPEKAP<br>KSLIYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFATYYC                                                                                                                                                                        |

| SEQ ID NO: | Description                          | Sequence                                                                                                                                                                                                                                                          |
|------------|--------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|            |                                      | QQYKSYPLTFGQGAKLEIKGGGSGGGGSGGGGSEVQLVESGGG<br>LVQPGESLRLSCAATGFTTFSSYWMHWVRQGPGLVWVSRINSD<br>GTSTVYADSVKGRFTISRDNKNTLFLQMNSLRAEDTAVYYCAR<br>GRGIMTYWGQGTTLVTVSS                                                                                                  |
| 44         | 51F4 VL-(G4S)3-VH                    | DIQMTQSPSSLSASVGDRVITITCRASQDIGSWLTWYQRKPEKAP<br>KSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC<br>QQYKSYPLTFGQGAKLEIKGGGSGGGGSGGGGSEVQLVESGGG<br>LVQPGGSLRLSCAATGFTTFSSYWMHWVRQGPGLVWVSRINSD<br>GTSTVYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAR<br>GRGIMTYWGQGTTLVTVSS |
| 45         | Leader peptide A                     | MSVPTQVLGLLLLLWLT DARC                                                                                                                                                                                                                                            |
| 46         | Leader peptide B                     | MEWSWVFLFFLSVT TGVHS                                                                                                                                                                                                                                              |
| 47         | CD8 hinge                            | TTTPAPRPPTPAPT IASQPLSLRPEACRPAAGGAVHTRGLDFAC<br>DIY                                                                                                                                                                                                              |
| 48         | CD8 signaling domain                 | IWAPLAGTCGVLLLSLVITLYCK                                                                                                                                                                                                                                           |
| 49         | 4-1BB intracellular domain           | RGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCEL                                                                                                                                                                                                                          |
| 50         | CD3-zeta intracellular domain        | RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPE<br>MGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG<br>LYQGLSTATKDTYDALHMQALPPR                                                                                                                                          |
| 51         | CD8 $\alpha$ Signal                  | MALPVTALLLPLALLLHAARP                                                                                                                                                                                                                                             |
| 52         | (G4S)3 linker                        | GGGGSGGGGSGGGGS                                                                                                                                                                                                                                                   |
| 53         | IgG1 (mut) hinge                     | EPKSCDKTHTCPPCAPPVAGPSVFLFPPKPKDTLMISRTPEVT<br>CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV<br>YTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK<br>TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHY<br>TQKSLSLSPGK         |
| 54         | CD28TM                               | FWVLVVVGGVLACYSLLVTVAFIIFWV                                                                                                                                                                                                                                       |
| 55         | CD28 intracellular signaling domain  | RSKRSRLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS                                                                                                                                                                                                                          |
| 56         | OX40L intracellular signaling domain | ERVQPLEENVGNAARPRFERNK                                                                                                                                                                                                                                            |
| 57         | CD3-zeta (CD3z) domain               | RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPE<br>MGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG<br>LYQGLSTATKDTYDALHMQALPPR                                                                                                                                          |
| 58         | T2A                                  | GSGEGRGSLTTCGDVEENPGP                                                                                                                                                                                                                                             |
| 59         | IL-15                                | MRISKPHLRSISIQCYLCLLLNSHFLTEAGIHVFILGCF SAGLP<br>KTEANWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAM<br>KCFLLELQVISLES GDASIHDTVENLIILANNSLSSNGNVTESG<br>CKECEELEEKNIKEFLQS FVHIVQMFINTS                                                                                 |
| 60         | CD8 $\alpha$ hinge                   | AKPTTTPAPRPPTPAPT IASQPLSLRPEACRPAAGGAVHTRGLD<br>FACD                                                                                                                                                                                                             |

| SEQ ID NO: | Description                                                                                                         | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
|------------|---------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 61         | Polypeptide Sequence for 47D7 VL-(G4S)3-VH- IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (with signal peptide) | MALPVTALLLPLALLLHAARPDIQMTQSPSSLSASVGDRVTTITC<br>RASQDIGSWLTWYQRKPEKAPKSLIYAASSLQSGVPSRFSGSGS<br>GTDFTLTIISSLQPEDFATYYCQQYKSYPLTFGQGAKLEIKGGGG<br>SGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAATGFTFSSYW<br>MHWVRQGPQKGLVWVSRINSDGTSTVYADSVKGRFTISRDNANK<br>TLYLQMNSLRAEDTAVYYCARGRGIMTYWGQGTLLTVTSSEPKSC<br>DKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVD<br>VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVL<br>HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP<br>SRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPV<br>LSDSGSFFLYSKLTVDKSRWQQGNVFSFSVMHEALHNHYTQKSL<br>SLSPGKFWVLVVGGLVACYSLLVTVAFIIFWVRSKRSRLHSD<br>YMNMTPRRPGPTRKHYQPYAPPRDFAAYRSEVRQPLEENVGNAA<br>RPRFERNKRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVL<br>DKRRGRDPKRRGRDPKRRGRDPKRRGRDPKRRGRDPKRRGRDP<br>EMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGER<br>RRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGEGRGSLTTC<br>GDVEENPGPMRISKPHLRSISIQCYLCLLLNSHFLTEAGIHVFI<br>LGCF SAGLPKTEANWVNVISDLKKIEDLIQSMHIDATLYTESDV<br>HPSCKVTAMKCFLEELQVISLES GDASIHDTVENLIILANNLSLS<br>SNGNVTESGCKECELEEKNIKEFLQSFVHIVQMFINITS |
| 62         | Polypeptide Sequence for 47D7 VL-(G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (with signal peptide)              | MALPVTALLLPLALLLHAARPDIQMTQSPSSLSASVGDRVTTITC<br>RASQDIGSWLTWYQRKPEKAPKSLIYAASSLQSGVPSRFSGSGS<br>GTDFTLTIISSLQPEDFATYYCQQYKSYPLTFGQGAKLEIKGGGG<br>SGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAATGFTFSSYW<br>MHWVRQGPQKGLVWVSRINSDGTSTVYADSVKGRFTISRDNANK<br>TLYLQMNSLRAEDTAVYYCARGRGIMTYWGQGTLLTVSSAKPTT<br>TPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDF<br>WVLVVGGLVACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPR<br>RPGPTRKHYQPYAPPRDFAAYRSEVRQPLEENVGNAA RPRFERN<br>KRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVL DKRRGRDP<br>EMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHD<br>GLYQGLSTATKDTYDALHMQALPPRGSGEGRGSLTTCGDVEENP<br>GPMRISKPHLRSISIQCYLCLLLNSHFLTEAGIHVFI LGCF SAG<br>LPKTEANWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVT<br>AMKCFLEELQVISLES GDASIHDTVENLIILANNLSLS SNGNVTE<br>SGCKECELEEKNIKEFLQSFVHIVQMFINITS                                                                                                                                                                                                                                            |
| 63         | Polypeptide Sequence for 45B6 VL-(G4S)3-VH- IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (with signal peptide) | MALPVTALLLPLALLLHAARPEIVMTQSPATLSLSPGERATLSCR<br>TSQSVFNSFLSWYQQKPGQAPRLLLYGTSTRATGIPARFSGSGSG<br>TDFTLTIISSLQPEDFAVYYCQQDYKLPLTFGGGKTVEIKGGGGSG<br>GGGSGGGGSQVQLLQSGAEVKKPGASVKVSCKASGYTFTTYGISW<br>VRQAPGQGLEWLGWITAYNRNTNYAQKLQGRVTMTTDTSTSTSM<br>VLRSLRSDDTAVYYCARVGDSDAFDIWGQGTMTVTSSEPKSCDKT<br>HTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE<br>DPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWL<br>NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |

| SEQ ID NO: | Description                                                                                                          | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
|------------|----------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|            |                                                                                                                      | KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSF<br>FLYSKLTVDKSRWQQGNVFSVMHEALHNHYTQKSLSLSPGKFW<br>VLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPRRP<br>GPTRKHYQPYAPPRDFAAYRSERVQPLEENVGNAARPRFERNKRV<br>KFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGG<br>KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGL<br>LSTATKDTYDALHMQALPPRGSGEGRGSLTTCGDVEENPGPMRIS<br>KPHLRSISIQCYLCLLLNSHFLTEAGIHVFILGCFASAGLPKTEAN<br>WVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLLE<br>LQVISLESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEEL<br>EENIKEFLQSFVHIVQMFINTS                                                                                                                                                                                                                                                                                                                                                                                |
| 64         | Polypeptide Sequence for 45B6 VL-(G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (with signal peptide)               | MALPVTALLLPLALLLHAARPEIVMTQSPATLSLSPGERATLSCR<br>TSQSVFNSFLSWYQQKPGQAPRLLLYGTSTRATGIPARFSGSGSG<br>TDFTLTISLQPEDFAVYYCQQDYKLPLTFGGGTKEIKGGGGSG<br>GGSGGGGSQVQLLQSGAEVKKPGASVKVSCKASGYTFTTYGISW<br>VRQAPGQGLEWLGWITAYNRNTNYAQKLQGRVTMTTDTSTSTSF<br>VLRLSRSDDTAVYYCARVGDSDAFDIWGQGTMTVTVSSAKPTTTPA<br>PRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFWVLV<br>VVGGLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPRRPGPT<br>RKHYQPYAPPRDFAAYRSERVQPLEENVGNAARPRFERNKRVKFS<br>RSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPR<br>RKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST<br>ATKDTYDALHMQALPPRGSGEGRGSLTTCGDVEENPGPMRISKPH<br>LRSISIQCYLCLLLNSHFLTEAGIHVFILGCFASAGLPKTEANWVN<br>VISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLLELQV<br>ISLESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEELEEK<br>NIKEFLQSFVHIVQMFINTS                                                                                                                                |
| 65         | Polypeptide Sequence for 20B12 VL-(G4S)3-VH- IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (with signal peptide) | MALPVTALLLPLALLLHAARPEIVLTQSPDFQSVTPKEKVTITCR<br>ASQSIGSNLHWYQQKPDQSPKLLIKYVSQSFSGVPSRFSGSGSGT<br>DFTLTINSLEAEDAATYYCHQSRSLPYTFGQGTKLEIKGGGGSGG<br>GGSGGGGSQVQLQESGPGLVKPSSETLSLTCTVSGGSISSYYWSWI<br>RQPPGKGLEWIGYIYYSGSTNYPNPSLKSRTVISVDTSKNQFSLKL<br>SSVTAADTAVYYCARTGYIPMDVWGQGTTVTVSSSEPKSCDKTHTC<br>PPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE<br>VKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGK<br>EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ<br>VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLY<br>SKLTVDKSRWQQGNVFSVMHEALHNHYTQKSLSLSPGKFWVLV<br>VVGGLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPRRPGPT<br>RKHYQPYAPPRDFAAYRSERVQPLEENVGNAARPRFERNKRVKFS<br>RSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPR<br>RKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST<br>ATKDTYDALHMQALPPRGSGEGRGSLTTCGDVEENPGPMRISKPH<br>LRSISIQCYLCLLLNSHFLTEAGIHVFILGCFASAGLPKTEANWVN<br>VISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLLELQV |

| SEQ ID NO: | Description                                                                                                         | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
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|            |                                                                                                                     | ISLESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEK<br>NIKEFLQSFVHIVQMFINTS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| 66         | Polypeptide Sequence for 20B12 VL-(G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (with signal peptide)             | MALPVTALLLPLALLLHAARPEIVLTQSPDFQSVTPKEKVTITCR<br>ASQSIGSNLHWYQQKPDQSPKLLIKYVSQSFSGVPSRFSGSGSGT<br>DFTLTINSLEAEDAATYYCHQSRSLPYTFGQGTKLEIKGGGGSGG<br>GGSGGGGSQVQLQESGPGLVKPSSETLSLTCTVSGGSISSYYWSWI<br>RQPPGKGLEWIGYIYSGSTNYPNPSLKSRTVISVDTSKNQFSLKL<br>SSVTAADTAVYYCARTGYIPMDVWGQGTITVTVSSAKPTTTPAPRP<br>PTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFWVLVVG<br>GVLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPRRPGPTRKH<br>YQPYAPPRDFAAYRSESVQPLEENVGNAARPRFERNKRVKFSRSA<br>DAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKN<br>PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATK<br>DTYDALHMQALPPRGSGEGRGSLLTCGDVEENPGPMRISKPHLRS<br>ISIQCYLCLLLNSHFLTEAGIHVFILGCFSAAGLPKTEANWVNVIS<br>DLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQVISL<br>ESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEKNIK<br>EFLQSFVHIVQMFINTS                                                                                                                                                                                                  |
| 67         | Polypeptide Sequence for 15B6 VL-(G4S)3-VH- IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (with signal peptide) | MALPVTALLLPLALLLHAARPQAVVTQESALTTSPGETVTLTCSR<br>STGAVTTGNYPNWWQEKPDHLFTGLIAGTNNRAPGVPARFSGSLI<br>GDKAALTITGAQTEDEAIYFCALWFSSHVVFSGGKLTVLGGGGGS<br>GGGGSGGGGSEVQLQQSGPVLVKPGASVKISCKASGYSTGYMH<br>WVRQSLVKRLEWIGRINPYTGVPYKHNFKDKASLTVDKSSSTAY<br>MELHSLTSEDSAVYYCARELGYYWGQGTTLTVSSEPKSCDKTHTC<br>PPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE<br>VKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGK<br>EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ<br>VSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLY<br>SKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGKFWVLV<br>VVGVLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPRRPGPT<br>RKHYQPYAPPRDFAAYRSESVQPLEENVGNAARPRFERNKRVKFS<br>RSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPR<br>RKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST<br>ATKDTYDALHMQALPPRGSGEGRGSLLTCGDVEENPGPMRISKPH<br>LRSISIQCYLCLLLNSHFLTEAGIHVFILGCFSAAGLPKTEANWVN<br>VISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLELQV<br>ISLESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEK<br>NIKEFLQSFVHIVQMFINTS |
| 68         | Polypeptide Sequence for 15B6 VL-(G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (with signal peptide)              | MALPVTALLLPLALLLHAARPQAVVTQESALTTSPGETVTLTCSR<br>STGAVTTGNYPNWWQEKPDHLFTGLIAGTNNRAPGVPARFSGSLI<br>GDKAALTITGAQTEDEAIYFCALWFSSHVVFSGGKLTVLGGGGGS<br>GGGGSGGGGSEVQLQQSGPVLVKPGASVKISCKASGYSTGYMH<br>WVRQSLVKRLEWIGRINPYTGVPYKHNFKDKASLTVDKSSSTAY<br>MELHSLTSEDSAVYYCARELGYYWGQGTTLTVSSAKPTTTPAPRP<br>PTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFWVLVVG                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |

| SEQ ID NO: | Description                                                                              | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
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|            |                                                                                          | GVLACYSLLVTVAFII FWVRSKRSRLHSDYMNMTPRRPGPTRKH<br>YQPYAPPRDFAAYRSEVRVQPLEENVGNAARPRFERNKRVKFSRSA<br>DAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN<br>PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATK<br>DTYDALHMQALPPRGSGEGRGSLLTCGDVEENPGPMRISKPHLRS<br>ISIQCYLCLLLNSHFLTEAGIHVFILGCF SAGLPKTEANWVNVIS<br>DLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLEELQVISL<br>ESGDASIHDTVENLIILANNSLSSNGNVTESGCKECEEELEEKNIK<br>EFLQSFVHIVQMFINTS                                                                                                                                                                    |
| 69         | scFv-polypeptide sequence for 47D7 clone CD8a Signal-VL-(G4S)3-VH (with signal peptide)  | MALPVTALLLPLALLLHAARPDIQMTQSPSSLSASVGDRVTITC<br>RASQDIGSWLTWYQRKPEKAPKSLIYAASSLQSGVPSRFSGSGS<br>GTDFTLTISSSLQPEDFATYYCQQYKSYPLTFGQGAKLEIKGGGG<br>SGGGSGGGGSEVQLVESGGGLVQPGGSLRLS CAATGFTFSSYW<br>MHWVRQGP GKGLVWVSRINSDGTSTVYADSVKGRFTISRDNAKN<br>TLYLQMNSLRAEDTAVYYCARGRGIMTYWGQGT LVTVSS                                                                                                                                                                                                                                                                                                       |
| 70         | scFv-polypeptide sequence for 45B6 clone CD8a Signal-VL-(G4S)3-VH (with signal peptide)  | MALPVTALLLPLALLLHAARPEIVMTQSPATLSLSPGERATLSCR<br>TSQSVFNSFLSWYQQKPGQAPRLLLYGTSTRATGIPARFSGSGSG<br>TDFTLTISSSLQPEDFAVYYCQQDYKLPLTFGGG TKVEIKGGGGSG<br>GGSGGGGSQVQLLQSGAEVKKPGASVKV SCKASGYTFTTYGISW<br>VRQAPGQGLEWLGWITAYNRNTNYAQKLQGRVTMTTDTSTSTSF<br>VLRSLRSDDTAVYYCARVGDSDAFDIWGQGTMTVTSS                                                                                                                                                                                                                                                                                                      |
| 71         | scFv-polypeptide sequence for 20B12 clone CD8a Signal-VL-(G4S)3-VH (with signal peptide) | MALPVTALLLPLALLLHAARPEIVLTQSPDFQSVTPKEKVTITCR<br>ASQSIGSNLHWYQQKPDQSPKLLIKYVSQSFSGVPSRFSGSGSGT<br>DFTLTINSLEAEDAATYYCHQSRSLPYTFGQGTKLEIKGGGGSGG<br>GGSGGGGSQVQLQESGPGLVKPSSETLSLTCTVSGGSISSYYWSWI<br>RQPPGKGLEWIGYIYYSGSTNYPNPSLKSRVTISVDTSKNQFSLKL<br>SSVTAADTAVYYCARTGYIPMDVWGQGT TTVTVSS                                                                                                                                                                                                                                                                                                      |
| 72         | scFv-polypeptide sequence for 15B6 clone CD8a Signal-VL-(G4S)3-VH (with signal peptide)  | MALPVTALLLPLALLLHAARPQAVVTQESALTTS PGETVTLTCRS<br>STGAVTTGNYPNWVQEKPDHLFTGLIAGTNNRAPGV PARFSGSLI<br>GDKAALTITGAQTEDEAIYFCALWFSSHVVF GGGTKLTVLGGGGS<br>GGGGSGGGGSEVQLQQSGPVLVKPGASVKISCKASGY SFTGYMH<br>WVRQSLVKRLEWIGRINPYTGVP SYKHNFKDKASLTVDKSSSTAY<br>MELHSLTSEDSAVYYCARELG GYGWQGT TTVTVSS                                                                                                                                                                                                                                                                                                   |
| 73         | Polypeptide sequence for IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15               | EPKSCDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVT<br>CVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV<br>YTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK<br>TTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMEALHNHY<br>TQKSLSLSPGKFWVLVVVGVLACYSLLVTVAFII FWVRSKRSR<br>LLHSDYMNMTPRRPGPTRKH YQPYAPPRDFAAYRSEVRVQPLEEN<br>VGNAARPRFERNKRVKFSRSADAPAYQQGQNQLYNELNLGRREE<br>YDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG<br>MKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPRGSGEGRG<br>SLLTCGDVEENPGPMRISKPHLRSISIQCYLCLLLNSHFLTEAG<br>IHVFILGCF SAGLPKTEANWVNVISDLKKIEDLIQSMHIDATLY |

