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(71) Applicants *(for all designated States except US):* **NOVARTIS AG** [CH/CH]; Lichtstrasse 35, CH-4056 Basel (CH). **THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA** [US/US]; 3160 Chestnut Street, Suite 200, Philadelphia, Pennsylvania 19104 (US).

(72) Inventors; and
(71) Applicants : **BEATTY, Gregory** [US/US]; 621 Spruce Street, Philadelphia, Pennsylvania 19106 (US). **ENGELS, Boris** [NL/US]; c/o Novartis Institutes for Biomedical Research Inc., 250 Massachusetts Avenue, Cambridge, Massachusetts 02 139 (US). **IDAMAKANTI, Neeraja** [US/US]; c/o Novartis Institutes for Biomedical Research Inc., 250 Massachusetts Avenue, Cambridge, Massachusetts 021 39 (US). **JUNE, Carl H.** [US/US]; 409 Baird Road, Merion Station, Pennsylvania 19066 (US). **LOEW, Andreas** [DE/US]; c/o Novartis Institutes for Biomedical Research Inc., 250 Massachusetts Avenue, Cambridge, Massachusetts 02139 (US). **SONG, Huijuan** [CN/CN]; c/o China Novartis Institutes for Biomedica Research, No. 8 Building, Lane 898 Halei Road, Zhangjiang Hi-Tech Park, Pudong New Area, Shanghai 201203 (CN). **WU, Qilong** [US/US]; c/o Novartis Institutes for Biomedical Research Inc., 250 Massachusetts Avenue, Cambridge, Massachusetts 02139 (US).

(74) Agent: **ZHONGZI LAW OFFICE;** 7F, New Era Building, 26 Pinganli Xidajie, Xicheng District, Beijing 100034 (CN).

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(54) Title: HUMAN MESOTHELIN CHIMERIC ANTIGEN RECEPTORS AND USES THEREOF

(57) Abstract: Provided are compositions and methods for treating diseases associated with expression of mesothelin. Also provided are a chimeric antigen receptor(CAR) specific to mesothelin, vectors encoding the same, and recombinant T cells comprising the mesothelin CAR. Further provided are methods of administering a genetically modified T cell expressing a CAR that comprises a mesothelin binding domain.

HUMAN MESOTHELIN CHIMERIC ANTIGEN RECEPTORS AND USES THEREOF

This application claims priority to International Application No. PCT/CN2013/089979, filed December 19, 2013, International Application No. PCT/CN2014/082610, filed July 21, 2014 and International Application No. PCT/CN2014/090509, filed November 6, 2014, the entire contents of each of these applications are incorporated herein by reference.

FIELD OF THE INVENTION

[001] The present invention relates generally to the use of T cells engineered to express a Chimeric Antigen Receptor (CAR) to treat a disease associated with expression of mesothelin.

BACKGROUND OF THE INVENTION

[002] Mesothelin was originally identified by Pastan and colleagues as a tumor associated antigen due to its limited expression by normal tissues and overexpression on tumors. Chang K, et al., *Cancer Res.* 1992;52(1):181-186 and Chang K, et al. *ProcNatlAcadSciUSA.* 1996;93(1):136-140. The mesothelin gene encodes a precursor 71-kDa protein that is processed to yield the 40-kDa protein, mesothelin, which is anchored at the cell membrane by a glycosylphosphatidyl inositol (GPI) linkage and an amino-terminal 31-kDa shed fragment, called megkaryocyte potentiating factor (MPF). Both fragments contain N-glycosylation sites. A soluble splice variant of the 40-kDa carboxyl-terminal fragment called "soluble mesothelin/MPF-related" has been found in the sera of patients with pancreatic ductal adenocarcinoma (PDA). Johnston, F, et al. *Clinical Cancer Research.* 2009;15(21):6511. Mesothelin is currently being explored both as a therapeutic target as well as a bio-marker for disease activity and therapeutic response. Argani P, et al. *Clin Cancer Res.* 2001;7(12):3862-3868.

[003] Mesothelin is a differentiation antigen that is also present on normal tissues. Using the mouse anti-human mesothelin antibody K1 that was developed by the Pastan group, strong K1 reactivity has been demonstrated within mesothelial cells that line the peritoneal, pleural, and

pericardial cavities, although at lower levels than usually seen for malignant tissues. Chang K, et al, *Cancer Res.* 1992;52(1):181-186. Weak K1 reactivity has been detected within the Fallopian tube epithelium, tracheal basal epithelium and tonsils epithelium. Mesothelin has also been found on all layers of the cornea. Jirsova K, et al. *Experimental eye research.* 2010;91(5):623-629. However, K1 reactivity has not been detected in the majority of normal tissues including the liver, kidneys, spleen, bone marrow, lymph nodes, thymus, cardiac muscle, tongue, skeletal muscle, skin, cerebral cortex, cerebellum, spinal cord, peripheral nerve, pituitary, adrenal, salivary gland, mammary gland, thyroid, parathyroid, testis, prostate, epididymis, cervical epithelium, lung parenchyma, esophagus, small-bowel epithelium, colon epithelium, bladder epithelium, gall-bladder epithelium. Chang K, et al., *Cancer Res.* 1992;52(1):181-186.

[004] Mesothelin is overexpressed in the vast majority of primary pancreatic adenocarcinomas with rare and weak expression seen in benign pancreatic tissue. Argani P, et al. *Clin Cancer Res.* 2001;7(12):3862-3868. Epithelial malignant pleural mesothelioma (MPM) universally expresses mesothelin while sarcomatoid MPM does not express mesothelin. Most serous epithelial ovarian carcinomas, and the related primary peritoneal carcinomas, express mesothelin.

[005] Mesothelin is a target of a natural immune response in ovarian cancer, and has been proposed to be a target for cancer immunotherapy. Bracci L, et al. *Clin Cancer Res.* 2007; 13(2 Pt l):644-653; Moschella F, et al. *Cancer Res.* 2011;71(10):3528-3539; Gross G, et al. *FASEBJ.* 1992;6(15):3370-3378; Sadelain M, et al. *NatRevCancer.* 2003;3(l):35-45; Muul LM, et al. *Blood.* 2003;101(7):2563-2569; Yee C, et al. *Proc Natl Acad Sci USA.* 2002;99(25): 16168-16173. The presence of mesothelin-specific CTLs in patients with pancreatic cancer correlates with overall survival. Thomas AM, et al. *J Exp Med.* 2004;200:297-306. In addition, Pastan and coworkers have used soluble antibody fragments of an anti-mesothelin antibody conjugated to immunotoxins to treat cancer patients with mesothelin-positive tumors. This approach has demonstrated adequate safety and some clinical activity in pancreatic cancer. Hassan R, et al. *Cancer Immun.* 2007;7:20 and Hassan R, et al. *Clin Cancer Res.* 2007;13(17):5144-5149. In ovarian cancer, this therapeutic strategy produced one minor response by RECIST criteria and stable disease in a second patient who also had complete resolution of their ascites.

SUMMARY OF THE INVENTION

[006] The invention features, e.g., methods of providing an immune response in patients by administering an immune effector cell that is engineered to express a Chimeric Antigen Receptor (CAR) that comprises an antibody (e.g., scFv) that specifically targets mesothelin. In particular, the invention pertains to the use of an immune effector cell such as, e.g., a T cell or NK cell, engineered to express a CAR that includes an antibody such as antigen binding fragment thereof to treat a cancer associated with expression of mesothelin (or MSLN). In particular, the invention pertains to adoptive cell transfer that may be particularly suitable for patients with mesothelin-expressing cancers, such as, e.g., mesothelioma (e.g., malignant pleural mesothelioma, lung cancer (e.g., non-small cell lung cancer, small cell lung cancer, squamous cell lung cancer, or large cell lung cancer), pancreatic cancer (e.g., pancreatic ductal adenocarcinoma, pancreatic metatstatic), ovarian cancer, colorectal cancer and bladder cancer, or any combination thereof.

[007] Accordingly, in one aspect, the invention pertains to an isolated nucleic acid molecule encoding a chimeric antigen receptor (CAR), wherein the CAR comprises an anti-mesothelin binding domain (e.g., a human anti-mesothelin binding domain), a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain. In one embodiment, the encoded anti-mesothelin binding domain comprises one or more (e.g., all three) light chain complementary determining region 1 (LC CDRl), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a human anti-mesothelin binding domain described herein, and one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDRl), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a human anti-mesothelin binding domain described herein. In one embodiment, the encoded human anti-mesothelin binding domain comprises, or consists of, a light chain variable region described herein (e.g., in Table 2, 4 or 5) and/or a heavy chain variable region described herein (e.g., in Table 2, 4, or 5). In one embodiment, the encoded anti-mesothelin binding domain is a scFv comprising or consisting of a light chain and a heavy chain of an amino acid sequence of Table 2. In an embodiment, the anti-mesothelin binding domain (e.g., an scFV) comprises or consists: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g.,

substitutions) of an amino acid sequence of a light chain variable region provided in Table 2, or a sequence with 95-99% identity with an amino acid sequence of Table 2; and/or a heavy chain variable region comprising, or consisting of, an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in Table 2, or a sequence with 95-99% identity to an amino acid sequence of Table 2. In one embodiment, the human anti-mesothelin binding domain comprises, or consists of, a sequence selected from the group consisting of SEQ ID NO: 39; SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, and SEQ ID NO: 62, or a sequence with 95-99% identity thereof. In one embodiment, the nucleic acid sequence encoding the human anti-mesothelin binding domain comprises, or consists of, a sequence selected from the group consisting of SEQ ID NO: 87; SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 109, and SEQ ID NO: 110, or a sequence with 95-99% identify thereof.

[008] In one embodiment, the isolated nucleic acid further comprises a sequence encoding a transmembrane domain, e.g., a transmembrane domain described herein. In one embodiment, the encoded transmembrane domain comprises, or consists of, a transmembrane domain of a protein selected from the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154. In one embodiment, the encoded transmembrane domain comprises, or consists of, a sequence of SEQ ID NO: 12. In one embodiment, the transmembrane domain comprises, or consists of, an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 12, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 12.

[009] In one embodiment, the encoded CAR includes an anti-mesothelin binding domain, e.g., an anti-mesothelin binding domain described herein, connected to the transmembrane domain by a hinge region, e.g., a hinge region described herein. In one embodiment, the hinge region comprises, or consists of, SEQ ID NO: 6 or SEQ ID NO: 8.

[0010] In one embodiment, the isolated nucleic acid molecule further comprises a sequence encoding a costimulatory domain, e.g., a costimulatory domain described herein. In one embodiment, the costimulatory domain is a functional signaling domain obtained from a protein selected from the group consisting of OX40, CD27, CD28, CDS, ICAM-1, LFA-1 (CDlla/CD18), ICOS (CD278), and 4-1BB (CD137). In one embodiment, the encoded costimulatory domain comprises, or consists of, a sequence of SEQ ID NO: 14. In one embodiment, the costimulatory domain comprises, or consists of, an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 14, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 14.