| SEQ ID NO: | Description                                                                         | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
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|            |                                                                                     | TESDVHPSCKVTAMKCFLELELQVISLES GDASIHDTVENLIILA<br>NNSLSSNGNVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINTS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| 74         | Polypeptide Sequence for CD8a hinge-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15                | AKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD<br>FACDFWVLVVVGGLACYSLLVTVAFIIFWVRSKRSRLLHSDYM<br>NMTPRRPGPTRKHYQPYAPPRDFAAYRSE RVQPLEENVGNAARP<br>RFERNKRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDR<br>RGRDPGEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRR<br>GKGHDGLYQGLSTATKDTYDALHMQALPPRGSGEGRGSLTTCGD<br>VEENPGPMRISKPHLRSISIQCYLCLLLNSHFLTEAGIHVFILG<br>CFSAGLPKTEANWVNVISDLKKIEDLIQSMHIDATLYTESDVHP<br>SCKVTAMKCFLELELQVISLES GDASIHDTVENLIILANNLSSN<br>GNVTESGCKECEEELEEKNIKEFLQSFVHIVQMFINTS                                                                                                                                                                                                                                                               |
| 75         | Polypeptide Sequence for 47D7 VL-(G4S)3-VH- IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z | DIQMTQSPSSLSASVGDRTTITCRASQDIGSWLTWYQRKPEKAP<br>KSLIYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFATYYC<br>QQYKSYPLTFGQGALEIKGGGSGGGGSGGGGSEVQLVESGGG<br>LVQPGGSLRLSCAATGFTTFSSYWMHWVRQGPGLVWVSRINSD<br>GTSTVYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAR<br>GRGIMTYWGQGTTLVTVSSEPKSCDKTHTCPPCPAPPVAGPSVFL<br>FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHN<br>AKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA<br>PIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYP<br>SDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQ<br>QGNVFSQSVMEALHNHYTQKSLSLSPGKFWVLVVVGGLACYS<br>LLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAP<br>PRDFAAYRSE RVQPLEENVGNAARPRFERNKRVKFSRSADAPAY<br>QQGQNQLYNELNLGRREEYDVLDRGRDPGEMGGKPRRKNPQEG<br>LYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTY<br>DALHMQALPPR |
| 76         | Polypeptide Sequence for 47D7 VL-(G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z              | DIQMTQSPSSLSASVGDRTTITCRASQDIGSWLTWYQRKPEKAP<br>KSLIYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFATYYC<br>QQYKSYPLTFGQGALEIKGGGSGGGGSGGGGSEVQLVESGGG<br>LVQPGGSLRLSCAATGFTTFSSYWMHWVRQGPGLVWVSRINSD<br>GTSTVYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAR<br>GRGIMTYWGQGTTLVTVSSAKPTTTPAPRPPTPAPTIASQPLSLR<br>PEACRPAAGGAVHTRGLDFACDFWVLVVVGGLACYSLLVTVAF<br>IIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY<br>RSE RVQPLEENVGNAARPRFERNKRVKFSRSADAPAYQQGQNQL<br>YNELNLGRREEYDVLDRGRDPGEMGGKPRRKNPQEGLYNELQK<br>DKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQA<br>LPPR                                                                                                                                                                                                       |
| 77         | Polypeptide Sequence for 45B6 VL-(G4S)3-VH- IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z | EIVMTQSPATLSLSPGERATLSCRTSQSVFNSFLSWYQQKPGQAP<br>RLLLYGTSTRATGIPARFSGSGSGTDFTLTISLQPEDFAVYYCQ<br>QDYKLPLTFGGGKVEIKGGGSGGGGSGGGGSGVQLLQSGAEVK<br>KPGASVKVSKASGYTFTTYGISWVRQAPGQGLEWLGWITAYNRN<br>TNYAQLQGRVTMTTDTSTSTSFVLRSLRSDDTAVYYCARVGD                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |

| SEQ ID NO: | Description                                                                          | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
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|            |                                                                                      | DAFDIWGQGTMTVTVSSEPKSCDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPGKFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSEERVQPLEENVGNAARPRFERNKRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQUALPPR                                                                                                                                                                                                                                |
| 78         | Polypeptide Sequence for 45B6 VL-(G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z               | EIVMTQSPATLSLSPGERATLSCRTSQSVFNSFLSWYQQKPGQAPRLLLYGTSTRATGIPARFSGSGSGTDFTLTITSSSLQPEDFAVYYCQQDYKLPLTFGGGKVEIKGGGGSGGGSGGGGSQVQLQSGAEVKKPGASVKVSCKASGYTFTTYGISWVRQAPGQGLEWLGWITAYNRNTNYAQLQGRVTMTTDTSTSTSFVLRSLRSDDTAVYYCARVGSDDAFDIWGQGTMTVTVSSAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSEERVQPLEENVGNAARPRFERNKRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQUALPPR                                                                                                                                                                                       |
| 79         | Polypeptide Sequence for 20B12 VL-(G4S)3-VH- IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z | EIVLTQSPDFQSVTPKEKVTITCRASQSIGSNLHWYQQKPDQSPKLLIKYVSQSFSGVPSRFSGSGSGTDFTLTINSLEAEDAATYYCHQSRSLPYTFGQGTKLEIKGGGGSGGGSGGGGSQVQLQESGPGLVKPSETLSLTCTVSGGSISSYYWSWIRQPPGKGLEWIGYIYYSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARTGYIPMDVWGQGTITVTVSSEPKSCDKTHTCPPCPAPPVAGPSVFLFPPKPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPGKFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSEERVQPLEENVGNAARPRFERNKRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQUALPPR |
| 80         | Polypeptide Sequence for 20B12 VL-(G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z              | EIVLTQSPDFQSVTPKEKVTITCRASQSIGSNLHWYQQKPDQSPKLLIKYVSQSFSGVPSRFSGSGSGTDFTLTINSLEAEDAATYYCHQSRSLPYTFGQGTKLEIKGGGGSGGGSGGGGSQVQLQESGPGLVKPSETLSLTCTVSGGSISSYYWSWIRQPPGKGLEWIGYIYYSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARTGYIPMDVWGQGTITVTVSSAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSEERVQPLEENVGNAARPRFERNKRVKFSRSADAPAYQQGQNQLYNELNLGR                                                                                                                                                                                                                                                                            |

| SEQ ID NO: | Description                                                                                               | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
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|            |                                                                                                           | EEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEI<br>GMKGERRRGKGHDLGLYQGLSTATKDTYDALHMQALPPR                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| 81         | Polypeptide Sequence for 15B6 VL-(G4S)3-VH- IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z                       | QAVVTQESALTTSPGETVTLTCRSSTGAVTTGNYPNWVQEKPDHL<br>FTGLIAGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFC<br>ALWFSSHWVFGGGTKLTVLGGGGSGGGSGGGGSEVQLQQSGPVL<br>VKPGASVKISCKASGYSFTGYMHVWRQSLVKRLEWIGRINPYTG<br>VPSYKHNFKDKASLTVDKSSSTAYMELHSLTSEDSAVYYCARELG<br>GYWGQGTTLTVSSEPKSCDKTHTCPPCPAPPVAGPSVFLFPPKPK<br>DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE<br>EQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK<br>AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWES<br>NGQPENNYKTTTPVLDSGSSFLLYSKLTVDKSRWQQGNVFCSSVM<br>HEALHNHYTQKSLSLSPGKFWVLVVVGGVLACYSLLVTVAFIIFW<br>VRSKRSLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSERV<br>QPLEENVGNAARPRFERNKRVKFSRSADAPAYQQGQNQLYNELNL<br>GRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAY<br>SEIGMKGERRRGKGHDLGLYQGLSTATKDTYDALHMQALPPR                                                                                 |
| 82         | Polypeptide Sequence for 15B6 VL-(G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z                                    | QAVVTQESALTTSPGETVTLTCRSSTGAVTTGNYPNWVQEKPDHL<br>FTGLIAGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFC<br>ALWFSSHWVFGGGTKLTVLGGGGSGGGSGGGGSEVQLQQSGPVL<br>VKPGASVKISCKASGYSFTGYMHVWRQSLVKRLEWIGRINPYTG<br>VPSYKHNFKDKASLTVDKSSSTAYMELHSLTSEDSAVYYCARELG<br>GYWGQGTTLTVSSAKPTTTPAPRPPTPAPTIASQPLSLRPEACRP<br>AAGGAVHTRGLDFACDFWVLVVVGGVLACYSLLVTVAFIIFWVRS<br>KRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSERVQPL<br>EENVGNAARPRFERNKRVKFSRSADAPAYQQGQNQLYNELNLGRR<br>EEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEI<br>GMKGERRRGKGHDLGLYQGLSTATKDTYDALHMQALPPR                                                                                                                                                                                                                                                                                      |
| 83         | Polynucleotide Sequence for 47D7 VL-(G4S)3-VH-IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (2628 bp) | ATGGCCTTGCCCGTCACTGCCCTGTTGCTGCCGCTTGCTCTGCT<br>TCTCCATGCAGCACGCCAGATATTCAGATGACTCAATCCCCGT<br>CTTCACTCAGCGCCTCAGTGGGGGATCGAGTCACGATCACATGC<br>CGAGCTTCTCAAGATATAGGGTCTTGCTGACGTGGTATCAACG<br>GAAACCGGAAAAGGCCCTAAATCACTCATCTATGCAGCGTCAT<br>CTTTGCAATCCGGCGTGCCAGCAGGTTTAGCGGTAGCGGAAGT<br>GGCACAGACTTCACACTGACAATCAGTTCCTTGCAGCCAGAGGA<br>CTTTGCCACTTATTATTGTCAGCAGTATAAGTCCTATCCACTTA<br>CCTTCGGACAGGGTGCCAAACTCGAGATTAAAGGCGGTGGCGGC<br>TCTGGAGGTGGAGGCTCAGGGGGTGGGGGGAGTGAGGTGCAATT<br>GGTGGAGAGTGGCGGGGGTCTCGTGCAACCCGGAGGATCACTGA<br>GATTGAGCTGCGCCGCACTGGGTTTACTTTTTCCAGCTATTGG<br>ATGCACTGGGTCCGCCAAGGACCCGGAAGGGCCTGGTTTGGGT<br>TTCTCGCATCAACTCTGACGGCACTAGCACGGTGTACGCTGACT<br>CCGTCAAAGGGCGGTTTACCATTAGTCGAGACAATGCAAAAAC<br>ACCTGTATTTGCAGATGAACTCCCTTAGGGCAGAAGACACGGC<br>AGTATACTATTGTGCGCGCGCAGGGGCATCATGACCTATTGGG |

| SEQ ID NO: | Description                          | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
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|            |                                      | GGCAAGGGACACTGGTAACGGTGTCAAGTGAGCCAAAGTCTTGC<br>GACAAGACTCATACCTGCCCGCCTTGTCCCGCACCACCTGTGGC<br>AGGTCCCAGCGTGTCTTCTGTTTCCGCCCAAGCCCAAGGACACAC<br>TCATGATCTCTAGGACACCCGAGGTACATGCGTCGTGGTTGAT<br>GTTTCTCATGAAGACCCCGAAGTGAAGTTAACTGGTATGTCTGA<br>CGGAGTTGAGGTCCATAATGCTAAAACAAAACCACGCGAGGAAC<br>AATATCAAAGCACCTATCGGGTCGTTAGTGTCTTACTGTACTT<br>CACCAGGACTGGCTCAACGGAAAGGAATATAAGTGCAAAGTCAG<br>CAATAAAGCCTTGCCAGCGCCCATCGAAAAAATATCAGCAAGG<br>CCAAGGGCCAACCCAGAGAACCCCAAGTGTACACACTCCCTCCT<br>AGCCGGGATGAATTGACCAAAAATCAaGTCtctCTCACTTGTCT<br>GGTAAAGGGATTCTATCCAAGTGATATTGCTGTCTGAATGGGAGA<br>GCAACGGGCAACCGGAGAACAATTACAAAACGACACCTCCCGTC<br>CTTGATAGTGACGGGTCTTCTTCTGTATTCAAAGCTTACAGT<br>CGACAAAAGTCGCTGGCAGCAGGGGAATGTCTTTAGCTGTAGTG<br>TCATGCACGAAGCTTTGCATAACCACTACACGCAAAAGTCTCTT<br>AGCCTGTCCCCAGGAAAGTTTGGGTCTTGGTGGTCTGTGGGAGG<br>GGTGCTGGCATGTTACTCACTGCTGGTCACCGTGGCCTTCATCA<br>TCTTCTGGGTGCGGAGCAAGAGGTCCCGCTGCTGCACAGCGAC<br>TATATGAACATGACCCACGAGACCGGGTCCCACTCGCAAACA<br>CTACCAGCCCTATGCTCCACCCCGGGACTTCGCGGCCTACAGGT<br>CAGAGAGAGTGCAGCCCCTGGAAGAGAATGTCGGCAACGCTGCC<br>CGCCCAAGATTTGAAAGGAACAAACGAGTGAAGTTCAGCAGGTC<br>CGCCGACGCTCCTGCATAACCAGCAGGGACAGAACCAGCTGTATA<br>ACGAGCTGAATCTGGGCCGGAGAGAGGAATACGACGTGCTGGAC<br>AAAAGGCGGGGCCGGGACCCCGAAATGGGAGGGGAAGCCACGACG<br>GAAAAACCCCCAGGAGGGCCTGTACAATGAGCTGCAAAAGGACA<br>AAATGGCCGAGGCTTATTCTGAAATCGGGATGAAGGGAGAGAGA<br>AGGCGCGGAAAAGGCCACGATGGCCTGTACCAGGGGCTGAGCAC<br>CGCTACAAAGGACACCTATGATGCACTGCACATGCAGGCCCTGC<br>CCCCTCGGGGCTCAGGTGAGGGGCGCGGGAGCCTGCTGACTTGT<br>GGGGATGTAGAGGAAAATCCTGGTCCTATGAGAATCAGCAAACC<br>ACACCTCCGGAGCATATCAATCCAGTGTTACTTGTGCCTTCTTT<br>TGAACTCCCATTTCTCTACCGAGGCAGGCATTTCATGTGTTCTATA<br>TTGGGGTGCTTTAGTGCTGGGCTTCCGAAAACGGAAGCTAACTG<br>GGTAAACGTCATCAGTGACCTTAAAAAAATTGAGGATCTTATCC<br>AATCAATGCACATCGACGCGACTCTCTACACAGAATCTGACGTA<br>CACCCGTCATGCAAAGTCACGGCAATGAAGTGTTTTCTTCTCGA<br>GCTCCAAGTAATTTCCCTGGAGTCTGGCGATGCCTCCATCCACG<br>ATACGGTTGAAAATCTGATTATATTGGCCAACAATAGCCTCAGT<br>TCTAACGGTAACGTGACTGAAAGTGGCTGCAAAGAGTGCGAAGA<br>GCTCGAAGAAAAGAATATCAAGGAGTTCCTCCAATCATTTGTTT<br>ACATTGTGCAATGTTTTATCAACACCTCTTGA |
| 84         | Polynucleotide Sequence for 47D7 VL- | ATGGCCTTGCCCGTCACTGCCCTGTTGCTGCCGCTTGCTCTGCTT<br>CTCCATGCAGCACGCCAGATATTCAGATGACTCAATCCCCGTCT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |

| SEQ ID NO: | Description                                               | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
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|            | (G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (2079 bp) | <p> TCACTCAGCGCCTCAGTGGGGGATCGAGTCACGATCACATGCCGA<br/> GCTTCTCAAGATATAGGGTCTTGGCTGACGTGGTATCAACGGAAA<br/> CCGGAAAAGGCCCTAAATCACTCATCTATGCAGCGTCATCTTTG<br/> CAATCCGGCGTGCCAGCAGGTTTAGCGGTAGCGGAAGTGGCACA<br/> GACTTCACACTGACAATCAGTTCCTTGCAGCCAGAGGACTTTGCC<br/> ACTTATTATTGTGTCAGCAGTATAAGTCCTATCCACTTACCTTCGGA<br/> CAGGGTGCCAACTCGAGATTAAAGGCGGTGGCGGCTCTGGAGGT<br/> GGAGGCTCAGGGGTGGGGGAGTGAGGTGCAATTGGTGGAGAGT<br/> GGCGGGGTCTCGTGCAACCCGGAGGATCACTGAGATTGAGCTGC<br/> GCCGCCACTGGGTTACTTTTTCCAGCTATTGGATGCACTGGGTC<br/> CGCCAAGGACCCGGAAAGGGCCTGGTTTGGGTTTCTCGCATCAAC<br/> TCTGACGGCACTAGCACGGTGACGCTGACTCCGTCAAAGGGCGG<br/> TTTACCATTAGTCGAGACAATGCAAAAAACACCTGTATTTGCAG<br/> ATGAACTCCCTTAGGGCAGAAGACACGGCAGTATACTATTGTGCG<br/> CGCGGCAGGGGCATCATGACCTATTGGGGGCAAGGGACACTGGTA<br/> ACGGTGTCAAGTGCAAAACCTACCACAACCTCTGCACCACGCCCC<br/> CCTACTCCAGCACCTACCATCGCATCTCAGCCACTGAGTCTGCGA<br/> CCAGAGGCCTGCCGGCCCGCCCGGGCGGGCCGTCCATACCAGA<br/> GGGCTGGACTTTGCCTGCGATTTTTGGGTCTGGTGGTCTGTGGGA<br/> GGGGTGCTGGCATGTTACTCACTGCTGGTCACCGTGGCCTTCATC<br/> ATCTTCTGGGTGCGGAGCAAGAGGTCCCGCCTGCTGCACAGCGAC<br/> TATATGAACATGACCCACGGAGACCGGGTCCCACTCGCAAACAC<br/> TACCAGCCCTATGCTCCACCCGGGACTTCGCGGCCCTACAGGTCA<br/> GAGAGAGTGACAGCCCCGGAAGAGAATGTGCGCAACGCTGCCCCG<br/> CCAAGATTTGAAAGGAACAAACGAGTGAAGTTCAGCAGGTCCGCC<br/> GACGCTCCTGCATACCAGCAGGGACAGAACCAGCTGTATAACGAG<br/> CTGAATCTGGGCCGGAGAGAGGAATACGACGTGCTGGACAAAAGG<br/> CGGGGCCGGGACCCCGAAATGGGAGGGAAGCCACGACGGAAAAAC<br/> CCCCAGGAGGGCCTGTACAATGAGCTGCAAAAGGACAAAATGGCC<br/> GAGGCTTATTCTGAAATCGGGATGAAGGGAGAGAGAAGGCGCGGA<br/> AAAGGCCACGATGGCCTGTACCAGGGGCTGAGCACCGCTACAAAG<br/> GACACCTATGATGCAC'TGCACATGCAGGCCCTGCCCCCTCGGGGC<br/> TCAGGTGAGGGGCGCGGGAGCCTGCTGACTTGTGGGGATGTAGAG<br/> GAAAATCCTGGTCTTATGAGAATCAGCAAACCACACCTCCGGAGC<br/> ATATCAATCCAGTGTTACTTGTGCCTTCTTTTGAAC'TCCCATTTTC<br/> CTCACCGAGGCAGGCATTTCATGTGTTTCATATTGGGGTGCTTTAGT<br/> GCTGGGCTTCCGAAAACGGAAGCTAACTGGGTAAACGTCATCAGT<br/> GACCTTAAAAAAATTGAGGATCTTATCCAATCAATGCACATCGAC<br/> GCGACTCTCTACACAGAATCTGACGTACACCCGTCATGCAAAGTC<br/> ACGGCAATGAAGTGTTTTCTTCTCGAGCTCCAAGTAATTTCCCTG<br/> GAGTCTGGCGATGCCTCCATCCACGATACGGTTGAAAATCTGATT<br/> ATATTGGCCAACAATAGCCTCAGTTCTAACGGTAACGTGACTGAA<br/> AGTGGCTGCAAAGAGTGCGAAGAGCTCGAAGAAAAGAATATCAAG<br/> GAGTTCCTCCAATCATTTGTTTACATTGTGCAAAATGTTTATCAAC<br/> ACCTCTTGA </p> |