[001 1] In one embodiment, the isolated nucleic acid comprises a sequence encoding an intracellular signaling domain, e.g., an intracellular signaling domain described herein. In one embodiment, the isolated nucleic acid encodes a functional signaling domain of 4-IBB and/or a functional signaling domain of CD3 zeta. In one embodiment, the encoded intracellular signaling domain comprises the sequence of SEQ ID NO: 7 and/or the sequence of SEQ ID NO: 9 or SEQ ID NO: 10. In one embodiment, the intracellular signaling domain comprises an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 7 and/or an amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 7 and/or an amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10. In one embodiment, the encoded intracellular signaling domain comprises, or consists of, the sequence of SEQ ID NO: 7 and the sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein the sequences comprising the intracellular signaling domain are expressed in the same frame and as a single polypeptide chain.

[0012] In another aspect, the invention pertains to an isolated nucleic acid molecule encoding a CAR construct comprising a leader sequence, e.g., of SEQ ID NO: 1; an anti-mesothelin

binding domain described herein, e.g., having an amino acid sequence of Table 2, or a sequence with 95-99% identify thereof; a hinge region, e.g., of SEQ ID NO: 2; a transmembrane domain, e.g., having a sequence of SEQ ID NO: 6; a costimulatory domain, e.g., a 4-1BB costimulatory domain having a sequence of SEQ ID NO: 7; and a primary signaling domain, e.g., CD3 zeta stimulatory domain having a sequence of SEQ ID NO: 9 or 10. In one embodiment, the isolated nucleic acid molecule comprises (e.g., consists of) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of Table 2. In one embodiment, the isolated nucleic acid molecule comprises (consists of) a nucleic acid encoding a polypeptide having an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of Table 2, or a sequence with 95-99% identity to an amino acid sequence of Table 2.

[0013] In another aspect, the invention pertains to an isolated polypeptide molecule encoded by the nucleic acid sequence, e.g., a nucleic acid described herein.

[0014] In another aspect, the invention pertains to an isolated polypeptide molecule comprising, or consisting of, a sequence selected from the group consisting of Table 2, an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in Table 2, or a sequence with 95-99% identity to an amino acid sequence of Table 2. In one embodiment, the isolated polypeptide comprises one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a human anti-mesothelin binding domain described herein, and one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a human anti-mesothelin binding domain described herein.

[0015] In another aspect, the invention pertains to an isolated chimeric antigen receptor (CAR) molecule comprising an anti-mesothelin binding domain described herein, e.g., a human anti-mesothelin binding domain described herein, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain.

[0016] In one embodiment, the anti-mesothelin binding domain does not compete for binding to human mesothelin with an antigen binding domain comprising a sequence comprising SEQ ID NO: 279.

[0017] In one embodiment, the anti-mesothelin binding domain competes for binding to human mesothelin with an antigen binding domain comprising a LC CDR1 , LC CDR2 and LC CDR3 of an anti-mesothelin light chain amino acid sequence selected from SEQ ID NO: 43 or SEQ ID NO: 49 and an HC CDR1, HC CDR2, and HC CDR3 of an anti-mesothelin heavy chain amino acid sequence selected from SEQ ID NO: 43 or SEQ ID NO: 49. In one embodiment, the anti-mesothelin binding domain competes for binding to human mesothelin with an antigen binding domain comprising SEQ ID NO: 43 or SEQ ID NO: 49.

[0018] In one embodiment, the anti-mesothelin binding domain binds to a different epitope of human mesothelin than the epitope of human mesothelin targeted by the antigen binding domain comprising a sequence comprising SEQ ID NO: 279. In an embodiment, the epitope comprises a sequence of amino acids selected from amino acids 314-315, 317-318, 346-349, and 369-375 of SEQ ID NO: 278, or any combination thereof. In an embodiment, the epitope comprises one or more amino acids selected from amino acids 314-315, 317-318, 346-349, and 369-375 of SEQ ID NO: 278, or any combination thereof.

[0019] In an embodiment, the anti-mesothelin binding domain described herein does not bind to the N-terminus of mesothelin as shown in SEQ ID NO: 278. In one embodiment, the anti-mesothelin binding domain binds to the C-terminus of human mesothelin. In one embodiment, the anti-mesothelin binding domain binds an epitope within amino acids 450-588 of SEQ ID NO: 278. In one embodiment, the epitope bound by the anti-mesothelin binding domain comprises a sequence selected from amino acids 485-490, 498-507, 532-537, and 545-572 of SEQ ID NO: 278, or a combination thereof. In one embodiment, the epitope bound by the anti-mesothelin binding domain comprises one or more amino acids selected from amino acids 485-490, 498-507, 532-537, and 545-572 of SEQ ID NO: 278, or any combination thereof. In these embodiments, SEQ ID NO: 278 represents amino acids 296-588 of human mesothelin, e.g., the first amino acid of SEQ ID NO: 278 is amino acid 296 and the last amino acid of SEQ ID NO: 278 is amino acid 588.

[0020] In one embodiment, the anti-mesothelin binding domain comprises one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a human anti-mesothelin binding domain described herein, and one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a human anti-mesothelin binding domain described herein. In one embodiment, the human anti-mesothelin binding domain comprises, or consists of, a light chain variable region described herein (e.g., in Table 2) and/or a heavy chain variable region described herein (e.g., in Table 2). In one embodiment, the anti-mesothelin binding domain is a scFv comprising, or consisting of, a light chain variable region and a heavy chain variable region of an amino acid sequence of Table 2. In an embodiment, the anti-mesothelin binding domain (e.g., an scFV) comprises, or consists of: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in Table 2, or a sequence with 95-99% identity to an amino acid sequence of Table 2; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in Table 2, or a sequence with 95-99% identity to an amino acid sequence of Table 2. In one embodiment, the human anti-mesothelin binding domain comprises, or consists of, a sequence selected from the group consisting of SEQ ID NO: 39; SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, and SEQ ID NO: 62, or a sequence with 95-99% identity thereof.

[0021] In one embodiment, the transmembrane domain is a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154. In one embodiment, the transmembrane domain comprises a

transmembrane domain described herein, e.g., having a sequence of SEQ ID NO: 6, an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 6, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 6.

[0022] In one embodiment, the anti-mesothelin binding domain is connected to the transmembrane domain by a hinge region. In one embodiment, the hinge region comprises a hinge region described herein, e.g., a hinge region of SEQ ID NO: 2.

[0023] In one embodiment, the isolated CAR molecule further comprises a costimulatory domain, e.g., a costimulatory domain described herein. In one embodiment, the costimulatory domain is a functional signaling domain obtained from a protein selected from the group consisting of OX40, CD27, CD28, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), and 4-IBB (CD137) or functional variant thereof. In one embodiment, the costimulatory domain comprises, or consists of, a sequence of SEQ ID NO: 7. In one embodiment, the costimulatory domain comprises, or consists of, an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO:7, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO:7.

[0024] In one embodiment, the isolated CAR molecule comprises an intracellular signaling domain, e.g., an intracellular signaling domain described herein. In one embodiment, the intracellular signaling domain comprises a functional signaling domain of 4-IBB and/or a functional signaling domain of CD3 zeta. In one embodiment, the intracellular signaling domain comprises, or consists of, the sequence of SEQ ID NO: 7 and/or the sequence of SEQ ID NO: 9 or SEQ ID NO: 10. In one embodiment, the intracellular signaling domain comprises, or consists of, an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 7 and/or an amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 7 and/or an amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10. In one embodiment, the intracellular signaling domain comprises, or consists of, the sequence of SEQ ID NO: 9 and the sequence of

SEQ ID NO: 9 or SEQ ID NO: 10, wherein the sequences comprising the intracellular signaling domain are expressed in the same frame and as a single polypeptide chain.

[0025] In another aspect, the invention pertains to an isolated CAR molecule comprising a leader sequence, e.g., of SEQ ID NO: 1; an anti-mesothelin binding domain described herein, e.g., having an amino acid sequence of Table 2, or a sequence with 95-99% identify thereof; a hinge region, e.g., of SEQ ID NO: 2; a transmembrane domain, e.g., having a sequence of SEQ ID NO: 6; a costimulatory domain, e.g., a 4-IBB costimulatory domain having a sequence of SEQ ID NO: 7; and a primary signaling domain, e.g., CD3 zeta stimulatory domain having a sequence of SEQ ID NO: 9 or SEQ ID NO: 10. In one embodiment, the isolated CAR molecule comprises (e.g., consists of) a polypeptide having an amino acid sequence of Table 2. In one embodiment, the isolated CAR molecule comprises (consists of) a polypeptide having an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of Table 2, or a sequence with 95-99% identity to an amino acid sequence of Table 2. In one embodiment, the isolated CAR molecule comprises, or consists of, an amino acid sequence selected from the group consisting of SEQ ID NO: 63; SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, 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, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, and SEQ ID NO: 86.

[0026] In another aspect, the invention pertains to a vector comprising a nucleic acid sequence described herein. In one embodiment, the nucleic acid sequence encodes a CAR molecule, e.g., a CAR molecule described herein. In one embodiment, the vector is selected from the group consisting of a DNA, a RNA, a plasmid, a lentivirus vector, adenoviral vector, or a retrovirus vector.

[0027] In one embodiment, the vector is a lentivirus vector, e.g., a lentivirus vector described herein. In one embodiment, the vector further comprises a promoter. In one embodiment, the promoter is an EF-1α promoter. In one embodiment, the EF-1α promoter comprises a sequence of SEQ ID NO: 11.

[0028] In one embodiment, the vector is an in vitro transcribed vector, e.g., a vector that transcribes RNA of a nucleic acid molecule described herein. In one embodiment, the RNA is transcribed from an in vitro transcription vector, wherein the vector is pD-A.anti-meso BD OF.2bg.150A, wherein the anti-meso BD is an anti-mesothelin binding domain described herein. In one embodiment, the nucleic acid sequence in the vector further comprises a poly(A) tail, e.g., a poly A tail described herein, e.g., comprising about 150 adenosine bases (SEQ ID NO: 271). In one embodiment, the nucleic acid sequence in the vector further comprises a 3'UTR, e.g., a 3'UTR described herein, e.g., comprising at least one repeat of a 3'UTR derived from human beta-globulin.

[0029] In another aspect, the invention pertains to a cell comprising the vector. The cell can be, e.g., a cell described herein. In one embodiment, the cell is a human T cell, e.g., a T cell described herein, or a human NK cell, e.g., a human NK cell described herein. In one embodiment, the human T cell is a CD8+ T cell. In one embodiment, the cell is an autologous T cell. In one embodiment, the cell is an allogeneic T cell. In one embodiment, the cell is a T cell and the T cell is diaglycerol kinase (DGK) deficient. In one embodiment, the cell is a T cell and the T cell is Ikaros deficient. In one embodiment, the cell is a T cell and the T cell is both DGK and Ikaros deficient.

[0030] In one aspect, the CAR-expressing cell described herein can further comprise a second CAR, e.g., a second CAR that includes a different antigen binding domain, e.g., to the same target (mesothelin) or a different target (e.g., a target other than mesothelin on stroma cells, e.g., FAP; a target other than mesothelin on prostate cancer cells, e.g., androgen receptor, OR51E2, PSMA, PSCA, PDGRF-β, TARP, GloboH, MAD-CT-1, or MAD-CT-2; a target other than mesothelin on ovararian cancer cells, e.g., Tn, PRSS21, CD171, Lewis Y, folate receptor a, claudin6, GloboH, or sperm protein 17; e.g., a target other than mesothelin on lung cancer cells, e.g.,VEGF, HER3, IGF-IR, EGFR, DLL4, or Trop-2). In one embodiment, the CAR-expressing cell comprises a first CAR that targets a first antigen and includes an intracellular signaling domain having a costimulatory signaling domain but not a primary signaling domain, and a second CAR that targets a second, different, antigen and includes an intracellular signaling domain having a primary signaling domain but not a costimulatory signaling domain. In one embodiment, the CAR expressing cell comprises a first mesothelin CAR that includes a mesothelin binding domain, a transmembrane domain and a costimulatory domain and a second

CAR that targets an antigen other than mesothelin (e.g., an antigen expressed on stroma cells, lung cancer cells, prostate cancer cells or ovarian cancer cells) and includes an antigen binding domain, a transmembrane domain and a primary signaling domain. In another embodiment, the CAR expressing cell comprises a first mesothelin CAR that includes a mesothelin binding domain, a transmembrane domain and a primary signaling domain and a second CAR that targets an antigen other than mesothelin (e.g., an antigen expressed on stroma cells, lung cancer cells, prostate cancer cells or ovarian cancer cells) and includes an antigen binding domain to the antigen, a transmembrane domain and a costimulatory signaling domain.

[003 1] In one embodiment, the CAR-expressing cell comprises a mesothelin CAR described herein and an inhibitory CAR. In one embodiment, the inhibitory CAR comprises an antigen binding domain that binds an antigen found on normal cells but not cancer cells, e.g., normal cells that also express mesothelin. In one embodiment, the inhibitory CAR comprises the antigen binding domain, a transmembrane domain and an intracellular domain of an inhibitory molecule. For example, the intracellular domain of the inhibitory CAR can be an intracellular domain of PD1, PD-L1, CTLA4, TFM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIRl, CD160, 2B4 and TGFR beta.

[0032] In another embodiment, the CAR-expressing cell described herein can further express another agent, e.g., an agent which enhances the activity or fitness of a CAR-expressing cell, e.g., an agent described herein. For example, in one embodiment, the agent can be an agent which inhibits a molecule that modulates or regulates, e.g., inhibits, T cell function. In some embodimemts, the molecule that modulates or regulates T cell function is an inhibitory molecule. Examples of inhibitory molecules include PD1, PD-L1, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIRl, CD160, 2B4 and TGFR beta. In embodiments, an agent, e.g., an inhibitory nucleic acid, e.g., a dsRNA, e.g., an siRNA or shRNA; or e.g., an inhibitory protein or system, e.g., a clustered regularly interspaced short palindromic repeats (CRISPR), a transcription-activator like effector nuclease (TALEN), or a zinc finger endonuclease (ZFN), e.g., as described herein, can be used to inhibit expression of a molecule that modulates or regulates, e.g., inhibits, T-cell function in the CAR-expressing cell. In an embodiment the agent is an shRNA, e.g., an shRNA described herein. In an embodiment, the agent that modulates or regulates, e.g., inhibits, T-cell function is inhibited within a CAR-expressing cell. For example, a dsRNA molecule that inhibits expression of a

molecule that modulates or regulates, e.g., inhibits, T-cell function is linked to the nucleic acid that encodes a component, e.g., all of the components, of the CAR.

[0033] In one embodiment, the agent which inhibits an inhibitory molecule comprises a first polypeptide, e.g., an inhibitory molecule, associated with a second polypeptide that provides a positive signal to the cell, e.g., an intracellular signaling domain described herein. In one embodiment, the agent comprises a first polypeptide, e.g., of an inhibitory molecule such as PDl, PD-L1, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, CTLA4, , VISTA, CD160, BTLA, LAIR1, TIM3, 2B4, TGFR beta and TIGIT, or a fragment of any of these (e.g., at least a portion of the extracellular domain of any of these), and a second polypeptide which is an intracellular signaling domain described herein (e.g., comprising a costimulatory domain (e.g., 41BB, CD27 or CD28, e.g., as described herein) and/or a primary signaling domain (e.g., a CD3 zeta signaling domain described herein)). In one embodiment, the agent comprises a first polypeptide of PDl or a fragment thereof (e.g., at least a portion of the extracellular domain of PDl), and a second polypeptide of an intracellular signaling domain described herein (e.g., a CD28 signaling domain described herein and/or a CD3 zeta signaling domain described herein).

[0034] In another aspect, the invention pertains to a method of making a cell comprising transducing a cell described herein, e.g., a T cell or a NK cell, with a vector of comprising a nucleic acid encoding a CAR molecule, e.g., a CAR molecule described herein. In one embodiment, the vector is a lentiviral vector described herein.

[0035] The present invention also provides a method of generating a population of RNA-engineered cells, e.g., cells described herein, e.g., T cells or NK cells, transiently expressing exogenous RNA. The method comprises introducing an in vitro transcribed RNA or synthetic RNA into a cell, where the RNA comprises a nucleic acid encoding a CAR molecule described herein.

[0036] In another aspect, the invention pertains to a method of providing anti-tumor immunity in a subject comprising administering to the subject an effective amount of a cell comprising a CAR molecule, e.g., a cell expressing a CAR molecule described herein, a cell described herein. In one embodiment, the cell is an autologous T cell or NK cell. In one

embodiment, the cell is an allogeneic T cell or NK cell. In one embodiment, the subject is a human.

[0037] In another aspect, the invention pertains to a method of treating a subject having a disease associated with expression of mesothelin *(e.g.,* a proliferative disease, a precancerous condition, and a noncancer related indication associated with the expression of mesothelin) comprising administering to the subject an effective amount of a cell comprising a CAR molecule, e.g., as described herein.

[0038] In one embodiment, the disease associated with mesothelin is cancer, e.g., a cancer described herein. In one embodiment, the disease associated with mesothelin is selected from the group consisting of: mesothelioma (e.g., malignant pleural mesothelioma), lung cancer (e.g., non-small cell lung cancer, small cell lung cancer, squamous cell lung cancer, or large cell lung cancer), pancreatic cancer (e.g., pancreatic ductal adenocarcinoma), ovarian cancer, colorectal cancer and bladder cancer or any combination thereof. In one embodiment, the disease is pancreatic cancer, e.g., metastatic pancreatic ductal adenocarcinoma (PDA), e.g., in a subject who has progressed on at least one prior standard therapy. In one embodiment, the disease is mesothelioma (e.g., malignant pleural mesothelioma), e.g., in a subject who has progressed on at least one prior standard therapy. In one embodiment, the disease is ovarian cancer, e.g., serous epithelial ovarian cancer, e.g., in a subject who has progressed after at least one prior regimen of standard therapy.

[0039] In one embodiment, the mesothelin CAR expressing cell, e.g., T cell or NK cell, is administered to a subject that has received a previous dose of melphalan.

[0040] In one embodiment, the cells expressing a CAR molecule, e.g., a CAR molecule described herein, are administered in combination with an agent that enhances the activity or fitness of a cell expressing a CAR molecule, e.g., an agent described herein.

[0041] In one embodiment, the cells expressing a CAR molecule, e.g., a CAR molecule described herein, are administered in combination with a low, immune enhancing dose of an mTOR inhibitor. While not wishing to be bound by theory, it is believed that treatment with a low, immune enhancing, dose (e.g., a dose that is insufficient to completely suppress the immune system but sufficient to improve immune function) is accompanied by a decrease in PD-1 positive T cells or an increase in PD-1 negative cells. PD-1 positive T cells, but not PD-1

negative T cells, can be exhausted by engagement with cells which express a PD-1 ligand, e.g., PD-L1 or PD-L2.

[0042] In an embodiment this approach can be used to optimize the performance of CAR cells described herein in the subject. While not wishing to be bound by theory, it is believed that, in an embodiment, the performance of endogenous, non-modified immune effector cells, e.g., T cells, is improved. While not wishing to be bound by theory, it is believed that, in an embodiment, the performance of a mesothelin CAR expressing cell is improved. In other embodiments, cells, e.g., T cells or NK cells, which have, or will be engineered to express a CAR, can be treated ex vivo by contact with an amount of an mTOR inhibitor that increases the number of PDl negative immune effector cells, e.g., T cells or increases the ratio of PDl negative immune effector cells, e.g., T cells/ PDl positive immune effector cells, e.g., T cells.

[0043] In an embodiment, administration of a low, immune enhancing, dose of an mTOR inhibitor, e.g., an allosteric inhibitor, e.g., RADOOl, or a catalytic inhibitor, is initiated prior to administration of an CAR expressing cell described herein, e.g., T cells or NK cells. In an embodiment, the CAR cells are administered after a sufficient time, or sufficient dosing, of an mTOR inhibitor, such that the level of PDl negative immune effector cells, e.g., T cells, or the ratio of PDl negative immune effector cells, e.g., T cells/ PDl positive immune effector cells, e.g., T cells, has been, at least transiently, increased.

[0044] In an embodiment, the cell, e.g., T cell or NK cell, to be engineered to express a CAR, is harvested after a sufficient time, or after sufficient dosing of the low, immune enhancing, dose of an mTOR inhibitor, such that the level of PDl negative immune effector cells, e.g., T cells, or the ratio of PDl negative immune effector cells, e.g., T cells/ PDl positive immune effector cells, e.g., T cells, in the subject or harvested from the subject has been, at least transiently, increased.

[0045] In one embodiment, the cells expressing a CAR molecule, e.g., a CAR molecule described herein, are administered in combination with an agent that ameliorates one or more side effect associated with administration of a cell expressing a CAR molecule, e.g., an agent described herein.

[0046] In one embodiment, the cells expressing a CAR molecule, e.g., a CAR molecule described herein, are administered in combination with an agent that treats the disease associated with mesothelin expression, e.g., an agent described herein.