| SEQ ID NO: | Description                                                                                               | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
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| 85         | Polynucleotide Sequence for 45B6 VL-(G4S)3-VH-IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (2637 bp) | ATGGCCTTGCCCGTCACTGCCCTGTTGCTGCCGCTTGCTCTGCTT<br>CTCCATGCAGCACGCCCAGAAATAGTCATGACGCAGTCACCTGCC<br>ACGCTGTCTCTTAGTCCTGGGGAACGCGCGACTTTGTTCATGTCGG<br>ACTAGCCAGTCAGTCTTCAACAGTTTCCTCAGTTGGTATCAGCAG<br>AAACCGGGGCAAGCCCCCGGCTCTTGTTGTACGGCACGTCTACG<br>CGAGCCACGGGTATTCAGCGAGATTTTCAGGAAGCGGCTCTGGC<br>ACTGATTTTCACTCACTATCAGTAGTCTCCAGCCGGAAGATTTTC<br>GCCGTTTACTATTGCCAACAAGACTATAAATTGCCGTTGACCTTT<br>GGCGGAGGTACAAAGGTCGAAATTAAGGGCGGTGGCGGCTCTGGA<br>GGTGGAGGCTCAGGGGGTGGGGGGAGTCAGGTTCAACTTCTTCAA<br>TCAGGGGCGGAGGTGAAAAAACCTGGTGCGTCAGTGAAGGTCTCA<br>TGCAAAGCAAGCGGGTACACTTTTACTACTTACGGTATAAGTTGG<br>GTACGCCAGGCTCCTGGCCAGGGTCTCGAATGGTTGGGATGGATA<br>ACGGCATACAACCGAAATACCAATTACGCTCAGAAACTCCAAGGG<br>CGAGTGACTATGACCACGGATACTAGCACTAGCACCTCCTTCATG<br>GTACTCAGATCACTTCGATCAGATGATACCGCAGTGTACTACTGC<br>GCTAGAGTGGGGGATAGCGACGCTTTTGACATCTGGGGCCAAGGT<br>ACAATGGTCACGGTTTTCATCAGAGCCAAAGTCTTGCGACAAGACT<br>CATACCTGCCCGCCTTGTCCCGCACCACCTGTGGCAGGTCCAGC<br>GTGTTTCTGTTTCCGCCCCAAGCCAAGGACACACTCATGATCTCT<br>AGGACACCCGAGGTTACATGCGTTCGTGGTTGATGTTTCTCATGAA<br>GACCCCGAAGTGAAGTTTAACTGGTATGTGACGGAGTTGAGGTC<br>CATAATGCTAAAAACAAACACGCGAGGAACAATATCAAAGCACC<br>TATCGGGTCGTTAGTGTCTTACTGTACTTACCAGGACTGGCTC<br>AACGGAAGGAATATAAGTGCAAAGTCAGCAATAAAGCCTTGCCA<br>GCGCCCATCGAAAAAAGTATCAGCAAGGCCAAGGGCCAACCCAGA<br>GAACCCCAAGGTACACACTCCCTCCTAGCCGGGATGAATTGACC<br>AAAAATCAaGTctctCTCACTTGTCTGGTAAAGGGATTCTATCCA<br>AGTGATATTGCTGTGCAATGGGAGAGCAACGGGCAACCGGAGAAC<br>AATTACAAAACGACACCTCCCGTCCTTGATAGTGACGGGTCCTTC<br>TTCCTGTATTCAAAGCTTACAGTCGACAAAAGTCGCTGGCAGCAG<br>GGGAATGTCTTTAGCTGTAGTGTTCATGCACGAAGCTTTGCATAAC<br>CACTACACGCAAAAAGTCTCTTAGCCTGTCCCAGGAAAGTTTGG<br>GTCTTGGTGGTCTGTTGGGAGGGGTGCTGGCATGTTACTCACTGCTG<br>GTCACCGTGGCCTTCATCATCTTCTGGGTGCGGAGCAAGAGGTCC<br>CGCCTGCTGCACAGCGACTATATGAACATGACCCACGGAGACCG<br>GGTCCCCTCGCAAACACTACCAGCCCCTATGCTCCACCCCGGGAC<br>TTCGCGGCCTACAGGTGAGAGAGAGTGCAGCCCCCTGGAAGAGAAT<br>GTGCGCAACGCTGCCCCGCCAAGATTTGAAAGGAACAAACGAGTG<br>AAGTTCAGCAGGTCCGCCGACGCTCCTGCATACCAGCAGGGACAG<br>AACCAGCTGTATAACGAGCTGAATCTGGGCGGAGAGAGGAATAC<br>GACGTGCTGGACAAAAGGCGGGGCCGGGACCCCGAAATGGGAGGG<br>AAGCCACGACGGAAAAACCCCCAGGAGGGCCTGTACAATGAGCTG<br>CAAAAGGACAAAATGGCCGAGGCTTATTCTGAAATCGGGATGAAG<br>GGAGAGAGAAGGCGCGGAAAAGGCCACGATGGCCTGTACCAGGGG |

| SEQ ID NO: | Description                                                                                   | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
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|            |                                                                                               | CTGAGCACCGCTACAAAGGACACCTATGATGCACTGCACATGCAG<br>GCCCTGCCCCCTCGGGGCTCAGGTGAGGGGCGCGGGAGCCTGCTG<br>ACTTGTGGGGATGTAGAGGAAAATCCTGGTCTATGAGAATCAGC<br>AAACCACACCTCCGGAGCATATCAATCCAGTGTTACTTGTGCCTT<br>CTTTTGAACCTCCATTTCTCACCGAGGCAGGCATTCATGTGTTT<br>ATATTGGGGTGCTTTAGTGCTGGGCTTCCGAAAACGGAAGCTAAC<br>TGGGTAAACGTCATCAGTGACCTTAAAAAATTGAGGATCTTATC<br>CAATCAATGCACATCGACGCGACTCTCTACACAGAATCTGACGTA<br>CACCCGTCATGCAAAGTCACGGCAATGAAGTGTTTCTTCTCGAG<br>CTCCAAGTAATTTCCCTGGAGTCTGGCGATGCCTCCATCCACGAT<br>ACGGTTGAAAATCTGATTATATTGGCCAACAATAGCCTCAGTTCT<br>AACGGTAACGTGACTGAAAGTGGCTGCAAAGAGTGCGAAGAGCTC<br>GAAGAAAAGAATATCAAGGAGTTCTTCCAATCATTTGTTACATT<br>GTGCAAATGTTTATCAACACCTCTTGA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| 86         | Polynucleotide Sequence for 45B6 VL-(G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (2088 bp) | ATGGCCTTGCCCCGTCACCTGCCCTGTTGCTGCCGCTTGCTCTGCTT<br>CTCCATGCAGCACGCCAGAAAATAGTCATGACGCAGTCACCTGCC<br>ACGCTGTCTCTTAGTCCTGGGGAACGCGCGACTTTGTTCATGTGCG<br>ACTAGCCAGTCAGTCTTCAACAGTTTCCTCAGTTGGTATCAGCAG<br>AAACCGGGGCAAGCCCCCGGCTCTTGTTGTACGGCACGTCTACG<br>CGAGCCACGGGTATTTCCAGCGAGATTTTCAGGAAGCGGCTCTGGC<br>ACTGATTTTACACTCACTATCAGTAGTCTCCAGCCGGAAGATTTT<br>GCCGTTTACTATTGCCAACAAGACTATAAATTGCCGTTGACCTTT<br>GGCGGAGGTACAAAGGTCGAAATTAAGGGCGGTGGCGGCTCTGGA<br>GGTGGAGGCTCAGGGGGTGGGGGGAGTCAGGTTCAACTTCTTCAA<br>TCAGGGGCGGAGGTGAAAAACCTGGTGCGTCAGTGAAGGTCTCA<br>TGCAAAGCAAGCGGGTACACTTTTACTACTTACGGTATAAGTTGG<br>GTACGCCAGGCTCCTGGCCAGGGTCTCGAATGGTTGGGATGGATA<br>ACGGCATAACAACCGAAATACCAATTACGCTCAGAACTCCAAGGG<br>CGAGTGACTATGACCACGGATACTAGCACTAGCACCTCCTTCATG<br>GTACTCAGATCACTTCGATCAGATGATACCGCAGTGTACTIONTGC<br>GCTAGAGTGGGGGATAGCGACGCTTTTGACATCTGGGGCCAAGGT<br>ACAATGGTCACGGTTTTCATCAGCAAAACCTACCACAACCTCTGCA<br>CCACGCCCCCTACTCCAGCACCTACCATCGCATCTCAGCCACTG<br>AGTCTGCGACCAGAGGCCTGCCGGCCCGCCCGCGGGGGCCGTC<br>CATACCAGAGGGCTGGACTTTGCCTGCGATTTTGGGTCTGCTG<br>GTCGTGGGAGGGGTGCTGGCATGTTACTCACTGCTGGTCAACCGTG<br>GCCTTCATCATCTTCTGGGTGCGGAGCAAGAGGTCCCGCCTGCTG<br>CACAGCGACTATATGAACATGACCCACGGAGACGGGTCCCACT<br>CGCAAACACTACCAGCCCTATGCTCCACCCCGGGACTTCGCGGCC<br>TACAGGTGAGAGAGAGTGCAGCCCCCTGGAAGAGAATGTCGGCAAC<br>GCTGCCCCGCCAAGATTTGAAAGGAACAAACGAGTGAAGTTCAGC<br>AGGTCCGCCGACGCTCCTGCATACCAGCAGGGACAGAACCAGCTG<br>TATAACGAGCTGAATCTGGGCCGGAGAGAGGAATACGACGTGCTG<br>GACAAAAGGCGGGGCGGGACCCCGAAATGGGAGGGAAGCCACGA<br>CGGAAAACCCCCAGGAGGGCCTGTACAATGAGCTGCAAAGGAC |

| SEQ ID NO: | Description                                                                                                | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
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|            |                                                                                                            | AAAATGGCCGAGGCTTATTCTGAAATCGGGATGAAGGGAGAGAGA<br>AGGCGCGGAAAAGGCCACGATGGCCTGTACCAGGGGCTGAGCACC<br>GCTACAAAGGACACCTATGATGCACTGCACATGCAGGCCCTGCCC<br>CCTCGGGGCTCAGGTGAGGGGCGCGGAGCCTGCTGACTTGTGGG<br>GATGTAGAGGAAAATCCTGGTCCTATGAGAATCAGCAAACCACAC<br>CTCCGGAGCATATCAATCCAGTGTTACTTGTGCCCTTCTTTTGAAC<br>TCCCATTTCTCACCGAGGCAGGCATTTCATGTGTTTCATATTGGGG<br>TGCTTTAGTGCTGGGCTTCCGAAAACGGAAGCTAACTGGGTAAAC<br>GTCATCAGTGACCTTAAAAAATTGAGGATCTTATCCAATCAATG<br>CACATCGACGCGACTCTCTACACAGAATCTGACGTACACCCGTCA<br>TGCAAAGTCACGGCAATGAAGTGTTTCTTCTCGAGCTCCAAGTA<br>ATTTCCCTGGAGTCTGGCGATGCCTCCATCCACGATACGGTTGAA<br>AATCTGATTATATTGGCCAACAATAGCCTCAGTTCACCGGTAAC<br>GTGACTGAAAGTGGCTGCAAAGAGTGCGAAGAGCTCGAAGAAAAG<br>AATATCAAGGAGTTCCTCCAATCATTTGTTACATTGTGCAAATG<br>TTTATCAACACCTCTTGA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| 87         | Polynucleotide Sequence for 20B12 VL-(G4S)3-VH-IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (2628 bp) | ATGGCCTTGCCCGTCACTGCCCTGTTGCTGCCGCTTGCTCTGCTT<br>CTCCATGCAGCACGCCCAGAAATCGTACTTACCCAGTCACCTGAC<br>TTCCAAAGTGTTACCCCAAAGGAGAAAGTAACAATAACATGCCGA<br>GCAAGCCAGTCTATAGGAAGCAATCTCCACTGGTATCAACAGAAG<br>CCCGATCAGAGTCCCAAGTTGCTCATTAATAACGTGAGCCAATCT<br>TTCTCAGGTGTGCCCAGCAGATTCTCAGGCAGCGGTTTCAGGAACC<br>GATTTACAGCTTACTATTAATTCCCTGGAGGCGGAAGACGCAGCT<br>ACCTATTATTGTCAACAGAGCCGGTCCCTTCCCTACACATTTGGC<br>CAGGGCACCAAACTTGAAATTAAGGGCGGTGGCGGCTCTGGAGGT<br>GGAGGCTCAGGGGGTGGGGGAGTCAGGTTCAACTTCAAGAATCA<br>GGCCCTGGACTGGTGAAACCAAGTGAAACTTTGTCACTCACTTGT<br>ACCGTTAGCGGTGGGTCCATAAGCAGTTATTACTGGAGTTGGATC<br>AGACAACCCCCAGGTAAAGGTCTCGAGTGGATTGGTTATATATAC<br>TACTCAGGCTCTACAACTATAATCCTTCCCTCAAGTCTCGGGTA<br>ACTATATCAGTTGATACGTCTAAAAATCAATTCAGCTTGAAGCTG<br>TCTAGTGTCACTGCTGCTGATACAGCAGTGTACTACTGTGCCCGG<br>ACTGGATATATTCCCATGGACGTCTGGGGGCAAGGTACAACTGTG<br>ACCGTGTCAAGCGAGCCAAAGTCTTGCGACAAGACTCATACCTGC<br>CCGCCTTGTCCCGCACCACTGTGGCAGGTCCCAGCGTGTTTCTG<br>TTTCCGCCCCAAGCCCAAGGACACACTCATGATCTCTAGGACACCC<br>GAGGTTACATGCGTCTGTGGTTGATGTTTCTCATGAAGACCCCGAA<br>GTGAAGTTTAACTGGTATGTGACGGAGTTGAGGTCCATAATGCT<br>AAAACAAAACCACGCGAGGAACAATATCAAAGCACCTATCGGGTC<br>GTTAGTGTCTTACTGTACTTCACCAGGACTGGCTCAACGGAAAG<br>GAATATAAGTGCAAAGTCAGCAATAAAGCCTTGCCAGCGCCCATC<br>GAAAAAATATCAGCAAGGCCAAGGGCCAACCCAGAGAACCCCCAA<br>GTGTACACACTCCCTCCTAGCCGGGATGAATTGACCAAAAATCAa<br>GTCtctCTCACTTGTCTGGTAAAGGGATTCTATCCAAGTGATATT<br>GCTGTGCAATGGGAGAGCAACGGGCAACCGGAGAACAATTACAAA |

| SEQ ID NO: | Description                                                                                    | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
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|            |                                                                                                | ACGACACCTCCCGTCCTTGATAGTGACGGGTCCTTCTTCCTGTAT<br>TCAAAGCTTACAGTCGACAAAAGTCGCTGGCAGCAGGGGAATGTC<br>TTTAGCTGTAGTGTCTATGCACGAAGCTTTGCATAACCACTACACG<br>CAAAAGTCTCTTAGCCTGTCCCCAGGAAAGTTTGGGTCCTGGTG<br>GTCGTGGGAGGGGTGCTGGCATGTTACTCACTGCTGGTCACCGTG<br>GCCTTCATCATCTTCTGGGTGCGGAGCAAGAGGTCCCGCCTGCTG<br>CACAGCGACTATATGAACATGACCCACGGAGACCGGGTCCCACT<br>CGCAAACACTACCAGCCCTATGCTCCACCCCGGGACTTCGCGGCC<br>TACAGGTGAGAGAGAGTGCAGCCCCTGGAAGAGAATGTCGGCAAC<br>GCTGCCCCGCCAAGATTTGAAAGGAACAAACGAGTGAAGTTCAGC<br>AGGTCCGCCGACGCTCCTGCATACCAGCAGGGACAGAACCAGCTG<br>TATAACGAGCTGAATCTGGGCCGGAGAGAGGAATACGACGTGCTG<br>GACAAAAGGCGGGGCCGGGACCCCGAAATGGGAGGGAAGCCACGA<br>CGGAAAAACCCCCAGGAGGGCCTGTACAATGAGCTGCAAAAGGAC<br>AAAATGGCCGAGGCTTATTCTGAAATCGGGATGAAGGGAGAGAGA<br>AGGCGCGGAAAAGGCCACGATGGCCTGTACCAGGGGCTGAGCACC<br>GCTACAAAGGACACCTATGATGCACTGCACATGCAGGCCCTGCCC<br>CCTCGGGGCTCAGGTGAGGGGCGCGGAGCCTGCTGACTTGTGGG<br>GATGTAGAGGAAAATCCTGGTCTTATGAGAATCAGCAAACCACAC<br>CTCCGGAGCATATCAATCCAGTGTTACTTGTGCCTTCTTTTGAAC<br>TCCCATTTCCTCACCAGGCAGGCATTTCATGTGTTTCATATTGGGG<br>TGCTTTTAGTGCTGGGCTTCCGAAAACGGAAGCTAACTGGGTAAAC<br>GTCATCAGTGACCTTAAAAAATGAGGATCTTATCCAATCAATG<br>CACATCGACGCGACTCTCTACACAGAATCTGACGTACACCCGTCA<br>TGCAAAGTCACGGCAATGAAGTGTTTTCTTCTCGAGCTCCAAGTA<br>ATTTCCCTGGAGTCTGGCGATGCCTCCATCCACGATACGGTTGAA<br>AATCTGATTATATTGGCCAACAATAGCCTCAGTTCTAACGGTAAC<br>GTGACTGAAAGTGGCTGCAAAGAGTGCGAAGAGCTCGAAGAAAAG<br>AATATCAAGGAGTTCCTCCAATCATTTGTTACATTGTGCAAATG<br>TTTATCAACACCTCTTGA |
| 88         | Polynucleotide Sequence for 20B12 VL-(G4S)3-VH-CD8a-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (2079 bp) | ATGGCCTTGCCCCGTCAGTGCCTGTTGCTGCCGCTTGCTCTGCT<br>TCTCCATGCAGCACGCCCAGAAATCGTACTTACCCAGTCACCTG<br>ACTTCCAAAGTGTTACCCCAAAGGAGAAAGTAACAATAACATGC<br>CGAGCAAGCCAGTCTATAGGAAGCAATCTCCACTGGTATCAACA<br>GAAGCCCGATCAGAGTCCCAAGTTGCTCATTAATAACGTGAGCC<br>AATCTTTCTCAGGTGTGCCAGCAGATTCTCAGGCAGCGGTTCA<br>GGAACCGATTTCACGCTTACTATTAATTCCCTGGAGGCGGAAGA<br>CGCAGCTACCTATTATTGTCACCAGAGCCGGTCCCTTCCCTACA<br>CATTTGGCCAGGGCACCAAACCTGAAATTAAGGGCGGTGGCGGC<br>TCTGGAGGTGGAGGCTCAGGGGGTGGGGGAGTCAGGTTCAACT<br>TCAAGAATCAGGCCCTGGACTGGTGAAACCAAGTGAACTTTGT<br>CACTCACTTGTACCGTTAGCGGTGGGTCCATAAGCAGTTATTAC<br>TGGAGTTGGATCAGACAACCCCCAGGTAAAGGTCTCGAGTGGAT<br>TGGTTATATATACTACTCAGGCTCTACAACTATAATCCTTCCC<br>TCAAGTCTCGGGTAACTATATCAGTTGATACGTCTAAAAATCAA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |

| SEQ ID NO: | Description                                                                                               | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
|------------|-----------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|            |                                                                                                           | TTCAGCTTGAAGCTGTCTAGTGTCACTGCTGCTGATACAGCAGT<br>GTACTACTGTGCCCCGACTGGATATATTCCCATGGACGTCTGGG<br>GGCAAGGTACAACCTGTGACCGTGTCAAGCGCAAAACCTACCACA<br>ACTCCTGCACCACGCCCCCTACTCCAGCACCTACCATCGCATC<br>TCAGCCACTGAGTCTGCGACCAGAGGCCTGCCGGCCCCGCCGCCG<br>GCGGGGCCGTCCATACCAGAGGGCTGGACTTTGCCTGCGATTTT<br>TGGGTCTTGGTGGTTCGTGGGAGGGGTGCTGGCATGTTACTCACT<br>GCTGGTCACCGTGGCCTTCATCATCTTCTGGGTGCGGAGCAAGA<br>GGTCCCCGCCTGCTGCACAGCGACTATATGAACATGACCCACGCG<br>AGACCGGTCCCCTCGCAAACACTACCAGCCCTATGCTCCACC<br>CCGGGACTTCGCGGCCTACAGGTCAGAGAGAGTGCAGCCCCCTGG<br>AAGAGAATGTCTGGCAACGCTGCCCGCCCAAGATTTGAAAGGAAC<br>AAACGAGTGAAGTTCAGCAGGTCCGCCGACGCTCCTGCATACCA<br>GCAGGGACAGAACCAGCTGTATAACGAGCTGAATCTGGGCCGGA<br>GAGAGGAATACGACGTGCTGGACAAAAGGCGGGGCCGGGACCCC<br>GAAATGGGAGGGAAGCCACGACGGAACCCAGGAGGGCCT<br>GTACAATGAGCTGCAAAAGGACAAAATGGCCGAGGCTTATTCTG<br>AAATCGGGATGAAGGGAGAGAGAAGGCGCGGAAAAGGCCACGAT<br>GGCCTGTACCAGGGGCTGAGCACCGCTACAAAGGACACCTATGA<br>TGCACTGCACATGCAGGCCCTGCCCCCTCGGGGCTCAGGTGAGG<br>GGCGCGGAGCCTGCTGACTTGTGGGGATGTAGAGGAAAATCCT<br>GGTCCTATGAGAATCAGCAAACCACACCTCCGGAGCATATCAAT<br>CCAGTGTACTTGTGCCTTCTTTTGAACCTCCATTTCTCACC<br>AGGCAGGCATTCATGTGTTTCATATTGGGGTGCTTTAGTGCTGGG<br>CTTCCGAAAACGGAAGCTAACTGGGTAAACGTCATCAGTGACCT<br>TAAAAAATTGAGGATCTTATCCAATCAATGCACATCGACGCGA<br>CTCTCTACACAGAATCTGACGTACACCCGTCATGCAAAGTCACG<br>GCAATGAAGTGTTCCTTCTCGAGCTCCAAGTAATTTCCCTGGA<br>GTCTGGCGATGCCTCCATCCACGATACGGTTGAAAATCTGATTA<br>TATTGGCCAACAATAGCCTCAGTTCTAACGGTAACGTGACTGAA<br>AGTGGCTGCAAAGAGTGCGAAGAGCTCGAAGAAAAGAATATCAA<br>GGAGTTCCTCCAATCATTTGTTTCACATTGTGCAAATGTTTATCA<br>ACACCTCTTGA |
| 89         | Polynucleotide Sequence for 15B6 VL-(G4S)3-VH-IgG1 (mut) hinge-CD28TM-CD28-Ox40L-CD3z-T2A-IL-15 (2628 bp) | ATGGCCTTGCCCCGTCCTGCCCCTGTTGCTGCCGCTTGCTCTGCT<br>TCTCCATGCAGCACGCCCCACAAGCGGTGGTAACCCAGGAATCAG<br>CACTCACAACATCACCAGGAGAGACAGTTACCTTGACTTGCCGG<br>TCTTCCACTGGCGCGGTAACACGGGTAAATTAACCTAACTGGGT<br>TCAGGAAAAGCCCGATCACTTGTTTACGGGTCTGATAGCTGGAA<br>CGAACAACCGGGCTCCAGGAGTTCCCGCCCGATTTTCTGGGTCA<br>TTGATTGGCGACAAAGCAGCTTTGACTATTACGGGTGCTCAAAC<br>CGAAGACGAAGCTATATACTTTTGTGCCCTCTGGTTCTCAAGTC<br>ACTGGGTATTTCGGAGGAGGGACCAAATTGACTGTTTGGGCGGT<br>GGCGGCTCTGGAGGTGGAGGCTCAGGGGGTGGGGGGAGTGAGGT<br>GCAGCTCCAACAATCCGGGCCCGTTCTTGTCAAACCAGGAGCCT<br>CAGTGAAAATTTTCATGTAAAGCCTCCGGGTATTCTTCTCACTGGA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |

| SEQ<br>ID<br>NO: | Description | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
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|                  |             | TACTACATGCACTGGGTGCGGCAAAGTTTGGTGAAGAGGCTGGA<br>ATGGATAGGACGCATTAATCCATACACGGGAGTGCTAGTTACA<br>AGCATAATTTCAAGGATAAAGCATCTCTCACCCTCGACAAAAGT<br>TCTTCTACAGCCTATATGGAACCTTCACTCCCTTACAAGTGAGGA<br>TAGTGCGGTTTATTATTGTGCACGAGAACTGGGTGGGTACTGGG<br>GGCAGGGCACGACACTCACGGTTAGCTCCGAGCCAAAGTCTTGC<br>GACAAGACTCATACCTGCCCGCCTTGTCCCGCACCACCTGTGGC<br>AGGTCCCAGCGTGTCTTCTGTTTCCGCCCCAAGCCAAGGACACAC<br>TCATGATCTCTAGGACACCCGAGGTTACATGCGTCGTGGTTGAT<br>GTTTCTCATGAAGACCCCGAAGTGAAGTTTAACTGGTATGTCTGA<br>CGGAGTTGAGGTCCATAATGCTAAAACAAAACCACGCGAGGAAC<br>AATATCAAAGCACCTATCGGGTCGTTAGTGTCCTTACTGTACTT<br>CACCAGGACTGGCTCAACGGAAAGGAATATAAGTGCAAAGTCAG<br>CAATAAAGCCTTGCCAGCGCCCATCGAAAAAATATCAGCAAGG<br>CCAAGGGCCAACCCAGAGAACCCCAAGTGTACACACTCCCTCCT<br>AGCCGGGATGAATTGACCAAAAATCAaGTctctCTCACTTGTCT<br>GGTAAAGGGATTCTATCCAAGTGATATTGCTGTCTGAATGGGAGA<br>GCAACGGGCAACCGGAGAACAATTACAAAACGACACCTCCCGTC<br>CTTGATAGTGACGGGTCTTCTTCTGTATTCAAAGCTTACAGT<br>CGACAAAAGTCGCTGGCAGCAGGGGAATGTCTTTAGCTGTAGTG<br>TCATGCACGAAGCTTTGCATAACCACTACACGCAAAAGTCTCTT<br>AGCCTGTCCCCAGGAAAGTTTGGGTCTTGGTGGTCTGTGGGAGG<br>GGTGCTGGCATGTTACTCACTGCTGGTCACCGTGGCCTTCATCA<br>TCTTCTGGGTGCGGAGCAAGAGGTCCCGCTGCTGCACAGCGAC<br>TATATGAACATGACCCACGAGACCGGGTCCCACTCGCAAACA<br>CTACCAGCCCTATGCTCCACCCCGGACTTCGCGGCCTACAGGT<br>CAGAGAGAGTGCAGCCCCTGGAAGAGAATGTCGGCAACGCTGCC<br>CGCCCAAGATTTGAAAGGAACAAACGAGTGAAGTTCAGCAGGTC<br>CGCCGACGCTCCTGCATAACGACAGGGACAGAACCAGCTGTATA<br>ACGAGCTGAATCTGGGCCGGAGAGAGGAATACGACGTGCTGGAC<br>AAAAGGCGGGGCCGGGACCCCGAAATGGGAGGGAAGCCACGACG<br>GAAAAACCCCCAGGAGGGCCTGTACAATGAGCTGCAAAAGGACA<br>AAATGGCCGAGGCTTATTCTGAAATCGGGATGAAGGGAGAGAGA<br>AGGCGCGGAAAAGGCCACGATGGCCTGTACCAGGGGCTGAGCAC<br>CGCTACAAAGGACACCTATGATGCACTGCACATGCAGGCCCTGC<br>CCCCTCGGGGCTCAGGTGAGGGGCGCGGGAGCCTGCTGACTTGT<br>GGGGATGTAGAGGAAAATCCTGGTCCTATGAGAATCAGCAAACC<br>ACACCTCCGGAGCATATCAATCCAGTGTTACTTGTGCCTTCTTT<br>TGAACCTCCATTTCTCACCAGGCAGGCATTTCATGTGTTTCATA<br>TTGGGGTGCTTTAGTGCTGGGCTTCCGAAAACGGAAGCTAACTG<br>GGTAAACGTCATCAGTGACCTTAAAAAAATTGAGGATCTTATCC<br>AATCAATGCACATCGACGCGACTCTCTACACAGAATCTGACGTA<br>CACCCTCATGCAAAGTCACGGCAATGAAGTGTTTTCTTCTCGA<br>GCTCCAAGTAATTTCCCTGGAGTCTGGCGATGCCTCCATCCACG<br>ATACGGTTGAAAATCTGATTATATTGGCCAACAATAGCCTCAGT |

| SEQ ID NO: | Description                                                                                                      | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|------------|------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|            |                                                                                                                  | TCTAACGGTAACGTGACTGAAAGTGGCTGCAAAGAGTGCGAAGA<br>GCTCGAAGAAAAGAATATCAAGGAGTTCCTCCAATCATTTGTTT<br>ACATTGTGCAAATGTTTATCAACACCTCTTGA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| 90         | Polynucleotide<br>Sequence for 15B6 VL-<br>(G4S)3-VH-CD8a-<br>CD28TM-CD28-<br>Ox40L-CD3z-T2A-IL-<br>15 (2079 bp) | ATGGCCTTGCCCGTCACTGCCCTGTTGCTGCCGCTTGCTCTGCTT<br>CTCCATGCAGCACGCCCACAAGCGGTGGTAACCCAGGAATCAGCA<br>CTCACAACATCACCAGGAGAGACAGTTACCTTGACTTGCCGGTCT<br>TCCACTGGCGCGGTAACCACGGGTAAATTACCCTAACTGGGTTCAG<br>GAAAAGCCCGATCACTTGTTTACGGGTCTGATAGCTGGAACGAAC<br>AACCGGGCTCCAGGAGTTCCCGCCCGATTTTCTGGGTCATTGATT<br>GGCGACAAAGCAGCTTTGACTATTACGGGTGCTCAAACCGAAGAC<br>GAAGCTATATACTTTTGTGCCCTCTGGTTCTCAAGTCACTGGGTA<br>TTCGGAGGAGGGACCAAATTGACTGTTTTGGGCGGTGGCGGCTCT<br>GGAGGTGGAGGCTCAGGGGGTGGGGGGAGTGAGGTGCAGCTCCAA<br>CAATCCGGGCCCCGTCTTGTCAAACCAGGAGCCTCAGTGAAAATT<br>TCATGTAAAGCCTCCGGGTATTCTTCACTGGATACTACATGCAC<br>TGGGTGCGGCAAAGTTTGGTGAAGAGGCTGGAATGGATAGGACGC<br>ATTAATCCATACACGGGAGTGCCTAGTTACAAGCATAATTTCAAG<br>GATAAAGCATCTCTCACCCTCGACAAAAGTTCTTCTACAGCCTAT<br>ATGGAACCTCACTCCCTTACAAGTGAGGATAGTGCGGTTTATTAT<br>TGTGCACGAGAACTGGGTGGGTACTGGGGGCAGGGCAGCAGACTC<br>ACGGTTAGCTCCGCAAACCTACCACAACCTCTGCACCACGCCCC<br>CCTACTCCAGCACCTACCATCGCATCTCAGCCACTGAGTCTGCGA<br>CCAGAGGCCTGCCGGCCCCGCCCGGGCGGGGCCGTCCATACCAGA<br>GGGCTGGACTTTGCCTGCGATTTTGGGTCTGGTGGTTCGTGGGA<br>GGGTGCTGGCATGTTACTCACTGCTGGTCACCGTGGCCTTCATC<br>ATCTTCTGGGTGCGGAGCAAGAGGTCCCGCCTGCTGCACAGCGAC<br>TATATGAACATGACCCACCGAGACCGGGTCCCACTCGCAAACAC<br>TACCAGCCCTATGCTCCACCCGGGACTTCGCGGCCCTACAGGTCA<br>GAGAGAGTGACGCCCCGGAAGAGAATGTGCGCAACGCTGCCCCG<br>CCAAGATTTGAAAGGAACAAACGAGTGAAGTTCAGCAGGTCCGCC<br>GACGCTCCTGCATACCAGCAGGGACAGAACCAGCTGTATAACGAG<br>CTGAATCTGGGCCGGAGAGAGGAATACGACGTGCTGGACAAAAGG<br>CGGGGCCGGGACCCCGAAATGGGAGGGAAGCCACGACGGAAAAAC<br>CCCCAGGAGGGCCTGTACAATGAGCTGCAAAAGGACAAAATGGCC<br>GAGGCTTATTCTGAAATCGGGATGAAGGGAGAGAGAAGGCGCGGA<br>AAAGGCCACGATGGCCTGTACCAGGGGCTGAGCACCGCTACAAAG<br>GACACCTATGATGCAC'TGCACATGCAGGCCCTGCCCCCTCGGGGC<br>TCAGGTGAGGGGCGCGGGAGCCTGCTGACTTGTGGGGATGTAGAG<br>GAAAATCCTGGTCCATGAGAATCAGCAAACCACACCTCCGGAGC<br>ATATCAATCCAGTGTTACTTGTGCCTTCTTTTGAAC'TCCCATTTT<br>CTCACCGAGGCAGGCATTATGTGTTTATATTGGGGTGCTTTAGT<br>GCTGGGCTTCCGAAAACGGAAGCTAACTGGGTAAACGTCATCAGT<br>GACCTTAAAAAAATTGAGGATCTTATCCAATCAATGCACATCGAC<br>GCGACTCTCTACACAGAATCTGACGTACACCCGTCATGCAAAGTC<br>ACGGCAATGAAGTGTTTTCTTCTCGAGCTCCAAGTAATTTCCCTG |

| SEQ ID NO: | Description                                                                                 | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
|------------|---------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|            |                                                                                             | GAGTCTGGCGATGCCTCCATCCACGATACGGTTGAAAATCTGATT<br>ATATTGGCCAACAATAGCCTCAGTTCTAACGGTAACGTGACTGAA<br>AGTGGCTGCAAAGAGTGCGAAGAGCTCGAAGAAAAGAATATCAAG<br>GAGTTCCTCCAATCATTTGTTACATTGTGCAAATGTTTATCAAC<br>ACCTCTTGA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| 91         | Polynucleotide<br>Sequence for IgG1 (mut)<br>hinge-CD28TM-CD28-<br>Ox40L-CD3z-T2A-IL-<br>15 | GAGCCAAAGTCTTGCGACAAGACTCATACCTGCCCGCCTTGTCC<br>CGCACCACCTGTGGCAGGTCCCAGCGTGTTCCTGTTTCCGCCCA<br>AGCCCAAGGACACACTCATGATCTCTAGGACACCCGAGGTTACA<br>TGCGTCGTGGTTGATGTTTCTCATGAAGACCCCGAAGTGAAGTT<br>TAAGTGGTATGTCGACGGAGTTGAGGTCCATAATGCTAAAACAA<br>AACCACGCGAGGAACAATATCAAAGCACCTATCGGGTCGTTAGT<br>GTCCTTACTGTACTTCACCAGGACTGGCTCAACGGAAAGGAATA<br>TAAGTGCAAAGTCAGCAATAAAGCCTTGCCAGCGCCCATCGAAA<br>AAACTATCAGCAAGGCCAAGGGCCAACCCAGAGAACCCCAAGTG<br>TACACACTCCCTCCTAGCCGGGATGAATTGACCAAAAATCAaGT<br>Ct ctCTCACTTGTCTGGTAAAGGGATTCTATCCAAGTGATATTG<br>CTGTGCAATGGGAGAGCAACGGGCAACCCGAGAACAATTACAAA<br>ACGACACCTCCCGTCCTTGATAGTGACGGGTCTTCTTCTTGTA<br>TTCAAAGCTTACAGTCGACAAAAGTCGCTGGCAGCAGGGGAATG<br>TCTTTAGCTGTAGTGTATGCACGAAGCTTTGCATAACCACTAC<br>ACGCAAAAGTCTCTTAGCCTGTCCCCAGGAAAGTTTGGGTCTT<br>GGTGGTCGTGGGAGGGGTGCTGGCATGTTACTCACTGCTGGTCA<br>CCGTGGCCTTCATCATCTTCTGGGTGCGGAGCAAGAGGTCCCGC<br>CTGCTGCACAGCGACTATATGAACATGACCCACGAGACCCGG<br>CCCTACACGGAAACATTACCAGCCCTATGCTCCACCCGGGACT<br>TCGCAGCTTACAGAAGTGAAAGAGTGACGCCCCGTGAAGAGAAT<br>GTCGGAATGCCGCTCGCCCAAGATTTGAAAGGAACAAACGAGT<br>GAAGTTCAGCAGGTCCGCCGACGCTCCTGCATACCAGCAGGGAC<br>AGAACCAGCTGTATAACGAGCTGAATCTGGGCCGAGAGAGGAA<br>TACGACGTGCTGGACAAAAGCGGGGCCGGACCCCGAAATGGG<br>AGGGAAGCCACGACGGAAAAACCCCAAGGAGGGCCTGTACAATG<br>AGCTGCAAAAGGACAAAATGGCCGAGGCTTATTCTGAAATCGGG<br>ATGAAGGGAGAGAGAAGGCGGGAAAGGCCACGATGGCCTGTA<br>CCAGGGGCTGAGCACCGCTACAAAGGACACCTATGATGCACTGC<br>ACATGCAGGCCCTGCCCCCTCGGGGCTCAGGTGAGGGGCGCGGG<br>AGCCTGCTGACTTGTGGGGATGTAGAGGAAAATCCTGGTCCTAT<br>GAGAATCAGCAAACCACACCTCCGAGCATATCAATCCAGTGTT<br>ACTTGTGCCTTCTTTTGAACCTCCATTTCTCACCAGGCAGGC<br>ATTCATGTGTTTCATATTGGGGTGCTTTAGTGCTGGGCTTCCGAA<br>AACGGAAGCTAACTGGGTAAACGTCATCAGTGACCTTAAAAAAA<br>TTGAGGATCTTATCCAATCAATGCACATCGACGCGACTCTCTAC<br>ACAGAATCTGACGTACACCCGTCATGCAAAGTCACGGCAATGAA<br>GTGTTTTCTTCTCGAGCTCCAAGTAATTTCCCTGGAGTCTGGCG<br>ATGCCTCCATCCACGATACGGTTGAAAATCTGATTATATTGGCC<br>AACAATAGCCTCAGTTCTAACGGTAACGTGACTGAAAGTGGCTG |