[0047] In one embodiment, the cells expressing a CAR molecule, e.g., a CAR molecule described herein, are administered at a dose and/or dosing schedule described herein.

[0048] In one embodiment, the cells expressing a CAR molecule, e.g., a CAR molecule described herein, are administered as a first line treatment for the disease, e.g., the cancer, e.g., the cancer described herein. In another embodiment, the cells expressing a CAR molecule, e.g., a CAR molecule described herein, are administered as a second, third, fourth line treatment for the disease, e.g., the cancer, e.g., the cancer described herein.

[0049] In one embodiment, a population of cells described herein is administered.

[0050] In one embodiment, the CAR molecule is introduced into T cells or NK cells, e.g., using in vitro transcription, and the subject (e.g., human) receives an initial administration of cells comprising a CAR molecule, and one or more subsequent administrations of cells comprising a CAR molecule, wherein the one or more subsequent administrations are administered less than 15 days, e.g., 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 days after the previous administration. In one embodiment, more than one administration of cells comprising a CAR molecule are administered to the subject (e.g., human) per week, e.g., 2, 3, or 4 administrations of cells comprising a CAR molecule are administered per week. In one embodiment, the subject (e.g., human subject) receives more than one administration of cells comprising a CAR molecule per week (e.g., 2, 3 or 4 administrations per week) (also referred to herein as a cycle), followed by a week of no administration of cells comprising a CAR molecule, and then one or more additional administration of cells comprising a CAR molecule (e.g., more than one administration of the cells comprising a CAR molecule per week) is administered to the subject. In another embodiment, the subject (e.g., human subject) receives more than one cycle of cells comprising a CAR molecule, and the time between each cycle is less than 10, 9, 8, 7, 6, 5, 4, or 3 days. In one embodiment, the cells comprising a CAR molecule are administered every other day for 3 administrations per week. In one embodiment, the cells comprising a CAR molecule are administered for at least two, three, four, five, six, seven, eight or more weeks.

[0051] In one aspect, the invention includes a population of autologous or allogenic cells that are transfected or transduced with a vector comprising a nucleic acid molecule encoding a mesothelin-CAR molecule, e.g., as described herein. In one embodiment, the vector is a retroviral vector. In one embodiment, the vector is a self-inactivating lentiviral vector as

described elsewhere herein. In one embodiment, the vector is delivered (e.g., by transfecting or electroporating) to a cell, e.g., a T cell or a NK cell, wherein the vector comprises a nucleic acid molecule encoding a mesothelin CAR molecule as described herein, which is transcribed as an mRNA molecule, and the mesothelin CAR molecule is translated from the RNA molecule and expressed on the surface of the cell.

[0052] In another aspect, the present invention provides a population of CAR-expressing cells, e.g., CART cells. In some embodiments, the population of CAR-expressing cells comprises a mixture of cells expressing different CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CAR having an anti-mesothelin binding domain described herein, and a second cell expressing a CAR having a different anti-mesothelin binding domain, e.g., an anti-mesothelin binding domain described herein that differs from the anti-mesothelin binding domain in the CAR expressed by the first cell. As another example, the population of CAR-expressing cells can include a first cell expressing a CAR that includes an anti-mesothelin binding domain, e.g., as described herein, and a second cell expressing a CAR that includes an antigen binding domain to a target other than mesothelin (e.g., a target other than mesothelin on stroma cells, e.g., FAP; a target other than mesothelin on prostate cancer cells, e.g., androgen receptor, OR51E2, PSMA, PSCA, PDGRF- β, TARP, GloboH, MAD-CT-1, or MAD-CT-2; a target other than mesothelin on ovararian cancer cells, e.g., Tn, PRSS21, CD171, Lewis Y, folate receptor a, claudin6, GloboH, or sperm protein 17; e.g., a target other than mesothelin on lung cancer cells, e.g.,VEGF, HER3, IGF-1R, EGFR, DLL4, or Trop-2). In one embodiment, the population of CAR-expressing cells includes, e.g., a first cell expressing a CAR that includes a primary intracellular signaling domain, and a second cell expressing a CAR that includes a secondary signaling domain.

[0053] In another aspect, the present invention provides a population of cells wherein at least one cell in the population expresses a CAR having an anti- mesothelin binding domain described herein, and a second cell expressing another agent, e.g., an agent which enhances the activity or function of a CAR-expressing cell. For example, in one embodiment, the agent can be an agent which inhibits a molecule that modulates or regulates, e.g., inhibits, T cell function. In some embodiments, the molecule that modulates or regulates T cell function is an inhibitory molecule, e.g., an agent described herein. Examples of inhibitory molecules include PD1, PD-L1, CTLA4, TEVI3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA,

TIGIT, LAIR1, CD160, 2B4 and TGFRbeta. In embodiments, an agent, e.g., an inhibitory nucleic acid, e.g., a dsRNA, e.g., an siRNA or shRNA; or e.g., an inhibitory protein or system, e.g., a clustered regularly interspaced short palindromic repeats (CRISPR), a transcription-activator like effector nuclease (TALEN), or a zinc finger endonuclease (ZFN), e.g., as described herein, can be used to inhibit expression of a molecule that modulates or regulates, e.g., inhibits, T-cell function in the CAR-expressing cell. In an embodiment the agent is an shRNA, e.g., an shRNA described herein. In an embodiment, the agent that modulates or regulates, e.g., inhibits, T-cell function is inhibited within a CAR-expressing cell. For example, a dsRNA molecule that inhibits expression of a molecule that modulates or regulates, e.g., inhibits, T-cell function is linked to the nucleic acid that encodes a component, e.g., all of the components, of the CAR.

[0054] In one embodiment, the agent which inhibits an inhibitory molecule comprises a first polypeptide, e.g., an inhibitory molecule, associated with a second polypeptide that provides a positive signal to the cell, e.g., an intracellular signaling domain described herein. In one embodiment, the agent comprises a first polypeptide, e.g., of an inhibitory molecule such as PDl, PD-L1, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, CTLA4, , VISTA, CD160, BTLA, LAIR1, TEVI3, 2B4, TGFR beta and TIGIT, or a fragment of any of these (e.g., at least a portion of an extracellular domain of any of these), and a second polypeptide which is an intracellular signaling domain described herein (e.g., comprising a costimulatory domain (e.g., 41BB, CD27 or CD28, e.g., as described herein) and/or a primary signaling domain (e.g., a CD3 zeta signaling domain described herein). In one embodiment, the agent comprises a first polypeptide of PDl or a fragment thereof (e.g., at least a portion of the extracellular domain of PDl), and a second polypeptide of an intracellular signaling domain described herein (e.g., a CD28 signaling domain described herein and/or a CD3 zeta signaling domain described herein).

[0055] In one embodiment, the nucleic acid molecule encoding a mesothelin CAR molecule, e.g., as described herein, is expressed as an mRNA molecule. In one embodiment, the genetically modified mesothelin CAR-expressing cells, e.g., T cells or NK cells, can be generated by transfecting or electroporating an RNA molecule encoding the desired CARs (e.g., without a vector sequence) into the cell. In one embodiment, a mesothelin CAR molecule is translated from the RNA molecule once it is incorporated and expressed on the surface of the recombinant cell.

[0056] In another aspect, the invention pertains to the isolated nucleic acid molecule encoding a CAR molecule, e.g., a CAR molecule described herein, a CAR molecule described herein, a vector comprising a CAR molecule described herein, and/or a cell comprising a CAR molecule described herein for use as a medicament.

[0057] In another aspect, the invention pertains to a the isolated nucleic acid molecule encoding a CAR molecule described herein, a CAR molecule described herein, a vector comprising a CAR molecule described herein, and/or a cell comprising a CAR molecule described herein for use in the treatment of a disease expressing mesothelin, e.g., as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0058] FIG. 1 is a schematic of the pD-A.anti-meso BD.OF.BBZ.2bg.150A plasmid. Figure discloses "150A" as SEQ ID NO: 271.

[0059] FIG. 2 depicts cell manufacturing and treatment schedules that can be used. **(A)** Autologous cells are obtained by leukocyte apheresis and T cells are enriched by expansion with anti-CD3/CD28 mAb coated magnetic beads. Cells are expanded for 8 to 12 days. On the last day of culture, the beads are removed using a magnetic field and the cells are washed, electroporated with human meso CAR mRNA construct, and cryopreserved in infusible medium. **(B)** Three treatment infusion schedules are depicted. On Schedule 1, patients receive lxl 0^8 human meso bearing CART cells by intravenous (i.v.) infusion on day 0 followed by lxl 0^9 human meso bearing CART cells one week later. Safety can be monitored for a minimum of one month before patients are eligible for Schedule 2. On Schedule 2, patients receive $1x10^8$ human meso bearing CART cells by i.v. infusion three times per week for one week followed by one week of rest and then lxl 0^9 human meso bearing CART cells administered three times per week for one week. On Schedule 3, patients receive $3xl0^8/m^2$ human meso bearing CART cells by i.v. infusion three times per week for three weeks followed by intra-tumoral injection into a primary lesion of $2xl0^8$ human meso bearing CART cells on days +35 and +57.

[0060] FIG. 3A and 3B are graphic representations of cytotoxicity as assayed donor 2 (healthy donor) T cell transduced with mouse SSI CAR or the anti-MSLN CARs Ml to M12 of

the invention and cultured with either control K562 cells that do not express MSLN as shown in FIG. 3A, or K562 cells transduced to express MSLN (K562-Meso) as shown in FIG. 3B.

[0061] FIG. 4A and 4B are graphs showing the IFNy secretion of the mouse SSI and CD 19 CART and the anti-MSLN CARTs upon stimulation by MSLN+ cells. FIG. 4A shows reactivity to the transduced cell line K562-Meso and its MSLN-negative parental line K562. FIG. 4B shows reactivity toward cancer cells naturally expressing MSLN; the ovarian cancer line Ovcar8 and the pancreatic cancer lines SW1990 and Panc0203.

[0062] FIG. 5 shows a clinical trial design for mesothelin CARTs made by transducing a CAR construct with a lentiviral vector.

[0063] FIG. 6A, 6B, 6C, and 6D show anti-tumor activity of CART-meso cells.

[0064] FIG. 7A, 7B, and 7C show the *in vivo* persistence of CARTmeso cells and trafficking to primary and metastatic tumor sites.

[0065] FIG. 8 shows cytokine and chemokines in the serum after CARTmeso cell infusion.

[0066] FIG. 9A and 9B show CARTmeso cell induction of anti-tumor antibodies. Sera was obtained from the MPM patient (FIG. 9A) and the pancreatic cancer patient (FIG. 9B).

[0067] FIG. 10 shows tumor growth in NSG mice injected with EMMESO tumor cells. After tumors grew to ~ 200 mm^3 in size, mesoCAR T cells were injected via tail vein and measured for 39 days post injection.

[0068] FIG. 11A and 1IB show the expression of mesoCAR by flow cytometry analysis at the time of injection (FIG. 11A) or after 40 days at the time of harvest from xenograft tumors.

[0069] FIG. 12 shows the functional capacity of mesoCAR T cells with regard to *in vitro* killing when isolated from the flank of NSG mice after 39 days, or cryo preserved after transduction.

[0070] FIG. 13 shows the expression of inhibitor enzymes DGK and SHP1 in TILs isolated from EMESO flank tumor compared to overnight-rested TILs.

[0071] FIG. 14A, 14B, 14C, 14D, 14E, and 14F show the effect of treatment with inhibitors (anti-PDLl, DGK inhibitor, and SSG) of inhibitory mechanisms that downregulate mesoCART

function on tumor cell killing (FIG. 14A, 14C and 14E) and IFNgamma cytokine secretion (FIG. 14B, 14C, and 14F).

[0072] FIG. 15A, 15B, 15C, and 15D show cytokine secretion from a small panel of human CART-MSLN after stimulation with various tumor cell lines. FIG. 15A shows IFNgamma secretion. FIG. 15B shows TNF. FIG. 15C shows IL-2. FIG. 15D shows IL-4.

[0073] FIG. 16A and 16B show the results of the killing assay of CART-MSLN-5, CART-MSLN-11, CART-MSLN17, and murine CART-MSLN-SS1 against Ovcar3 (FIG. 16A) and U87mg (FIG. 16B) tumor cells.

[0074] FIG. 17A and 17B show the results of the killing assay of the panel of CART-MSLN against Ovcar3 tumor cells.

[0075] FIG. 18 shows the anti-tumor activity of a first set of CART-MSLN (including M5, M11, M17, and M21) in the Ovcar8 xenograft model.

[0076] FIG. 19 shows the anti-tumor activity of a second set of CART-MSLN (including M12, M14, M16, and M23) in the Ovcar8 xenograft model.

[0077] FIG. 20A, 20B, and 20C depicts the loss of fucntionality of mesoCAR T cells in the tumor microenvironment (TILs) over time compared to fresh or thawed mesoCAR T cells. A) Cytotoxicity assay; B) IFNγ release assay; and C) western blot analysis of ERK signaling (via phosphorylation).

[0078] FIG. 21 depicts the effect of deletion of DGK on cytotoxicity of mesoCAR T cells. Percent target cell killing is assessed at different effector:target ratios.

[0079] FIG. 22 depicts the effect of deletion of DGK on IFNγ production and release from mesoCAR T cells. Concentration of IFNγ is assessed at different effector:target ratios.

[0080] FIG. 23 depicts the effect of deletion of DGK on ERK signaling, or T cell activation, mesoCAR T cells. B: albumin, M: mesothelin, 3/28: CD3/CD28 stimulated cells.

[0081] FIG. 24 depicts the effect of deletion of DGK on TGFβ sensitivity of mesoCAR T cells with regard to cytotoxic activity.

[0082] FIG. 25A and 25B depict the effect of deletion of DGK on therapeutic efficacy of mesoCAR T cells in a tumor mouse model. A) Effect on anti-tumor activity is shown by tumor volume over time. B) Persistance and proliferation of tumor infiltrating cells.

[0083] FIG. 26A, 26B, 26C, 26D, 26E, and 26F shows the cytokine production and cytotoxic mediator release in CAR-expressing T cells with reduced levels of Ikaros. FIG. 26A shows Ikaros expression in wild-type and *Ikzf1+I-* CAR T cells as measured by flow cytometry (left panel) and western blot (right panel). Following stimulation with mesothelin-coated beads, PMA/Ionomycin (PMA/I), or BSA-coated beads (control), the percentage of cells producing IFN-γ (FIG. 26B), TNF-a (FIG. 26C), and IL-2 (FIG. 26D), the cytotoxic mediator granzyme B (FIG. 26E), and CD107a expression (FIG. 26F) was determined.

[0084] FIG. 27A, 27B, and 27C shows cytokine production and cytotoxic mediator release in CAR-expressing T cells with a dominant negative allele of Ikaros (IkDN). Following stimulation with mesothelin-coated beads, PMA/Ionomycin (PMA/I), or BSA-coated beads (control), the percentage of cells producing IFN-γ (FIG. 27A), IL-2 (FIG. 27B), and CD107a expression (FIG. 27C) was determined.

[0085] FIG. 28A, 28B, 28C, 28D, and 28E shows that the depletion of Ikaros did not augment activation and signaling of CAR T cells following antigen stimulation. The levels of CD69 (FIG. 28A), CD25 (FIG. 28B), and 4-IBB (FIG. 28C) was determined by flow cytometry at the indicated time points in *Ikzf1+I-* CAR T cells. In FIG. 28D, the RAS/ERK signaling pathways were examined in wild-type (WT) and Ikaros dominant negative cells (IkDN) after TCR stimulation with CD3/CD28 antibodies. The levels of phosphorylated TCR signaling proteins such as phosphorylated PLCy, phosphorylated Lck, phosphorylated JNK, phosphorylated Akt, phosphorylated ERK, phosphorylated IKKa, and IκBα were assessed by western blot. In FIG. 28E, WT and IkDN cells transduced with mesoCAR were stimulated with BSA or mesothelin-coated beads, and downstream signaling pathways were examined by western blot by assessing the levels of phosphorylated ERK and phosphorylated PLCy.

[0086] FIG. 29A, 29B, 29C, 29D, and 29E shows that the reduction of Ikaros in CAR T cells augments the response against target cells AE17 or mesothelin-expressing AE17 (AE17 meso)*in vitro*. FIG. 29A depicts IFNy production in WT and *Ikzf1+I-* meso CART cells at the indicated effectortarget cell ratios. Cytolysis of meso CAR-expressing WT and *Ikzf1+I-* (FIG. 29B) and IkDN (FIG. 29C) was measured at the indicated effector: target cell ratios. IFNy production (FIG. 29D) and cytolysis (FIG. 29E) of WT and *Ikzf1+I-* transduced with FAP-CAR was measured at the indicated effectortarget cell ratios, where the target cells were FAP-expressing 3T3 cells.

[0087] FIG. 3OA, 3OB, and 30C shows the efficacy of CAR T cells with depletion of Ikaros against established tumors *in vivo.* CAR T cells were administered to mice bearing established mesothelin-expressing AE17 tumors. Tumor volume was measured after administration with mesoCAR-expressing WT and *Ikzfl+I-* (FIG. 30A) or IkDN (FIG. 30B). Tumor volume was measured after administration of FAP-CAR-expressing WT and *Ikzfl+I-* (FIG. 30C).

[0088] FIG. 31A, 3IB, 31C, 3ID, 3IE, and 3IF shows the increased persístence and resistance of *Ikzfl+I-* CAR T cells in the immunosuppressive tumor microenvironment compared to WT CAR T cells. The percentage of CAR-expressing WT or *Ikzfl+I-* cells (GFP positive) were detected by flow cytometry from harvested from the spleen (FIG. 31A) and the tumors (FIG. 3IB). The functional capacity of the CAR T cells harvested 3 days after infusion from the spleen or tumors was assessed by measuring IFNy production after stimulation with CD3/CD28 antibodies (FIG. 31C) or PMA/Ionomycin (PMA/I) (FIG. 3ID). Regulatory T cells (CD4+FoxP3+ expression) and macrophages (CD206 expression) were assessed by measuring the expression of Treg or macrophage markers on CAR T cells harvested 9 days after infusion from the spleen or tumors.

[0089] FIG. 32A and 32B shows that T cells with reduced Ikaros levels are less sensitive to soluble inhibitory factors TGFP and adenosine. MesoCAR-expressing WT, *Ikzfl+I-*, and IkDN cells were tested for their ability to produce IFNy (FIG. 32A) and cytotoxicity (FIG. 32B) in response to TGF-β or adenosine.

[0090] FIG. 33A and 33B are graphs showing an increase in titers to influenza vaccine strains as compared to placebo. In FIG. 33A, the increase above baseline in influenza geometric mean titers to each of the 3 influenza vaccine strains (H1N1 A/California/ 07/2009, H3N2 A/Victoria/21 0/2009, B/Brisbane/60/ 2008) relative to the increase in the placebo cohort 4 weeks after vaccination is shown for each of the RADOOl dosing cohorts in the intention to treat population. The bold black line indicates the 1.2 fold increase in titers relative to placebo that is required to be met for 2 out of 3 influenza vaccine strains to meet the primary endpoint of the study. The star "*" indicates that the increase in GMT titer relative to placebo exceeds 1 with posterior probability of at least 80%. FIG 33B is a graph of the same data as in FIG. 33A for the subset of subjects with baseline influenza titers <= 1:40.

[0091] FIG. 34 shows a scatter plot of RADOOl concentration versus fold increase in geometric mean titer to each influenza vaccine strain 4 weeks after vaccination. RADOOl

concentrations (1 hour post dose) were measured after subjects had been dosed for 4 weeks. All subjects who had pharmacokinetic measurements were included in the analysis set. The fold increase in geometric mean titers at 4 weeks post vaccination relative to baseline is shown on the y axis.

[0092] FIG. 35 is a graphic representation showing increase in titers to heterologous influenza strains as compared to placebo. The increase above baseline in influenza geometric mean titers to 2 heterologous influenza strains (A/HINI strain A/New Jersey/8/76 and A/H3N2 strain A/Victoria/361/1 1) not contained in the influenza vaccine relative to the increase in the placebo cohort 4 weeks after vaccination is shown for each of the RADOOl dosing cohorts in the intention to treat population. * indicates increase in titer relative to placebo exceeds 1 with a posterior probability of at least 80%.

[0093] FIG. 36A and 36B are graphic representations of IgG and IgM levels before and after influenza vaccination. Levels of anti-A/HlNl/California/07/2009 influenza IgG and IgM were measured in serum obtained from subjects before and 4 weeks post influenza vaccination. No significant difference in the change from baseline to 4 weeks post vaccination in anti-HlNl influenza IgG and IgM levels were detected between the RADOOl and placebo cohorts (all p values > 0.05 by Kruskal-Wallis rank sum test).