| SEQ ID NO: | Description                                                                      | Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
|------------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|            |                                                                                  | CAAAGAGTGCGAAGAGCTCGAAGAAAAGAATATCAAGGAGTTCC<br>TCCAATCATTTGTTTCACATTGTGCAAATGTTTATCAACACCTCT<br>TGA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| 92         | Polynucleotide<br>Sequence for CD8a-<br>CD28TM-CD28-<br>Ox40L-CD3z-T2A-IL-<br>15 | GCAAAACCTACCACAACCTCCTGCACCACGCCCCCTACTCCAGC<br>ACCTACCATCGCATCTCAGCCACTGAGTCTGCGACCAGAGGCCT<br>GCCGCCCCGCCGCCGCGGGGCCGTCCATACCAGAGGGCTGGAC<br>TTTGCTGCGATTTTTTGGGTCTTGGTGGTTCGTGGGAGGGGTGCT<br>GGCATGTTACTCACTGCTGGTCACCGTGGCCTTCATCATCTTCT<br>GGGTGCGGAGCAAGAGGTCCCGCCTGCTGCACAGCGACTATATG<br>AACATGACCCACGGAGACCCGGCCCTACACGGAAACATTACCA<br>GCCCTATGCTCCACCCCGGGACTTCGCAGCTTACAGAAGTGAAA<br>GAGTGCAGCCCCCTGGAAGAGAATGTGCGGAATGCCGCTCGCCCA<br>AGATTTGAAAGGAACAAACGAGTGAAGTTCAGCAGGTCCGCCGA<br>CGCTCCTGCATACCAGCAGGGACAGAACCAGCTGTATAACGAGC<br>TGAATCTGGGCCGGAGAGAGGAATACGACGTGCTGGACAAAAGG<br>CGGGGCCGGGACCCCGAAATGGGAGGGGAAGCCACGACGGAAAAA<br>CCCCCAGGAGGGCCTGTACAATGAGCTGCAAAAGGACAAAATGG<br>CCGAGGCTTATTTCTGAAATCGGGATGAAGGGAGAGAGAAGGCGC<br>GGAAAAGGCCACGATGGCCTGTACCAGGGGCTGAGCACCGCTAC<br>AAAGGACACCTATGATGCACTGCACATGCAGGCCCTGCCCCCTC<br>GGGGCTCAGGTGAGGGGCGCGGGAGCCTGCTGACTTGTGGGGAT<br>GTAGAGGAAAATCCTGGTCCTATGAGAATCAGCAAACCACACCT<br>CCGGAGCATATCAATCCAGTGTTACTTGTGCCTTCTTTTGAACCT<br>CCCATTTCTCTACCGAGGCAGGCATTTCATGTGTTTCATATTGGGG<br>TGCTTTAGTGCTGGGCTTCCGAAAACGGAAGCTAACTGGGTAAA<br>CGTCATCAGTGACCTTAAAAAAATTGAGGATCTTATCCAATCAA<br>TGCACATCGACGCGACTCTCTACACAGAATCTGACGTACACCCG<br>TCATGCAAAGTCACGGCAATGAAGTGTTTTCTTCTCGAGCTCCA<br>AGTAATTTCCCTGGAGTCTGGCGATGCCTCCATCCACGATACGG<br>TTGAAAATCTGATTATATTGGCCAACAATAGCCTCAGTTCTAAC<br>GGTAACGTGACTGAAAGTGGCTGCAAAGAGTGCGAAGAGCTCGA<br>AGAAAAGAATATCAAGGAGTTCCTCCAATCATTTGTTTCACATTG<br>TGCAAATGTTTATCAACACCTCTTGA |
| 93         | Polynucleotide<br>Sequence for IL-15                                             | ATGAGAATCAGCAAACCACACCTCCGGAGCATATCAATCCAGTG<br>TTACTTGTGCCTTCTTTTGAACCTCCCATTTCTCTACCGAGGCAG<br>GCATTTCATGTGTTTCATATTGGGGTGCTTTAGTGCTGGGCTTCCG<br>AAAACGGAAGCTAACTGGGTAAACGTCATCAGTGACCTTAAAAA<br>AATTGAGGATCTTATCCAATCAATGCACATCGACGCGACTCTCT<br>ACACAGAATCTGACGTACACCCGTCATGCAAAGTCACGGCAATG<br>AAGTGTTTTCTTCTCGAGCTCCAAGTAATTTCCCTGGAGTCTGG<br>CGATGCCTCCATCCACGATACGGTTGAAAATCTGATTATATTGG<br>CCAACAATAGCCTCAGTTCTAACGGTAACGTGACTGAAAGTGGC<br>TGCAAAGAGTGCGAAGAGCTCGAAGAAAAGAATATCAAGGAGTT<br>CCTCCAATCATTTGTTTCACATTGTGCAAATGTTTATCAACACCT<br>CTTGA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |

| SEQ ID NO: | Description                                           | Sequence                                                                                                                             |
|------------|-------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|
| 94         | Polynucleotide Sequence for T2A                       | GGCTCAGGTGAGGGGCGCGGAGCCTGCTGACTTGTGGGGATGT<br>AGAGGAAAATCCTGGTCCT                                                                   |
| 95         | (G4S)n linker                                         | (G4S)n, wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10                                                                                |
| 96         | (GxS)n linker                                         | (GxS)n linker, wherein x and n, independently can be an integer between 3 and 12, including 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more |
| 97         | Amino acid residues 296-315 of recombinant mesothelin | EVEKTACPSGKKAREIDESL                                                                                                                 |
| 98         | Amino acid residues 326-337 of recombinant mesothelin | CVDAALLATQMD                                                                                                                         |
| 99         | Amino acid residues of recombinant mesothelin         | RVNAIPFTYEQL                                                                                                                         |
| 100        | Amino acid residues 314-325 of recombinant mesothelin | SLIFYKKWELEA                                                                                                                         |
| 101        | Amino acid residues 337-349 of recombinant mesothelin | DRVNAIPFTYEQL                                                                                                                        |
| 102        | Amino acid residues 369-376 of recombinant mesothelin | IQHLGYLF                                                                                                                             |
| 103        | Amino acid residues 385-392 of recombinant mesothelin | RKWNVTSL                                                                                                                             |
| 104        | Amino acid residues 423-433 of recombinant mesothelin | IDRFVKGRGQL                                                                                                                          |
| 105        | Amino acid residues 546-558 of recombinant mesothelin | EVQKLLGPHVEGL                                                                                                                        |
| 106        | Amino acid residues 573-588 of recombinant mesothelin | RQRQDDLDTLGLGLQG                                                                                                                     |

**WHAT IS CLAIMED**

1. A chimeric antigen receptor (CAR) comprising a polypeptide comprising:
  - (a) an extracellular antigen binding domain comprising a human mesothelin (MSLN) binder;
  - (b) a transmembrane domain; and
  - (c) an intracellular signaling domain;wherein the human MSLN binder comprises:
  - (i) a heavy chain variable domain (VH) comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 3, 27, 28, or 29 and a light chain variable domain (VL) comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 4;
  - (ii) a VH comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 11 and a VL comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 12;
  - (iii) a VH comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 19 and a VL comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 20; or
  - (iv) a VH comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 30 and a VL comprising a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 31.
2. The CAR of claim 1, wherein the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 5, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 6, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 7; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 8, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 9, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 10.
3. The CAR of claim 1, wherein the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 13, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 14,

and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 15; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 16, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 17, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 18.

4. The CAR of claim 1, wherein the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 21, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 22, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 23; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 24, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 25, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 26.

5. The CAR of claim 1, wherein the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 32, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 33, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 34; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 35, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 36, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 37.

6. The CAR of claim 1, wherein the VH comprises the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 27, SEQ ID NO: 28, or SEQ ID NO: 29 and the VL comprises the amino acid sequence of SEQ ID NO: 4.

7. The CAR of claim 1, wherein the VH comprises the amino acid sequence of SEQ ID NO: 11 and the VL comprises the amino acid sequence of SEQ ID NO: 12.

8. The CAR of claim 1, wherein the VH comprises the amino acid sequence of SEQ ID NO: 19 and the VL comprises the amino acid sequence of SEQ ID NO: 20.

9. The CAR of claim 1, wherein the VH comprises the amino acid sequence of SEQ ID NO: 30 and the VL comprises the amino acid sequence of SEQ ID NO: 31.

10. The CAR of claim 1, wherein the VH comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 3, SEQ ID NO: 27, SEQ ID NO: 28, or

SEQ ID NO: 29; and the VL comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 4, and wherein:

(i) the VH comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 3, 27, 28, or 29 and the VL comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 4; or

(ii) the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 5, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 6, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 7; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 8, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 9, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 10.

11. The CAR of claim 1, wherein the VH comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 11; and the VL comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 12, and wherein:

(i) the VH comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 11 and the VL comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 12; or

(ii) the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 13, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 14, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 15; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 16, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 17, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 18.

12. The CAR of claim 1, wherein the VH comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 19; and the VL comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 20, and wherein:

(i) the VH comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 19 and the VL comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 20; or

(ii) the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 21, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 22, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 23; and the VL comprises a CDR 1 comprising the amino

acid sequence of SEQ ID NO: 24, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 25, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 26.

13. The CAR of claim 1, wherein the VH comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 30; and the VL comprises an amino acid sequence with at least 90% identity to the amino acid sequence of SEQ ID NO: 31, and wherein:

(i) the VH comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VH comprising the amino acid sequence of SEQ ID NO: 30 and the VL comprises a CDR 1, a CDR 2 and a CDR 3 as set forth in the VL comprising the amino acid sequence of SEQ ID NO: 31; or

(ii) the VH comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 32, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 33, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 34; and the VL comprises a CDR 1 comprising the amino acid sequence of SEQ ID NO: 35, a CDR 2 comprising the amino acid sequence of SEQ ID NO: 36, and a CDR 3 comprising the amino acid sequence of SEQ ID NO: 37.

14. The CAR of any one of claims 1 to 13, wherein the human MSLN binder comprises a single chain Fv (scFv) comprising the VH and the VL.

15. The CAR of claim 14, wherein the VL is N-terminal to the VH.

16. The CAR of claim 14, wherein the VL is C-terminal to the VH.

17. The CAR of any one of claims 14 to 16, wherein the VL is joined to the VH via a flexible linker.

18. The CAR of claim 17, wherein the flexible linker comprises the amino acid sequence of SEQ ID NO: 52.

19. The CAR of any one of claims 1 to 18, wherein the scFv comprises:

(i) the amino acid sequence of SEQ ID NO: 38; or an amino acid sequence having at least 85% identity to SEQ ID NO: 38;

(ii) the amino acid sequence of SEQ ID NO: 39; an amino acid sequence having at least 85% identity to SEQ ID NO: 39; or the amino acid sequence of SEQ ID NO: 39 wherein the 1st amino acid residue of SEQ ID NO: 39 is pyroglutamate;

(iii) the amino acid sequence of SEQ ID NO: 40; an amino acid sequence having at least 85% identity to SEQ ID NO: 40; or the amino acid sequence of SEQ ID NO: 40 wherein the 1st amino acid residue of SEQ ID NO: 40 is pyroglutamate;

(iv) the amino acid sequence of SEQ ID NO: 41; an amino acid sequence having at least 85% identity to SEQ ID NO: 41; or the amino acid sequence of SEQ ID NO: 41 wherein the 1st amino acid residue of SEQ ID NO: 41 is pyroglutamate;

(v) the amino acid sequence of SEQ ID NO: 42; or an amino acid sequence having at least 85% identity to SEQ ID NO: 42;

(vi) the amino acid sequence of SEQ ID NO: 43; or an amino acid sequence having at least 85% identity to SEQ ID NO: 43; or

(vii) the amino acid sequence of SEQ ID NO: 44; or an amino acid sequence having at least 85% identity to SEQ ID NO: 44.

20. The CAR of any one of claims 1 to 19, wherein the transmembrane domain is derived from a molecule selected from a group consisting of CD8 $\alpha$ , CD4, CD28, CD137, CD80, CD86, CD152, and PD1.

21. The CAR of claim 20, wherein the transmembrane domain is derived from a CD28 transmembrane domain.

22. The CAR of claim 21, wherein the CD28 transmembrane domain comprises the amino acid sequence of SEQ ID NO: 54 or an amino acid sequence having at least 85% identity to SEQ ID NO: 54.

23. The CAR of any one of claims 1 to 22, wherein the intracellular signaling domain comprises a primary intracellular signaling domain of an immune effector cell.

24. The CAR of claim 23, wherein the primary intracellular signaling domain is derived from CD3-zeta (CD3 $\zeta$ ).

25. The CAR of claim 24, wherein the primary intracellular signaling domain derived from CD3-zeta comprises the amino acid sequence of SEQ ID NO: 57 or an amino acid sequence having at least 85% identity to SEQ ID NO: 57.
26. The CAR of any one of claims 1 to 25, wherein the intracellular signaling domain further comprises a co-stimulatory signaling domain.
27. The CAR of claim 26, wherein the co-stimulatory signaling domain is derived from a molecule selected from the group consisting of CD27, CD28, CD137, OX40, OX40L, CD30, CD40, CD3, LFA-1, CD2, CD7, LIGHT, NKG2C, B7-H3, ligands of CD83 and combinations thereof.
28. The CAR of claim 27, wherein the co-stimulatory signaling domain comprises an OX40L intracellular signaling domain.
29. The CAR of claim 28, wherein the OX40L intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 56 or an amino acid sequence having at least 85% identity to SEQ ID NO: 56.
30. The CAR of claim 28 or 29, wherein the co-stimulatory signaling domain further comprises a CD28 intracellular signaling domain.
31. The CAR of claim 30, wherein the CD28 intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 55 or an amino acid sequence having at least 85% identity to SEQ ID NO: 55.
32. The CAR of any one of claims 1 to 31, further comprising a hinge domain located between the C-terminus of the extracellular antigen binding domain and the N-terminus of the transmembrane domain.
33. The CAR of claim 32, wherein the hinge domain is derived from CD8 $\alpha$ .

34. The CAR of claim 33, wherein the hinge domain derived from CD8 $\alpha$  comprises the amino acid sequence of SEQ ID NO: 60 or an amino acid sequence having at least 85% identity to SEQ ID NO: 60.
35. The CAR of claim 32, wherein the hinge domain comprises at least a portion of an IgG1 hinge domain.
36. The CAR of claim 35, wherein the IgG1 hinge domain comprises the amino acid sequence of SEQ ID NO: 53 or an amino acid sequence having at least 85% identity to SEQ ID NO: 53.
37. The CAR of any one of claims 1 to 36, further comprising a signal peptide located at the N-terminus of the polypeptide.
38. The CAR of claim 37, wherein the signal peptide is derived from CD8 $\alpha$ .
39. The CAR of claim 38, wherein the signal peptide derived from CD8 $\alpha$  comprises the amino acid sequence of SEQ ID NO: 51 or an amino acid sequence having at least 85% identity to SEQ ID NO: 51.
40. The CAR of any one of claims 1 to 39, comprising
- (i) the amino acid sequence of SEQ ID NO: 75 or an amino acid sequence having at least 85% identity to SEQ ID NO: 75;
  - (ii) the amino acid sequence of SEQ ID NO: 76 or an amino acid sequence having at least 85% identity to SEQ ID NO: 76;
  - (iii) the amino acid sequence of SEQ ID NO: 77; an amino acid sequence having at least 85% identity to SEQ ID NO: 77; or the amino acid sequence of SEQ ID NO: 77 wherein the 1st amino acid residue of SEQ ID NO: 77 is pyroglutamate;
  - (iv) the amino acid sequence of SEQ ID NO: 78; an amino acid sequence having at least 85% identity to SEQ ID NO: 78; or the amino acid sequence of SEQ ID NO: 78 wherein the 1st amino acid residue of SEQ ID NO: 78 is pyroglutamate;
  - (v) the amino acid sequence of SEQ ID NO: 79; an amino acid sequence having at least 85% identity to SEQ ID NO: 79; or the amino acid sequence of SEQ ID NO: 79 wherein the 1st amino acid residue of SEQ ID NO: 79 is pyroglutamate;

(vi) the amino acid sequence of SEQ ID NO: 80; an amino acid sequence having at least 85% identity to SEQ ID NO: 80; or the amino acid sequence of SEQ ID NO: 80 wherein the 1st amino acid residue of SEQ ID NO: 80 is pyroglutamate;

(vii) the amino acid sequence of SEQ ID NO: 81; an amino acid sequence having at least 85% identity to SEQ ID NO: 81; or the amino acid sequence of SEQ ID NO: 81 wherein the 1st amino acid residue of SEQ ID NO: 81 is pyroglutamate; or

(viii) the amino acid sequence of SEQ ID NO: 82; an amino acid sequence having at least 85% identity to SEQ ID NO: 82; or the amino acid sequence of SEQ ID NO: 82 wherein the 1st amino acid residue of SEQ ID NO: 82 is pyroglutamate.