[0094] FIG. 37A, 37B, and 37C are graphic representations of the decrease in percent of PD-1-positive CD4 and CD8 and increase in PD-l-negative CD4 T cells after RADOOl treatment. The percent of PD-1 -positive CD4, CD8 and PD-l-negative CD4 T cells was determined by FACS analysis of PBMC samples at baseline, after 6 weeks of study drug treatment (Week 6) and 6 weeks after study drug discontinuation and 4 weeks after influenza vaccination (Week 12). FIG. 37A shows there was a significant decrease (-37.1 - -28.5%) in PD-l-positive CD4 T cells at week 12 in cohorts receiving RADOOl at dose levels 0.5mg/Day (n=25), 5mg/Week (n=29) and 20 mg/Week (n=30) as compared to the placebo cohort (n=25) with p=0.002 (0.02), p=0.003 (q=0.03), and p= 0.01 (q=0.05) respectively. FIG. 37B shows there was a significant decrease (-43.3 - -38.5%) in PD-l-positive CD8 T cells at week 12 in cohorts receiving RADOOl (n=109) at dose levels 0.5mg/Day (n=25), 5mg/Week (n=29) and 20 mg/Week (n=30) as compared to the placebo cohort (n=25) with p=0.01 (0.05), p=0.007 (q=0.04), and p= 0.01 (q=0.05) respectively. FIG. 37C shows was a significant increase (3.0 - 4.9%) in PD-l-negative CD4 T cells at week 12 in cohorts receiving RADOOl (n=109) at dose levels 0.5mg/Day (n=25), 5mg/Week (n=29)

and 20 mg/Week (n=30) as compared to the placebo cohort (n=25) with p=0.0007 (0.02), p=0.03 (q=0.07), and p= 0.03 (q=0.08) respectively.

[0095] FIG. 38A and 38B are graphic representations of the decrease in percent of PD-1-positive CD4 and CD8 and increase in PD-1-negative CD4 T cells after RADOOl treatment adjusted for differences in baseline PD-1 expression. The percent of PD-1-positive CD4, CD8 and PD-1-negative CD4 T cells was determined by FACS analysis of PBMC samples at baseline, after 6 weeks of study drug treatment (Week 6) and 6 weeks after study drug discontinuation and 4 weeks after influenza vaccination (Week 12). FIG. 38A shows a significant decrease of 30.2% in PD-1+ CD4 T cells at week 6 in the pooled RAD cohort (n=84) compared to placebo cohort (n=25) with p=0.03 (q=0. 13). The decrease in PD- 1-positive CD4 T cells at week 12 in the pooled RAD as compared to the placebo cohort is 32.7% with p=0.05 (q=0.19). FIG. 38B shows a significant decrease of 37.4% in PD-1-positive CD8 T cells at week 6 in the pooled RADOOl cohort (n=84) compared to placebo cohort (n=25) with p=0.008 (q=0.07). The decrease in PD-1-positive CD8 T cells at week 12 in the pooled RADOOl as compared to the placebo cohort is 41.4% with p=0.066 (q=0.21). FIG. 38A and 38B represent the data in FIG. 37A, 37B, and 37C but with the different RADOOl dosage groups of FIG. 37A, 37B, and 37C pooled into the single RADOOl-treated group in FIG. 38A and 38B.

[0096] FIG. 39 depicts increases in exercise and energy in elderly subjects in response to RADOOl.

[0097] FIG. 40A and 40B depict the predicted effect of RADOOl on P70 S6K activity in cells. FIG. 40A depicts P70 S6 kinase inhibition with higher doses of weekly and daily RADOOl; FIG. 40B depicts P70 S6 kinase inhibition with lower doses of weekly RADOOl.

[0098] FIG. 41A, 41B, and 41C are Biacore T200 SPR sensograms for the scFvs SSI (FIG. 41A), M5 (FIG. 41B), and Mil (FIG. 41C).

[0099] FIG. 42A, 42B, and 42C are epitope binning SPR sensograms for the anti-human mesothelin scFvs in comparison to the murine SSI scFv. Competitive binding was observed for scFvs M12, M14, Ml6, Ml7, M21, and M23 (FIG. 42A). ScFv M5 (FIG. 42B) and Ml1 (FIG. 42C) bind to a different epitope than SSI.

[00100] FIG. 43 is a graph depicting tumor growth after various mesothelin CAR T treatments in the OVCAR8 tumor model. Mean tumor volume +/- SEM to day 62 post tumor implantation. T cells were administered on days 14 and 19. Small circles: mice treated with lOOul of PBS via

the lateral tail vein; black squares: mice treated with Isotype control T cells; gray triangles: mice treated with one dose of SSI CAR T cells; inverted triangles: mice treated with a double dose of SSI CAR T cells; diamonds: mice treated with a single dose of M5 CAR T cells; large circles: mice treated with a double dose of M5 CAR T cells; gray squares: mice treated with a single dose of M11 CAR T cells; and black triangles: mice treated with a double dose of M11 CAR T cells.

[00101] FIG. 44 is a schematic representation of the human mesothelin peptide coverage in hydrogen deuterium exchange mass spectrometry analysis. Each black bar represents a peptide.

[00102] FIG. 45A and 45B are graphic representations showing the difference in deuterium uptake of human mesothelin when in complex with SSI (black bars) and M5 (grey bars). The difference in deuterium uptake upon antibody binding (represented on the y-axis) is depicted for each peptide fragment detected (represented on the x-axis), with peptides at amino acids 297-464 in FIG. 45A and peptides at amino acids 458-586 in FIG. 45B. All differences are relative to the deuterium uptake of unbound mesothelin (control). * denote regions of statistical significance using the Tukey test for peptides with a difference less than 0.75 Da.

[00103] FIG. 46 is a schematic representation showing the primary sequence of antigen human mesothelin (amino acids 296-588) and the regions protected by SSI and M5. The black bars designate the amino acids protected when complexed with SSI (amino acids 314-315, 317-318, 346-349, and 369-375). The grey bars designate the amino acids protected when complexed with M5 (amino acids 485-490, 498-507, 532-537, and 545-572). FIG. 47 shows a generic map showing different configurations of constructs encoding a CAR with a shRNA for coexpression of the CAR and an shRNA. Fig. 47A-47D show the various configurations on a single vector, e.g., where the U6 regulated shRNA is upstream or downstream of the EF1 alpha regulated CAR encoding elements. In the exemplary constructs depicted in Fig. 47A and 47B, the transcription occurs through the U6 and EF1 alpha promoters in the same direction. In the exemplary constructs depicted in Fig. 47C and 47D, the transcription occurs through the U6 and EF1 alpha promoters in different directions. In Figure 47E, the shRNA (and corresponding U6 promoter) is on a first vector, and the CAR (and corresponding EF1 alpha promoter) is on a second vector (Fig. 16E).

[00104] FIG. 48 depicts the structures of two exemplary RCAR configurations. The antigen binding members comprise an antigen binding domain, a transmembrane domain, and a switch domain. The intracellular binding members comprise a switch domain, a co-stimulatory signaling domain and a primary signaling domain. The two configurations demonstrate that the first and second switch domains described herein can be in different orientations with respect to the antigen binding member and the intracellular binding member. Other RCAR configurations are further described herein.

DETAILED DESCRIPTION

Definitions

[00105] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

[00106] The term "a" and "an" refers to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

[00107] The term "about" when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or in some instances ±10%, or in some instances ±5%, or in some instances ±1%, or in some instances ±0. 1% from the specified value, as such variations are appropriate to perform the disclosed methods.

[00108] The term "Chimeric Antigen Receptor" or alternatively a "CAR" refers to a set of polypeptides, typically two in the simplest embodiments, which when in an immune effector cell, provides the cell with specificity for a target cell, typically a cancer cell, and with intracellular signal generation. In some embodiments, a CAR comprises at least an extracellular antigen binding domain, a transmembrane domain and a cytoplasmic signaling domain (also referred to herein as "an intracellular signaling domain") comprising a functional signaling domain derived from a stimulatory molecule and/or costimulatory molecule as defined below. In some aspects, the set of polypeptides are contiguous with eachother. In some embodiments, the set of polypeptides include a dimerization switch that, upon the presence of a dimerization molecule,

can couple the polypeptides to one another, e.g., can couple an antigen binding domain to an intracellular signaling domain. In one aspect, the stimulatory molecule is the zeta chain associated with the T cell receptor complex. In one aspect, the cytoplasmic signaling domain further comprises one or more functional signaling domains derived from at least one costimulatory molecule as defined below. In one aspect, the costimulatory molecule is chosen from the costimulatory molecules described herein, e.g., 4-1BB (i.e., CD137), CD27 and/or CD28. In one aspect, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain derived from a stimulatory molecule. In one aspect, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain derived from a costimulatory molecule and a functional signaling domain derived from a stimulatory molecule. In one aspect, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising two functional signaling domains derived from one or more costimulatory molecule(s) and a functional signaling domain derived from a stimulatory molecule. In one aspect, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising at least two functional signaling domains derived from one or more costimulatory molecule(s) and a functional signaling domain derived from a stimulatory molecule. In one aspect the CAR comprises an optional leader sequence at the amino-terminus (N-ter) of the CAR fusion protein. In one aspect, the CAR further comprises a leader sequence at the N-terminus of the extracellular antigen binding domain, wherein the leader sequence is optionally cleaved from the antigen binding domain (e.g., a scFv) during cellular processing and localization of the CAR to the cellular membrane.

[00109] The term "signaling domain" refers to the functional portion of a protein which acts by transmitting information within the cell to regulate cellular activity via defined signaling pathways by generating second messengers or functioning as effectors by responding to such messengers.

[001 10] As used herein, the term "mesothelin" refers to the 40-kDa protein, mesothelin, which is anchored at the cell membrane by a glycosylphosphatidyl inositol (GPI) linkage and an amino-

terminal 31-kDa shed fragment, called megkaryocyte potentiating factor (MPF). Both fragments contain N-glycosylation sites. The term also refers to a soluble splice variant of the 40-kDa carboxyl-terminal fragment also called "soluble mesothelin/MPF-related". Preferably, the term refers to a human mesothelin of GenBank accession number AAH03512.1, and naturally cleaved portions thereof, e.g., as expressed on a cell membrane, e.g., a cancer cell membrane.

[001 11] The term "antibody" as used herein, refers to a protein, or polypeptide sequence derived from an immunoglobulin molecule which specifically binds with an antigen. Antibodies can be polyclonal or monoclonal, multiple or single chain, or intact immunoglobulins, and may be derived from natural sources or from recombinant sources. Antibodies can be tetramers of immunoglobulin molecules.

[001 12] The term "antibody fragment" refers to at least one portion of an antibody, that retains the ability to specifically interact with (e.g., by binding, steric hinderance, stabilizing/destabilizing, spatial distribution) an epitope of an antigen. Examples of antibody fragments include, but are not limited to, Fab, Fab', $F(ab')_2$, Fv fragments, scFv antibody fragments, disulfide-linked Fvs (sdFv), a Fd fragment consisting of the VH and CHI domains, linear antibodies, single domain antibodies such as sdAb (either VL or VH), camelid VHH domains, multi-specific antibodies formed from antibody fragments such as a bivalent fragment comprising two Fab fragments linked by a disulfide brudge at the hinge region, and an isolated CDR or other epitope binding fragments of an antibody. An antigen binding fragment can also be incorporated into single domain antibodies, maxibodies, minibodies, nanobodies, intrabodies, diabodies, triabodies, tetrabodies, v-NAR and bis-scFv (see, e.g., Hollinger and Hudson, Nature Biotechnology 23:1126-1136, 2005). Antigen binding fragments can also be grafted into scaffolds based on polypeptides such as a fibronectin type III (Fn3)(see U.S. Patent No.: 6,703,199, which describes fibronectin polypeptide minibodies).

[001 13] The term "scFv" refers to a fusion protein comprising at least one antibody fragment comprising a variable region of a light chain and at least one antibody fragment comprising a variable region of a heavy chain, wherein the light and heavy chain variable regions are contiguously linked, e.g., via a synthetic linker, e.g., a short flexible polypeptide linker, and capable of being expressed as a single chain polypeptide, and wherein the scFv retains the specificity of the intact antibody from which it is derived. Unless specified, as used herein an

scFv may have the VL and VH variable regions in either order, e.g., with respect to the N-terminal and C-terminal ends of the polypeptide, the scFv may comprise VL-linker-VH or may comprise VH-linker-VL.

[001 14] The portion of the CAR of the invention comprising an antibody or antibody fragment thereof may exist in a variety of forms where the antigen binding domain is expressed as part of a contiguous polypeptide chain including, for example, a single domain antibody fragment (sdAb), a single chain antibody (scFv) a humanized antibody or bispecific antibody (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426). In one aspect, the antigen binding domain of a CAR composition of the invention comprises an antibody fragment. In a further aspect, the CAR comprises an antibody fragment that comprises a scFv.

[001 15] The term "antibody heavy chain" refers to the larger of the two types of polypeptide chains present in antibody molecules in their naturally occurring conformations, and which normally determines the class to which the antibody belongs.

[001 16] The term "antibody light chain" refers to the smaller of the two types of polypeptide chains present in antibody molecules in their naturally occurring conformations. Kappa (κ) and lambda (λ) light chains refer to the two major antibody light chain isotypes.

[001 17] The term "recombinant antibody" refers to an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage or yeast expression system. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using recombinant DNA or amino acid sequence technology which is available and well known in the art.

[001 18] The term "antigen" or "Ag" refers to a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. Furthermore,

antigens can be derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an "antigen" as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene. It is readily apparent that the present invention includes, but is not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are arranged in various combinations to encode polypeptides that elicit the desired immune response. Moreover, a skilled artisan will understand that an antigen need not be encoded by a "gene" at all. It is readily apparent that an antigen can be generated synthesized or can be derived from a biological sample, or might be macromolecule besides a polypeptide. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a fluid with other biological components.

[001 19] The term "compete" refers to the ability of an antigen binding domain, e.g., an antibody or fragment thereof, to interfere with binding directly or indirectly of another antigen binding domain, e.g., an antigen binding domain provided herein, e.g., an antibody or fragment thereof provided herein, to the target, e.g., mesothelin. The extent to which an antigen binding domain, e.g., an antibody or fragment thereof, is able to interfere with the binding of another antigen binding domain, e.g., an antibody or fragment thereof, to the target, and therefore whether it can be said to compete, can be determined using a competition binding assay. In some embodiments, a competition binding assay is a quantitative competition assay. For example, one particularly suitable quantitative competition assay uses a surface plasmon resonance (SPR)-based approach to measure binding, e.g., competition, between one antibody or fragment thereof and another antibody or fragment thereof for binding to an immobilized target. An exemplary SPR-based competition assay is described in Example 2 herein. Another suitable quantitative competition assay uses a FACS-based approach to measure competition between a labelled (e.g., His tagged, biotinylated or radioactively labeled, among others) antibody or fragment thereof and another antibody or fragment thereof for binding to the target.

[00120] The term "anti-cancer effect" refers to a biological effect which can be manifested by various means, including but not limited to, e.g., a decrease in tumor volume, a decrease in the number of cancer cells, a decrease in the number of metastases, an increase in life expectancy, decrease in cancer cell proliferation, decrease in cancer cell survival, or amelioration of various

physiological symptoms associated with the cancerous condition. An "anti-cancer effect" can also be manifested by the ability of the peptides, polynucleotides, cells and antibodies in prevention of the occurrence of cancer in the first place. The term "anti-tumor effect" refers to a biological effect which can be manifested by various means, including but not limited to, e.g., a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in tumor cell proliferation, or a decrease in tumor cell survival.

[00121] The term "autologous" refers to any material derived from the same individual to whom it is later to be re-introduced into the individual.

[00122] The term "allogeneic" refers to any material derived from a different animal of the same species as the individual to whom the material is introduced. Two or more individuals are said to be allogeneic to one another when the genes at one or more loci are not identical. In some aspects, allogeneic material from individuals of the same species may be sufficiently unlike genetically to interact antigenically.

[00123] The term "xenogeneic" refers to a graft derived from an animal of a different species.

[00124] The term "cancer" refers to a disease characterized by the uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers are described herein and include, but are not limited to, mesothelioma, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer and the like.

[00125] The phrase "disease associated with expression of mesothelin" includes, but is not limited to, a disease associated with expression of mesothelin or condition associated with cells which express mesothelin including, e.g., proliferative diseases such as a cancer or malignancy or a precancerous condition such as a mesothelial hyperplasia; or a noncancer related indication associated with cells which express mesothelin. Examples of various cancers that express mesothelin include but are not limited to, mesothelioma, lung cancer, ovarian cancer, pancreatic cancer, and the like.

[00126] The term "conservative sequence modifications" refers to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody or antibody

fragment containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody or antibody fragment of the invention by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues within a CAR of the invention can be replaced with other amino acid residues from the same side chain family and the altered CAR can be tested, e.g., for the ability to bind mesothelin using the functional assays described herein.

[00127] The term "stimulation," refers to a primary response induced by binding of a stimulatory molecule (e.g., a TCR/CD3 complex or CAR) with its cognate ligand (or tumor antigen in the case of a CAR) thereby mediating a signal transduction event, such as, but not limited to, signal transduction via the TCR/CD3 complex or signal transduction via the appropriate NK receptor or signaling domains of the CAR. Stimulation can mediate altered expression of certain molecules.

[00128] The term "stimulatory molecule," refers to a molecule expressed by an immune cell (e.g., T cell, NK cell, B cell) that provides the cytoplasmic signaling sequence(s) that regulate activation of the immune cell in a stimulatory way for at least some aspect of the immune cell signaling pathway. In one aspect, the signal is a primary signal that is initiated by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, and which leads to mediation of a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A primary cytoplasmic signaling sequence (also referred to as a "primary signaling domain") that acts in a stimulatory manner may contain a signaling motif which is known as immunoreceptor tyrosine-based activation motif or ITAM. Examples of an ITAM containing cytoplasmic signaling sequence that is of particular use in the invention includes, but is not limited to, those derived from CD3 zeta, common FcR gamma (FCER1 G),

Fc gamma RIIa,, FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta , CD3 epsilon, , CD79a, CD79b, DAP10, and DAP12. In a specific CAR of the invention, the intracellular signaling domain in any one or more CARS of the invention comprises an intracellular signaling sequence, e.g., a primary signaling sequence of CD3-zeta. In a specific CAR of the invention, the primary signaling sequence of CD3-zeta is the sequence provided as SEQ ID NO:9, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like. In a specific CAR of the invention, the primary signaling sequence of CD3-zeta is the sequence as provided in SEQ ID NO: 10, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like.

[00129] The term "antigen presenting cell" or "APC" refers to an immune system cell such as an accessory cell (e.g., a B-cell, a dendritic cell, and the like) that displays a foreign antigen complexed with major histocompatibility complexes (MHC's) on its surface. T-cells may recognize these complexes using their T-cell receptors (TCRs). APCs process antigens and present them to T-cells.

[00130] An "intracellular signaling domain," as the term is used herein, refers to an intracellular portion of a molecule. The intracellular signaling domain generates a signal that promotes an immune effector function of the CAR containing cell, e.g., a CART cell. Examples of immune effector function, e.g., in a CART cell, include cytolytic activity and helper activity, including the secretion of cytokines.

[0013 1] In an embodiment, the intracellular signaling domain can comprise a primary intracellular signaling domain. Exemplary primary intracellular signaling domains include those derived from the molecules responsible for primary stimulation, or antigen dependent simulation. In an embodiment, the intracellular signaling domain can comprise a costimulatory intracellular domain. Exemplary costimulatory intracellular signaling domains include those derived from molecules responsible for costimulatory signals, or antigen independent stimulation. For example, in the case of a CART, a primary intracellular signaling domain can comprise a cytoplasmic sequence of a T cell receptor, and a costimulatory intracellular signaling domain can comprise cytoplasmic sequence from co-receptor or costimulatory molecule.

[00132] A primary intracellular signaling domain can comprise a signaling motif which is known as an immunoreceptor tyrosine-based activation motif or ITAM. Examples of ITAM

containing primary cytoplasmic signaling sequences include, but are not limited to, those derived from CD3 zeta, common FcR gamma (FCER1G), Fc gamma RIIa,, FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta , CD3 epsilon, , CD79a, CD79b, DAP10, and DAP12.

[00133] The term "zeta" or alternatively "zeta chain", "CD3-zeta" or "TCR-zeta" is defined as the protein provided as GenBan Acc. No. BAG36664.1, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like, and a "zeta stimulatory domain" or alternatively a "CD3-zeta stimulatory domain" or a "TCR-zeta stimulatory domain" is defined as the amino acid residues from the cytoplasmic domain of the zeta chain, or functional derivatives thereof, that are sufficient to functionally transmit an initial signal necessary for T cell activation. In one aspect the cytoplasmic domain of zeta comprises residues 52 through 164 of GenBank Acc. No. BAG36664.1 or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like, that are functional orthologs thereof. In one aspect, the "zeta stimulatory domain" or a "CD3-zeta stimulatory domain" is the sequence provided as SEQ ID NO: 9. In one aspect, the "zeta stimulatory domain" or a "CD3-zeta stimulatory domain" is the sequence provided as SEQ ID NO: 10.