41. A polyprotein comprising the CAR of any one of claims 1 to 40 and an IL-15, wherein the CAR and the IL-15 are linked by a self-cleaving peptide, wherein optionally:

(i) the IL-15 comprises the amino acid sequence of SEQ ID NO: 59; and/or

(ii) the self-cleaving peptide comprises the amino acid sequence of SEQ ID NO: 58, wherein optionally the polyprotein comprises:

(a) the amino acid sequence of SEQ ID NO: 61 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 61,

(b) the amino acid sequence of SEQ ID NO: 62 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 62,

(c) the amino acid sequence of SEQ ID NO: 63 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 63,

(d) the amino acid sequence of SEQ ID NO: 64 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 64,

(e) the amino acid sequence of SEQ ID NO: 65 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 65,

(f) the amino acid sequence of SEQ ID NO: 66 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 66,

(g) the amino acid sequence of SEQ ID NO: 67 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 67, or

(h) the amino acid sequence of SEQ ID NO: 68 or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 68.

42. A polynucleotide comprising a nucleic acid encoding the CAR of any one of claims 1 to 40.

43. The polynucleotide of claim 42, further comprising a nucleic acid encoding an IL-15.

44. The polynucleotide of claim 43, wherein the IL-15 comprises the amino acid sequence of SEQ ID NO: 59.

45. The polynucleotide of claim 43 or 44, wherein the nucleic acid encoding the IL-15 comprises the nucleic acid sequence of SEQ ID NO: 93.

46. The polynucleotide of any one of claims 42 to 45, wherein the polynucleotide encodes a polyprotein comprising the CAR and the IL-15.

47. The polynucleotide of any one of claims 43 to 46, further comprising a nucleic acid encoding a self-cleaving peptide, optionally a T2A self-cleaving peptide.

48. The polynucleotide of claim 47, wherein the CAR is joined to the IL-15 by the self-cleaving peptide.

49. The polynucleotide of claim 48, wherein the self-cleaving peptide is capable of inducing ribosomal skipping between the CAR and the IL-15.

50. The polynucleotide of claim 48 or 49, wherein the self-cleaving peptide comprises the sequence of SEQ ID NO: 58.

51. The polynucleotide of any one of claims 47 to 50, where the nucleic acid encoding the self-cleaving peptide comprises the nucleic acid sequence of SEQ ID NO: 94.
52. The polynucleotide of any one of claims 42 to 51, wherein the polynucleotide encodes a polyprotein comprising the amino acid sequence of SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, or SEQ ID NO: 68.
53. The polynucleotide of any one of claims 42 to 52, wherein the polynucleotide comprises the nucleic acid sequence of SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, or SEQ ID NO: 90.
54. A vector comprising the polynucleotide of any one of claims 42 to 53.
55. The vector of claim 54, wherein the vector is a viral vector, and wherein optionally the viral vector is a retroviral vector or a lentiviral vector.
56. An engineered cell comprising the polynucleotide of any one of claims 42 to 53 or the vector of any one of claims 54 to 55.
57. An engineered cell expressing the chimeric antigen receptor encoded by the polynucleotide of any one of claims 42 to 53 or the vector of any one of claims 54 to 55.
58. An engineered cell expressing the chimeric antigen receptor and the IL-15 encoded by the polynucleotide of any one of claims 42 to 53 or the vector of any one of claims 54 to 55.
59. The engineered cell of any one of claims 56 to 58, wherein the engineered cell is a lymphocyte.
60. The engineered cell of claim 59, wherein the lymphocyte is a natural killer (NK) cell.
61. The engineered cell of claim 60, wherein the lymphocyte is a T cell.
62. The engineered cell of any one of claims 56 to 61, wherein the engineered cell is derived from a human cell.

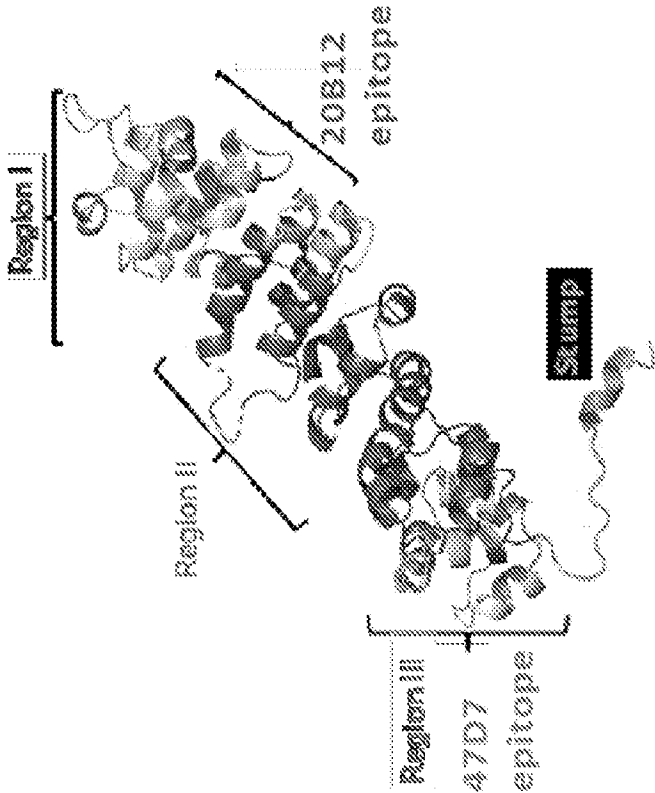
63. The engineered cell of any one of claims 56 to 62, wherein the engineered cell is from a primary cell obtained from a subject.
64. The engineered cell of any one of claims 56 to 63, wherein the engineered cell is from a primary cell obtained from cord blood.
65. The engineered cell of any one of claims 56 to 64, wherein the engineered cell comprises a KIR-B haplotype.
66. The engineered cell of any one of claims 56 to 65, wherein the engineered cell expresses CD16 having the V/V polymorphism at F158.
67. A population of cells comprising a plurality of the engineered cells of any one of claims 56 to 66.
68. The population of cells of claim 67, wherein at least 10% of the cells comprise the CAR of any one of claims 1 to 40, the polynucleotide of any one of claims 42 to 53, or the vector of any one of claims 54 to 55.
69. The population of cells of claim 67 or 68, wherein at least 10% of the cells express the polyprotein of claim 41, the polynucleotide of any one of claims 42 to 53, or the vector of any one of claims 54 to 55.
70. A pharmaceutical composition comprising the population of cells of any one of claims 67 to 69.
71. The pharmaceutical composition of claim 70, further comprising a pharmaceutically acceptable excipient.
72. A method of treatment comprising administering the engineered cell of any one of claims 56 to 66, the population of cells of any one of claims 67 to 69, or the pharmaceutical composition of claim 70 or 71 to a subject having a disease or condition associated with MSLN.

73. Use of the engineered cell of any one of claims 56 to 66, the population of cells of any one of claims 67 to 69, or the pharmaceutical composition of claim 70 or 71 in the manufacture of a medicament for treating a disease or condition associated with MSLN.

74. Use of the engineered cell of any one of claims 56 to 66, the population of cells of any one of claims 67 to 69, or the pharmaceutical composition of claim 70 or 71 in the manufacture of a medicament for treating a disease or condition associated with MSLN.

75. The method or use of any one of claims 72 to 74, wherein the disease or condition associated with MSLN is cancer.

Human MSLN protein  
(HDX data overlay on AlphaFold model)



45B6: Region 1, low affinity  
47D7: Region 3 (membrane proximal), high affinity  
20B12: Region 1-2 junction (tentative), lower affinity

FIG. 1A

| Campaign             | Clone ID | huMSLN-CHOK1 EC50 nM, Cell ELISA | OVCAR3 Binding EC50 nM, Cell ELISA | HuMSLN (87BGR) nM, ELISA | Domain binding, ELISA | KD Human (M) PH 7.4, SPR |
|----------------------|----------|----------------------------------|------------------------------------|--------------------------|-----------------------|--------------------------|
| SV018                | 45B6     | 0.02                             | 0.01                               | 9.16                     | 1                     | N.D.                     |
| SV017                | 47D7     | 0.18                             | 0.06                               | 0.18                     | 3                     | 1.10E-09                 |
| SV018                | 20B12    | 0.04                             | 0.06                               | 0.70                     | N.D.                  | 3.00E-08                 |
| N.D.: Not determined |          |                                  |                                    |                          |                       |                          |

FIG. 1B

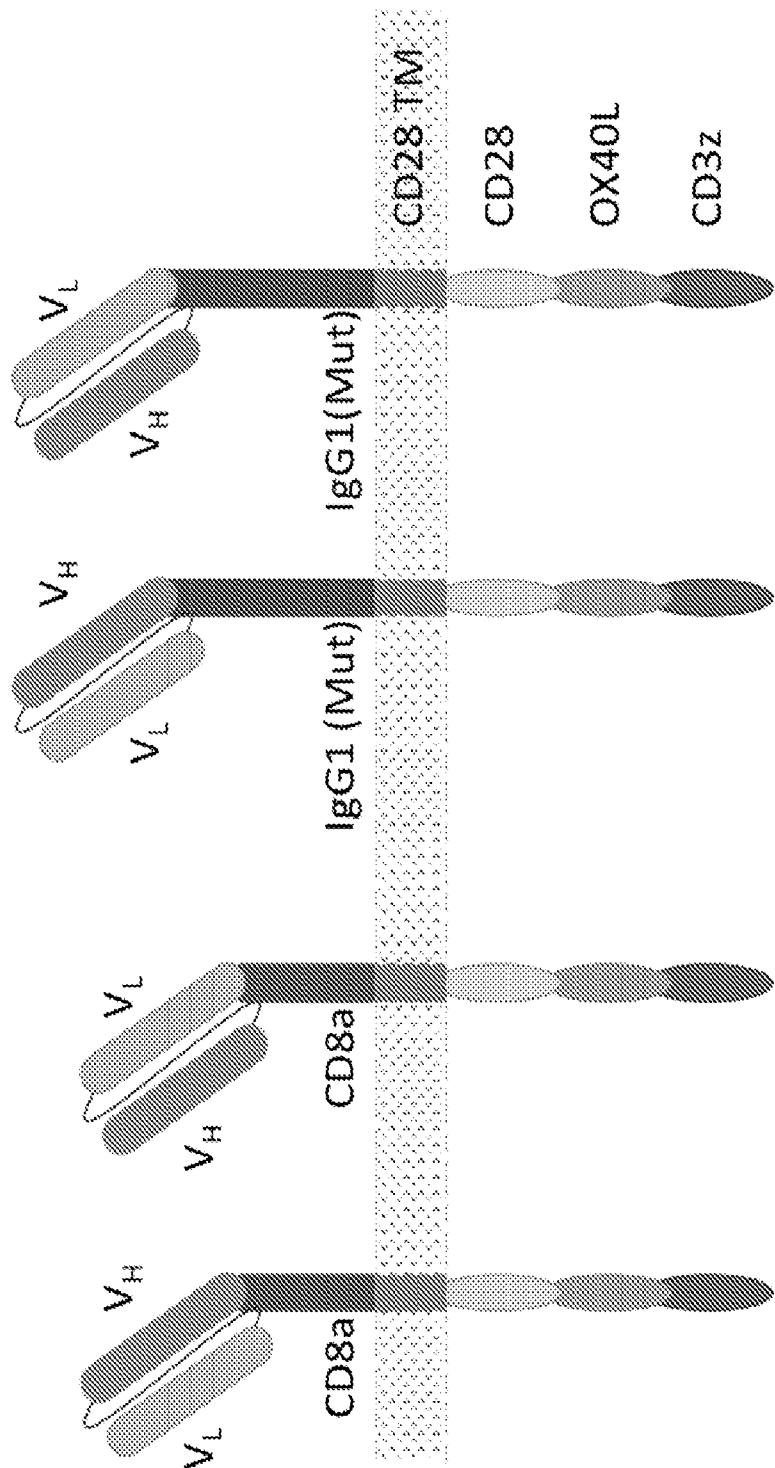


FIG. 2

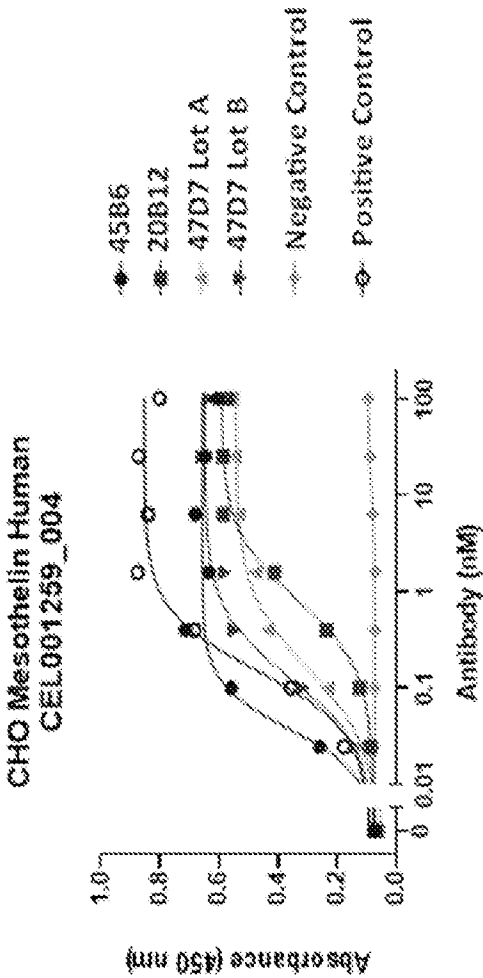


FIG. 3B

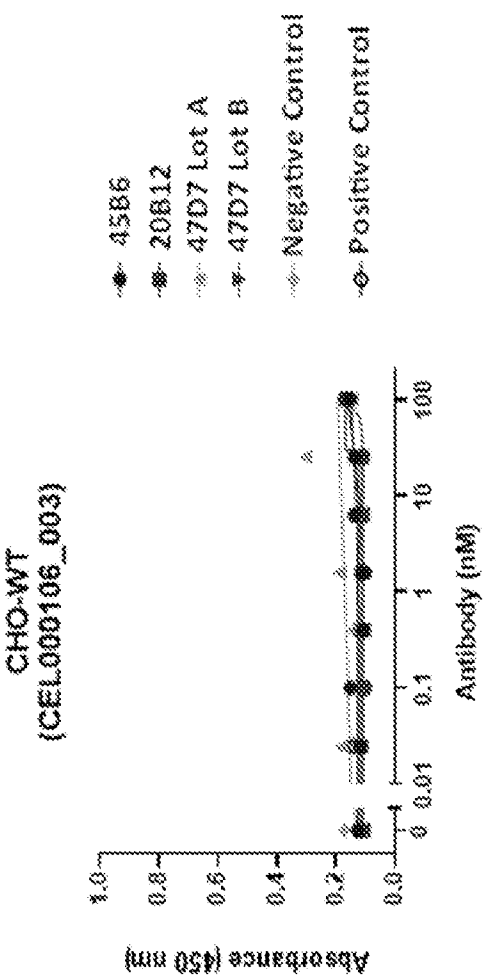


FIG. 3C

| Protein Name                                                                          | EC50 (nM)<br>CHO MSLN Hu | EC50 (nM)<br>CHO-WT |
|---------------------------------------------------------------------------------------|--------------------------|---------------------|
| 45B6_VL-VH-MutIgG1Fc                                                                  | 0.04                     | NB                  |
| 20B12_VL-VH-MutIgG1Fc                                                                 | 0.81                     | NB                  |
| 47D7_VL-VH-MutIgG1Fc                                                                  | 0.16                     | NB                  |
| 47D7_VH-VL-MutIgG1Fc                                                                  | 0.12                     | NB                  |
| Negative control<br>Humanized [RSV] mAb<br>(Synagis)                                  | NB                       | NB                  |
| Positive control<br>Mouse Human Chimera x<br>[MSLN_H] mAb (MORab-009)<br>IgG1 / Kappa | 0.15                     | NB                  |

NB = No binding

FIG. 3A

47D7 ScFv-Fc

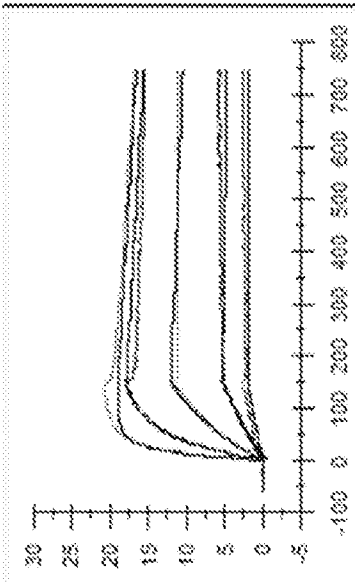


FIG. 4C

45B6 ScFv-Fc

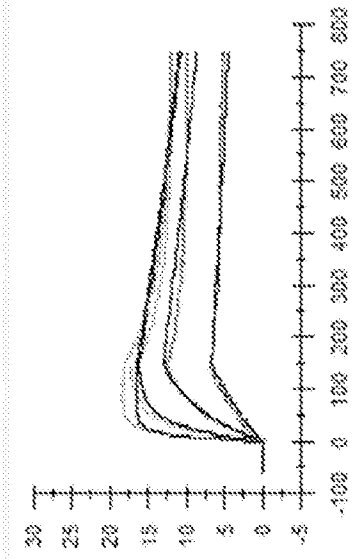


FIG. 4B

45B6-Fab

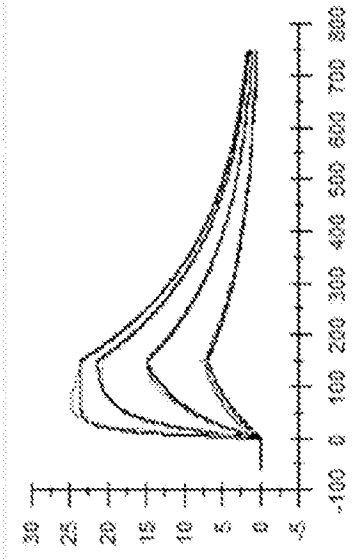
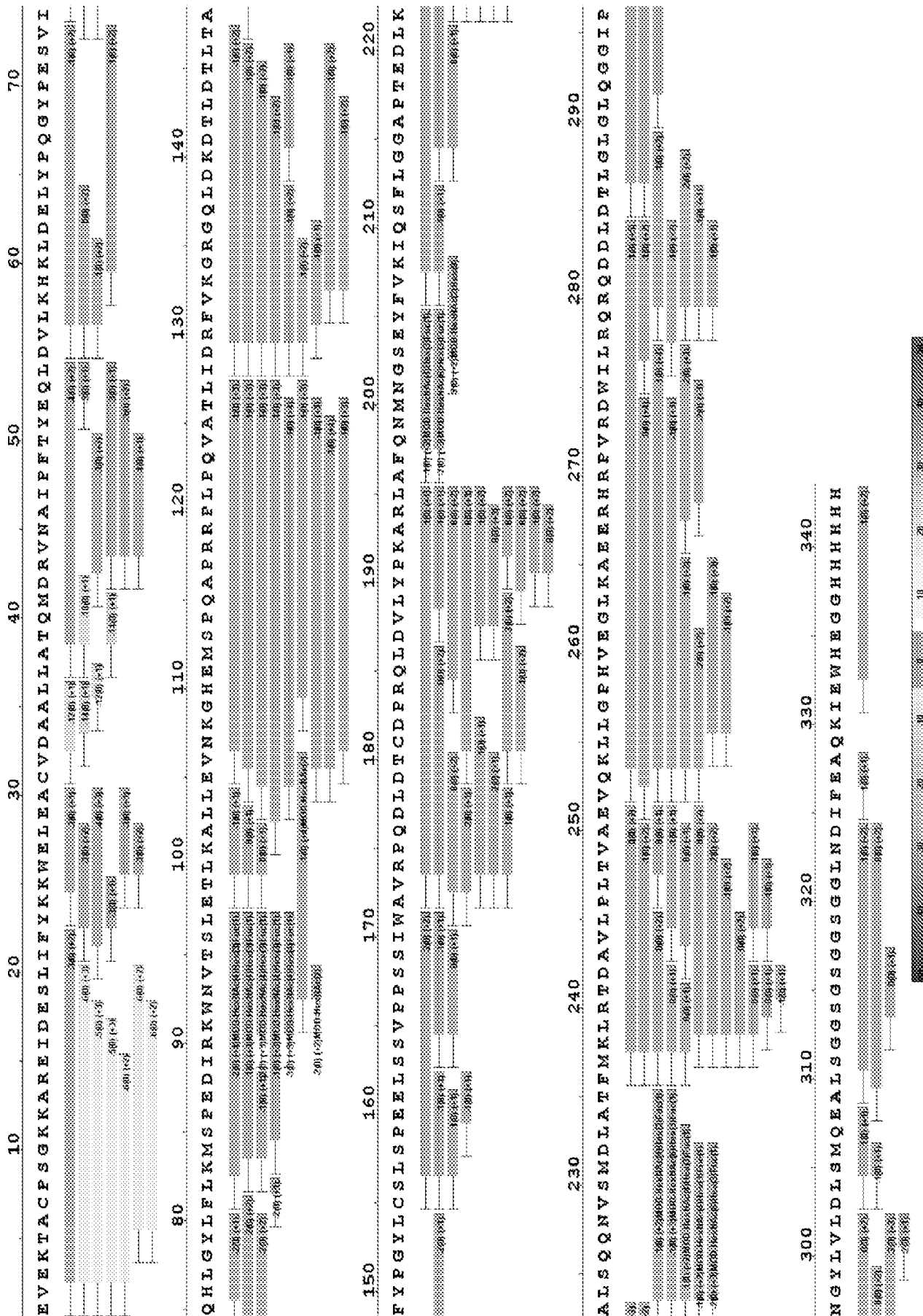


FIG. 4A

| Sample/Analyte | $k_a$<br>(1/Ms) | $k_d$<br>(1/s) | $K_D$<br>(M) | $K_D$<br>(pM) | $R_{max}$<br>(RU) | Ligand<br>(RU) | Binding Capacity (%) |
|----------------|-----------------|----------------|--------------|---------------|-------------------|----------------|----------------------|
| 45B6 Fab       | $7.30E+06$      | $4.67E-03$     | $6.40E-10$   | 640           | 30.0              | 25.6           | 95%                  |
| 45B6 ScFv-Fc   | $8.37E+06$      | $6.93E-04$     | $8.28E-11$   | 82.8          | 22.0              | 8.5            | 99%                  |
| 47D7 ScFv-Fc   | $4.83E+06$      | $2.23E-04$     | $4.95E-11$   | 49.5          | 23.5              | 8.2            | 109%                 |

FIG. 4D



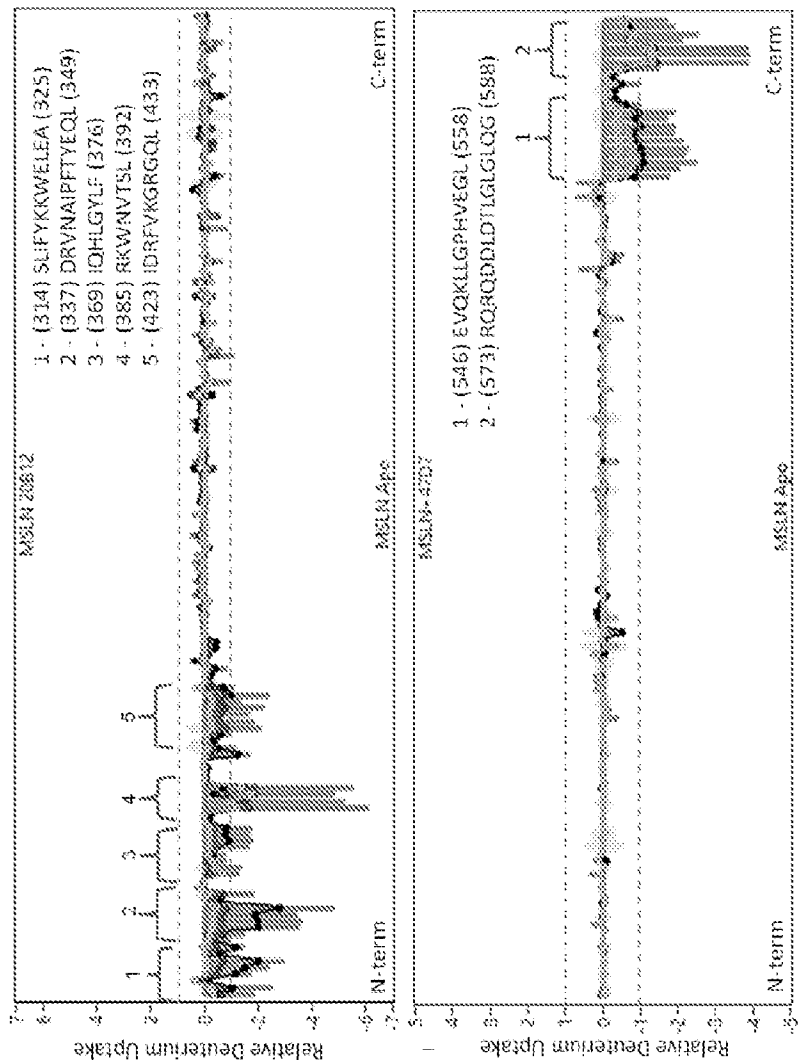
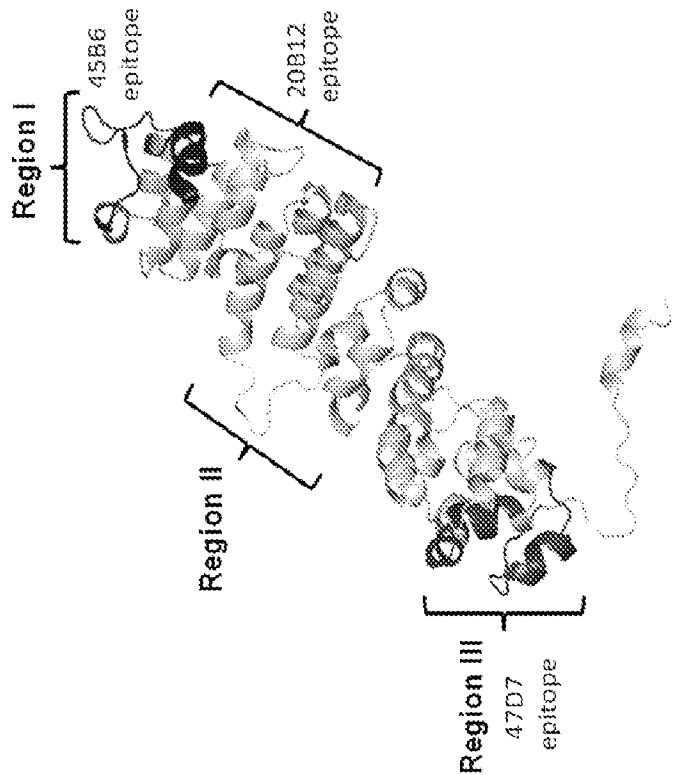
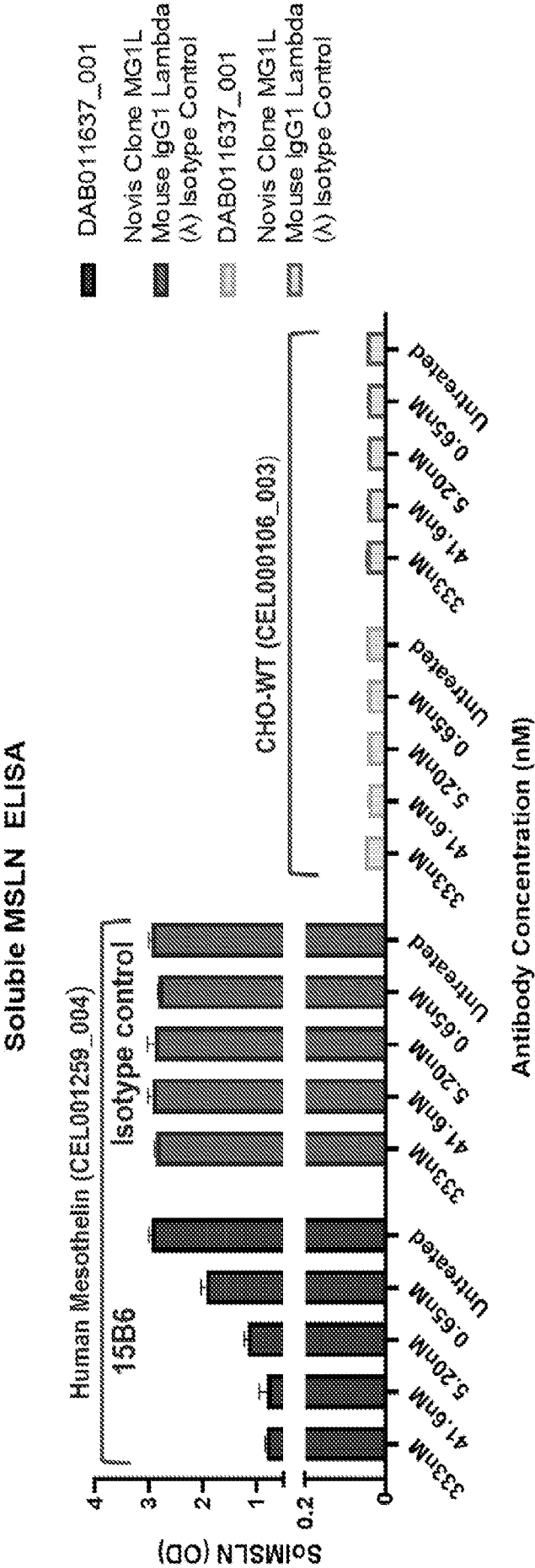


FIG. 5C

FIG. 5B





**FIG. 6**

|                   | A431<br>MSLN neg |    | NCI-N87<br>MSLN copy ~3200 |    | KLM-1<br>MSLN pos |    | OVCAR3<br>MSLN copy ~7800 |    |
|-------------------|------------------|----|----------------------------|----|-------------------|----|---------------------------|----|
|                   | -                | +  | -                          | +  | -                 | +  | -                         | +  |
| 500ng/ml sMSLN    | 0                | 0  | 41                         | 54 | 0                 | 12 | 54                        | 63 |
| CAR-NK Candidates | NK Cells         |    |                            |    |                   |    |                           |    |
|                   | 4586-CD8H        | 10 | 7                          | 97 | 97                | 99 | 99                        | 99 |
|                   | 20812-CD8H       | 10 | 3                          | 97 | 97                | 99 | 98                        | 98 |
|                   | 20812-4gG1mH     | 11 | 7                          | 97 | 96                | 96 | 89                        | 99 |
|                   | 4707-CD8H        | 4  | 12                         | 93 | 63                | 97 | 88                        | 94 |
| 4707-4gG1mH       | 9                | 6  | 61                         | 37 | 87                | 67 | 98                        | 94 |

% kill at end of 5-day study; E:T ratio 1:1 A431, NCI-N87, OVCAR3. E:T ratio KLM-1 3:1

% kill at end of 5-day study; E:T ratio 1:1 A431, NCI-N87, OVCAR3, E:T ratio KLM-1 3:1

FIG. 7

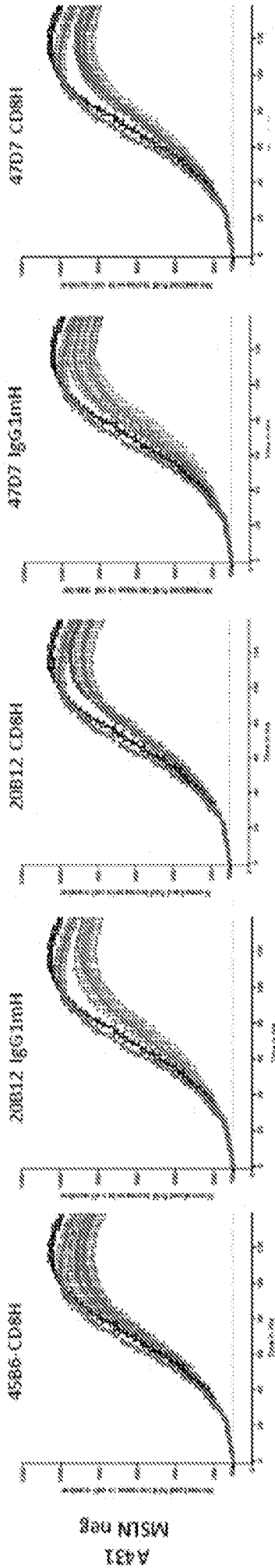


FIG. 8A

FIG. 8B

FIG. 8C

FIG. 8D

FIG. 8E

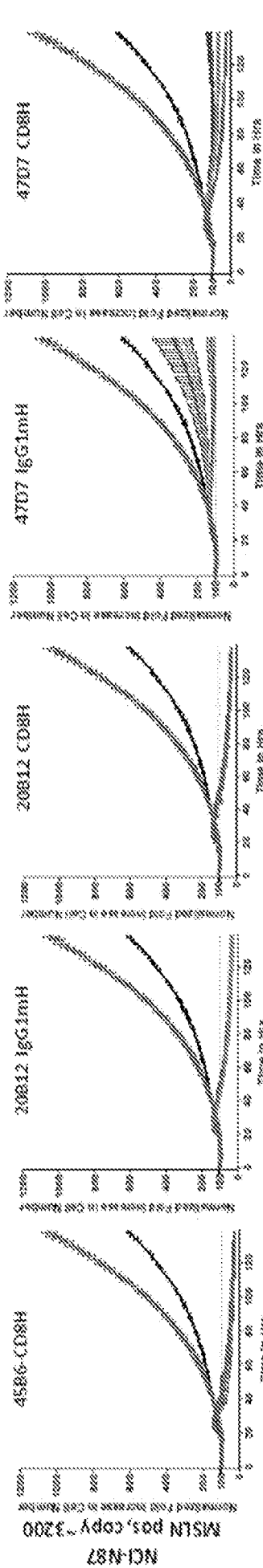


FIG. 8F

FIG. 8G

FIG. 8H

FIG. 8I

FIG. 8J

Target cells alone  
Non-CAR NK cells  
CAR-NK  
CAR-NK + 100ng/ml sMSLN  
CAR-NK + 500ng/ml sMSLN

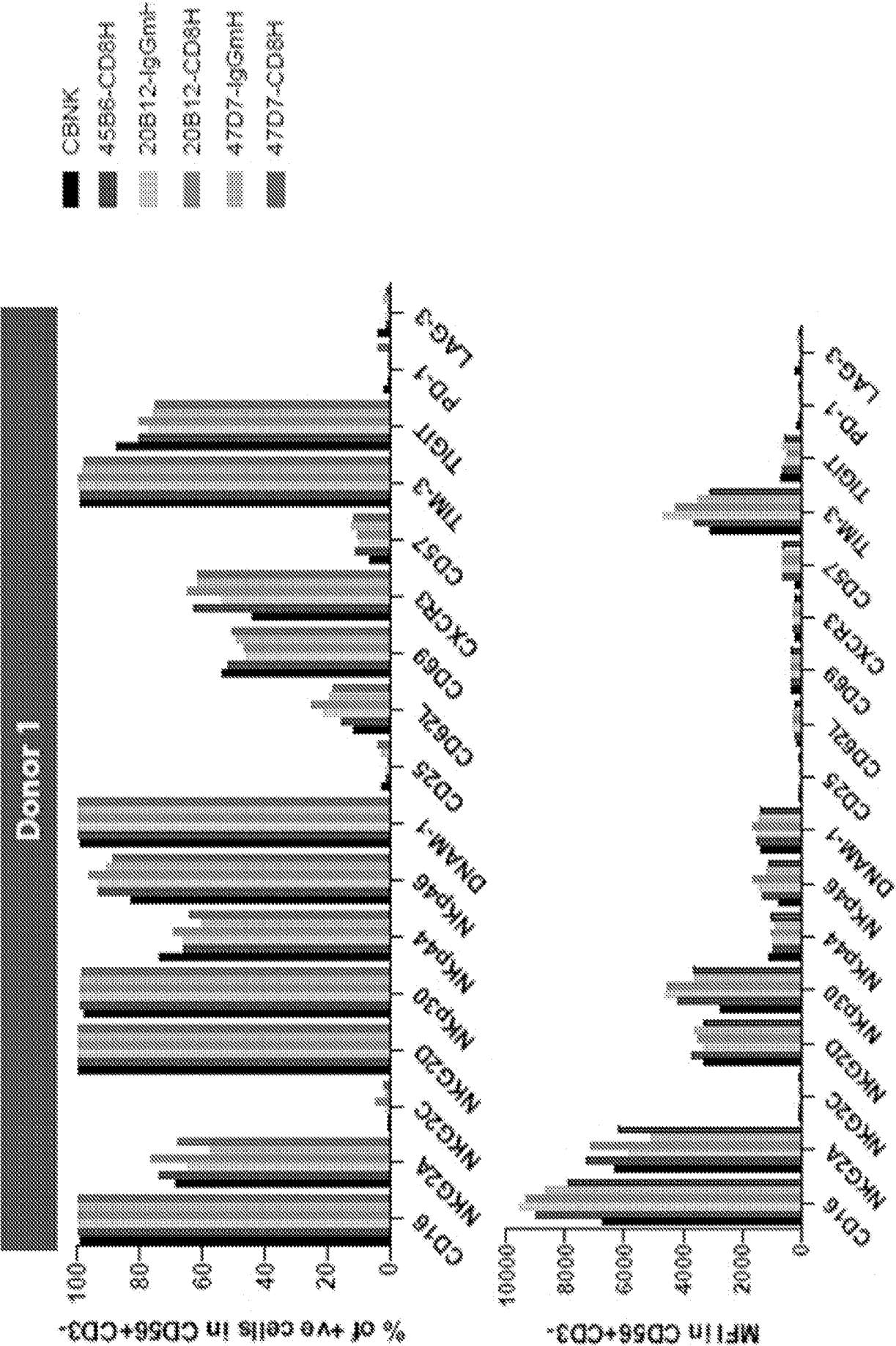


FIG. 9A

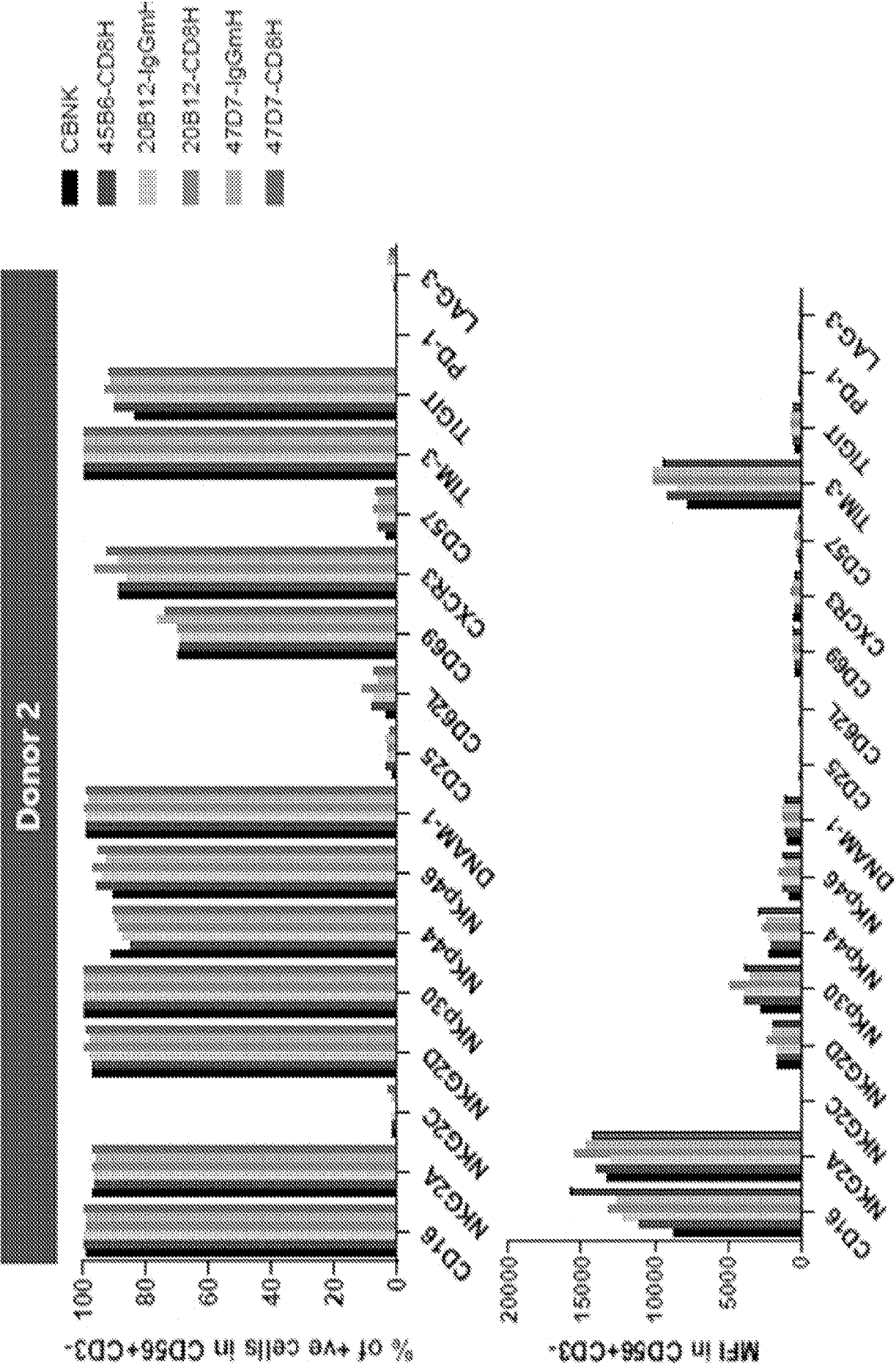
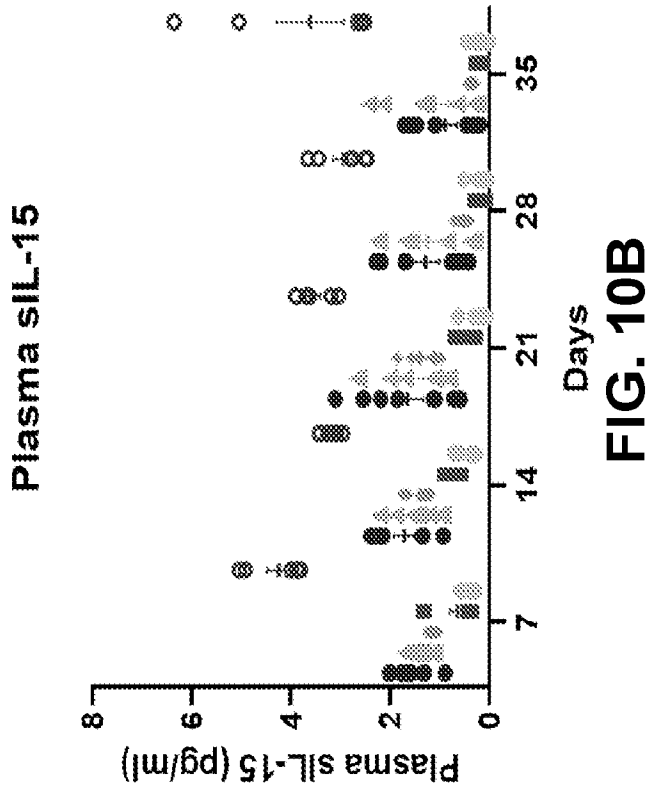
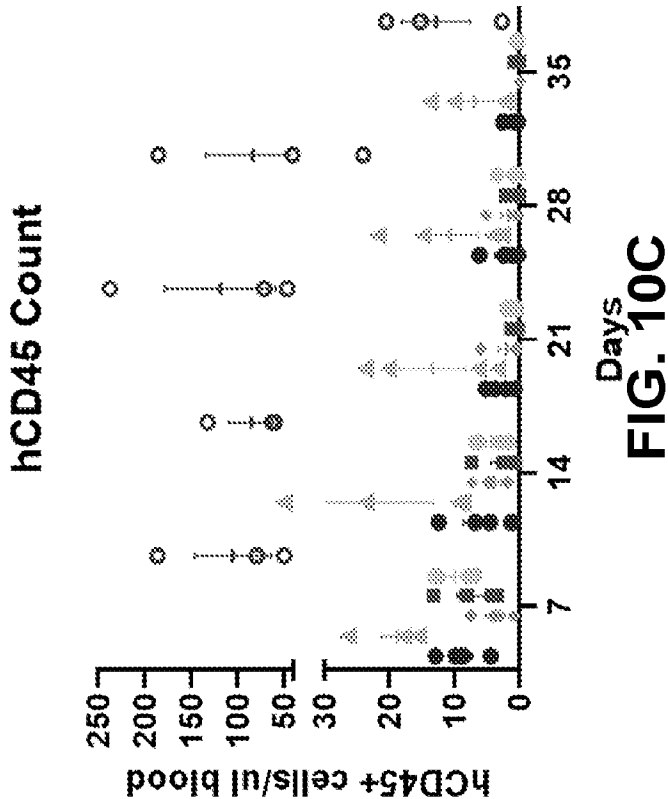
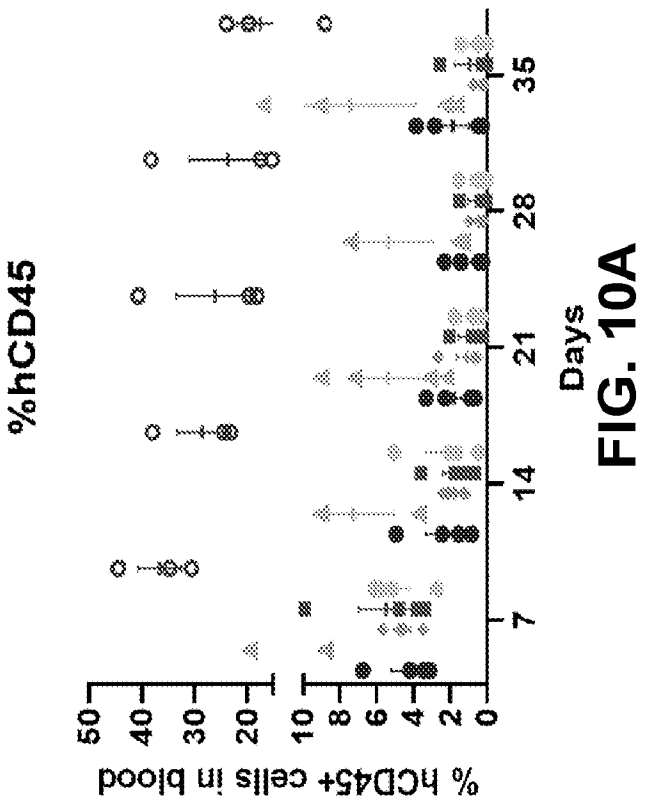
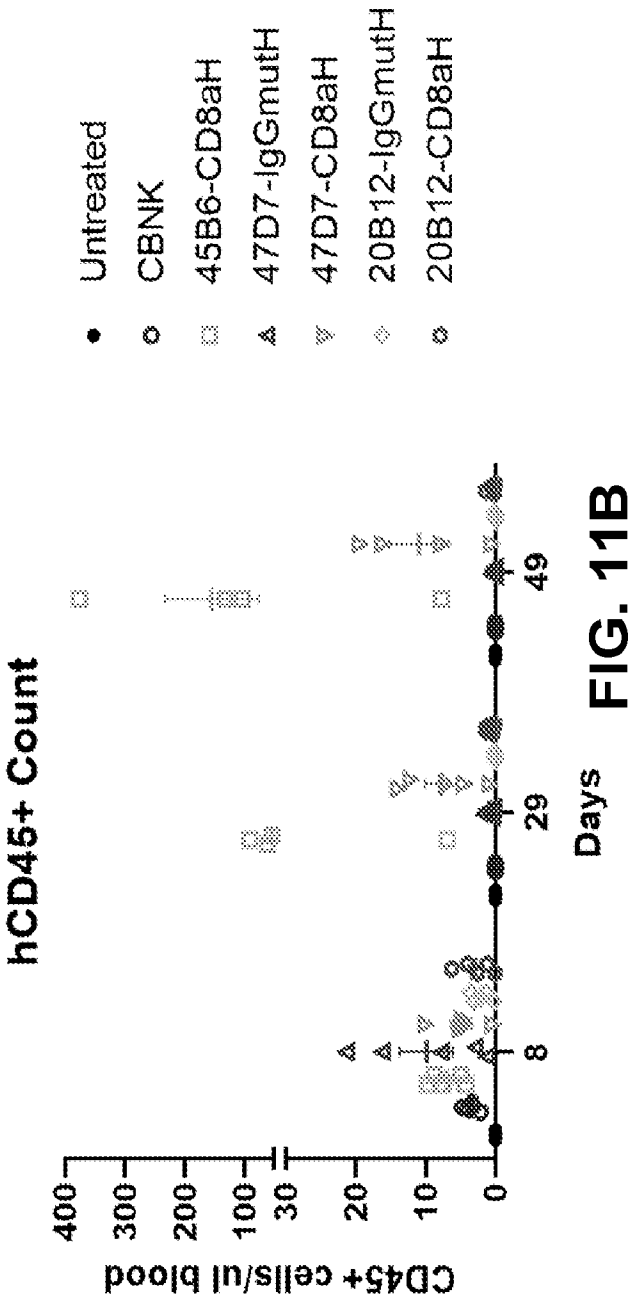
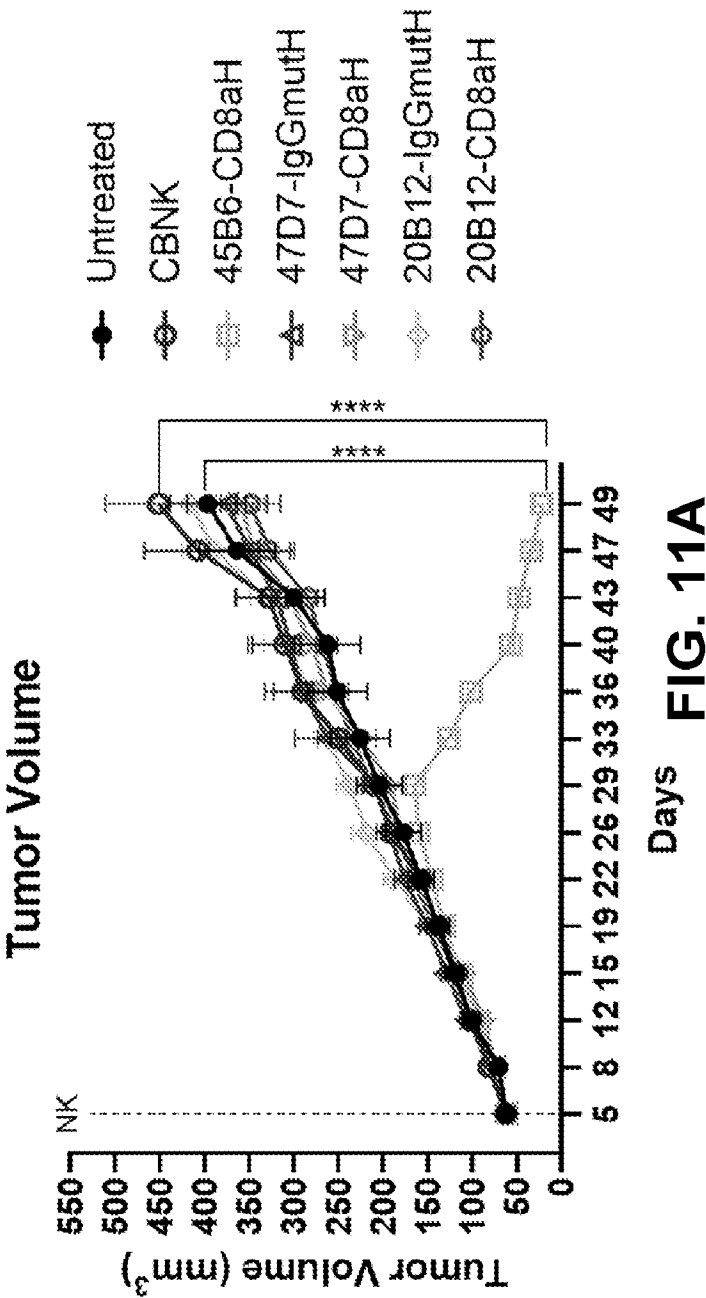


FIG. 9B



- 5700 45B6-CD8aH
- ▲ 5700 20B12-CD8aH
- ◆ 504415P 45B6-CD8aH
- 504415P 20B12-CD8aH
- ◆ 4500 20B12-CD8aH
- 315339P 45B6-CD8aH





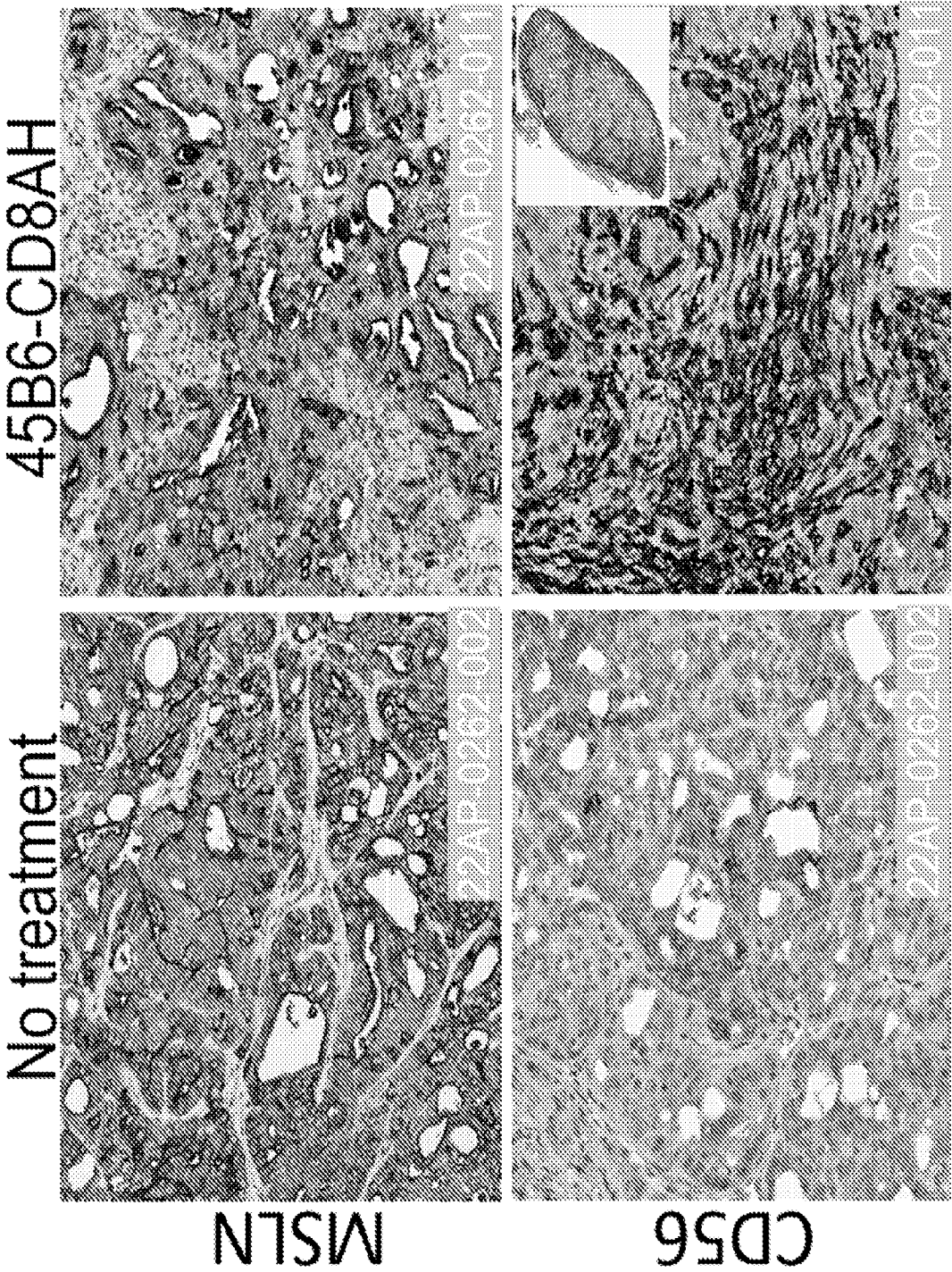


FIG. 11C