[00134] The term a "costimulatory molecule" refers to a cognate binding partner on a T cell that specifically binds with a costimulatory ligand, thereby mediating a costimulatory response by the T cell, such as, but not limited to, proliferation. Costimulatory molecules are cell surface molecules other than antigen receptors or their ligands that are contribute to an efficient immune response. Costimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and a Toll ligand receptor, as well as OX40, CD27, CD28, CDS, ICAM-1, LFA-1 (CDlla/CD18), ICOS (CD278), and 4-1BB (CD137). Further examples of such costimulatory molecules include CDS, ICAM-1, GITR, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CDlld, ITGAE, CD103, ITGAL, CDl la, LFA-1, ITGAM, CDllb, ITGAX, CDl lc, ITGB1, CD29, ITGB2, CD 18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAMl (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, and a ligand that specifically binds with CD83

[00135] A costimulatory intracellular signaling domain can be an intracellular portion of a costimulatory molecule. A costimulatory molecule can be represented in the following protein families: TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SLAM proteins), and activating NK cell receptors. Examples of such molecules include CD27, CD28, 4-lBB (CD137), OX40, GITR, CD30, CD40, ICOS, BAFFR, HVEM, ICAM-1, lymphocyte function-associated antigen-1 (LFA-1), CD2, CDS, CD7, CD287, LIGHT, NKG2C, SLAMF7, NKp80, CD160, B7-H3, and a ligand that specifically binds with CD83, and the like.

[00136] The intracellular signaling domain can comprise the entire intracellular portion, or the entire native intracellular signaling domain, of the molecule from which it is derived, or a functional fragment or derivative thereof.

[00137] The term "4-IBB" refers to a member of the TNFR superfamily with an amino acid sequence provided as GenBank Acc. No. AAA62478.2, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like. In one aspect a "4-IBB costimulatory domain" is defined as amino acid residues 214-255 of GenBank Acc. No.. AAA62478.2, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like. In one aspect, the "4-IBB costimulatory domain" is the sequence provided as SEQ ID NO: 7 or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like.

[00138] An "antigen presenting cell," as used herein, means an immune system cell such as an accessory cell (e.g., a B-cell, a dendritic cell, and the like) that displays foreign antigens complexed with major histocompatibility complexes (MHC's) on their surfaces. T-cells may recognize these complexes using their T-cell receptors (TCRs). APCs process antigens and present them to T-cells.

[00139] 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, cDNA, or RNA, 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.

[00140] 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. The phrase nucleotide sequence that encodes a protein or a RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

[00141] The term "effective amount" or "therapeutically effective amount" is used interchangeably herein, and refer to an amount of a compound, formulation, material, or composition, as described herein effective to achieve a particular biological result. The term "endogenous" refers to any material from or produced inside an organism, cell, tissue or system.

[00142] The term "exogenous" refers to any material introduced from or produced outside an organism, cell, tissue or system.

[00143] The term "expression" refers to the transcription and/or translation of a particular nucleotide sequence driven by its promoter.

[00144] The term "transfer vector" refers to a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "transfer vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to further include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, a polylysine compound, liposome, and the like. Examples of viral transfer vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, and the like.

[00145] The term "expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide

sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, including cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

[00146] The term "lentivirus" refers to a genus of the Retroviridae family. Lentiviruses are unique among the retroviruses in being able to infect non-dividing cells; they can deliver a significant amount of genetic information into the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. HIV, SIV, and FIV are all examples of lentiviruses. The term "lentiviral vector" refers to a vector derived from at least a portion of a lentivirus genome, including especially a self-inactivating lentiviral vector as provided in Milone et al., Mol. Ther. 17(8): 1453-1464 (2009). Other examples of lentivirus vectors that may be used in the clinic include but are not limited to, e.g., the LENTrVECTOR® gene delivery technology from Oxford BioMedica, the LENTIMAX™ vector system from Lentigen and the like. Nonclinical types of lentiviral vectors are also available and would be known to one skilled in the art.

[00147] The term "homologous" or "identity" refers to the subunit sequence identity between two polymeric molecules, e.g., between two nucleic acid molecules, such as, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit; e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous or identical at that position. The homology between two sequences is a direct function of the number of matching or homologous positions; e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two sequences are homologous, the two sequences are 50% homologous; if 90% of the positions (e.g., 9 of 10), are matched or homologous, the two sequences are 90% homologous.

[00148] The term "humanized" refers to those forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies

and antibody fragments thereof are human immunoglobulins (recipient antibody or antibody fragment) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, a humanized antibody/antibody fragment can comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications can further refine and optimize antibody or antibody fragment performance. In general, the humanized antibody or antibody fragment thereof will comprise a significant portion of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody or antibody fragment can also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al, Nature, 321: 522-525, 1986; Reichmann et al., Nature, 332: 323-329, 1988; Presta, Curr. Op. Struct. Biol, 2: 593-596, 1992.

[00149] The term "fully human" refers to an immunoglobulin, such as an antibody or antibody fragment, where the whole molecule is of human origin or consists of an amino acid sequence identical to a human form of the antibody or immunoglobulin.

[00150] The term "isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

[00151] In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

[00152] The term "operably linked" or "transcriptional control" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic

acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Operably linked DNA sequences can be contiguous with each other and, where necessary to join two protein coding regions, are in the same reading frame.

[00153] The term "parenteral" administration of an immunogenic composition includes, e.g., subcutaneous (s.c), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, intratumoral, or infusion techniques.

[00154] The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolim et al, Mol. Cell. Probes 8:91-98 (1994)).

[00155] The terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs,

fusion proteins, among others. A polypeptide includes a natural peptide, a recombinant peptide, a recombinant peptide, or a combination thereof.

[001 56] The term "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

[00157] The term "promoter/regulatory sequence" refers to a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

[00158] The term "constitutive" promoter refers to a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

[00159] The term "inducible" promoter refers to a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

[00160] The term "tissue-specific" promoter refers to a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

[00161] The term "flexible polypeptide linker" as used in the context of a scFv refers to a peptide linker that consists of amino acids such as glycine and/or serine residues used alone or in combination, to link variable heavy and variable light chain regions together. In one embodiment, the flexible polypeptide linker is a Gly/Ser linker and comprises the amino acid sequence $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_n$ (SEQ ID NO: 38), where n is a positive integer equal to or greater than 1. For example, n=l, n=2, n=3. n=4, n=5 and n=6, n=7, n=8, n=9 and n=10. In one embodiment, the flexible polypeptide linkers include, but are not limited to, $(Gly_4\ Ser)_4$ (SEQ ID NO: 27)or $(Gly_4$

$Ser)_3$ (SEQ ID NO: 28)In another embodiment, the linkers include multiple repeats of (Gly_2Ser), (GlySer) or (Gly_3Ser) (SEQ ID NO: 29). Also included within the scope of the invention are linkers described in WO2012/138475, incorporated herein by reference).

[00162] As used herein, a 5' cap (also termed an RNA cap, an RNA 7-methylguanosine cap or an RNA m^7G cap) is a modified guanine nucleotide that has been added to the "front" or 5' end of a eukaryotic messenger RNA shortly after the start of transcription. The 5' cap consists of a terminal group which is linked to the first transcribed nucleotide. Its presence is critical for recognition by the ribosome and protection from RNases. Cap addition is coupled to transcription, and occurs co-transcriptionally, such that each influences the other. Shortly after the start of transcription, the 5' end of the mRNA being synthesized is bound by a cap-synthesizing complex associated with RNA polymerase. This enzymatic complex catalyzes the chemical reactions that are required for mRNA capping. Synthesis proceeds as a multi-step biochemical reaction. The capping moiety can be modified to modulate functionality of mRNA such as its stability or efficiency of translation.

[00163] As used herein, "in vitro transcribed RNA" refers to RNA, preferably mRNA, that has been synthesized in vitro. Generally, the in vitro transcribed RNA is generated from an in vitro transcription vector. The in vitro transcription vector comprises a template that is used to generate the in vitro transcribed RNA.

[00164] As used herein, a "poly(A)" is a series of adenosines attached by polyadenylation to the mRNA. In the preferred embodiment of a construct for transient expression, the polyA is between 50 and 5000 (SEQ ID NO: 30), preferably greater than 64, more preferably greater than 100, most preferably greater than 300 or 400. poly(A) sequences can be modified chemically or enzymatically to modulate mRNA functionality such as localization, stability or efficiency of translation.

[00165] As used herein, "polyadenylation" refers to the covalent linkage of a polyadenylyl moiety, or its modified variant, to a messenger RNA molecule. In eukaryotic organisms, most messenger RNA (mRNA) molecules are polyadenylated at the 3' end. The 3' poly(A) tail is a long sequence of adenine nucleotides (often several hundred) added to the pre-mRNA through the action of an enzyme, polyadenylate polymerase. In higher eukaryotes, the poly(A) tail is added onto transcripts that contain a specific sequence, the polyadenylation signal. The poly(A)

tail and the protein bound to it aid in protecting mRNA from degradation by exonucleases. Polyadenylation is also important for transcription termination, export of the mRNA from the nucleus, and translation. Polyadenylation occurs in the nucleus immediately after transcription of DNA into RNA, but additionally can also occur later in the cytoplasm. After transcription has been terminated, the mRNA chain is cleaved through the action of an endonuclease complex associated with RNA polymerase. The cleavage site is usually characterized by the presence of the base sequence AAUAAA near the cleavage site. After the mRNA has been cleaved, adenosine residues are added to the free 3' end at the cleavage site.

[00166] As used herein, "transient" refers to expression of a non-integrated transgene for a period of hours, days or weeks, wherein the period of time of expression is less than the period of time for expression of the gene if integrated into the genome or contained within a stable plasmid replicon in the host cell.

[00167] As used herein, the terms "treat", "treatment" and "treating" refer to the reduction or amelioration of the progression, severity and/or duration of a proliferative disorder, or the amelioration of one or more symptoms (preferably, one or more discernible symptoms) of a proliferative disorder resulting from the administration of one or more therapies (e.g., one or more therapeutic agents such as a CAR of the invention). In specific embodiments, the terms "treat", "treatment" and "treating" refer to the amelioration of at least one measurable physical parameter of a proliferative disorder, such as growth of a tumor, not necessarily discernible by the patient. In other embodiments the terms "treat", "treatment" and "treating" -refer to the inhibition of the progression of a proliferative disorder, either physically by, e.g., stabilization of a discernible symptom, physiologically by, e.g., stabilization of a physical parameter, or both. In other embodiments the terms "treat", "treatment" and "treating" refer to the reduction or stabilization of tumor size or cancerous cell count.

[00168] The term "signal transduction pathway" refers to the biochemical relationship between a variety of signal transduction molecules that play a role in the transmission of a signal from one portion of a cell to another portion of a cell. The phrase "cell surface receptor" includes molecules and complexes of molecules capable of receiving a signal and transmitting signal across the membrane of a cell.

[00169] The term "subject" is intended to include living organisms in which an immune response can be elicited (e.g., mammals, human).

[00170] The term a "substantially purified" cell refers to a cell that is essentially free of other cell types. A substantially purified cell also refers to a cell which has been separated from other cell types with which it is normally associated in its naturally occurring state. In some instances, a population of substantially purified cells refers to a homogenous population of cells. In other instances, this term refers simply to cell that have been separated from the cells with which they are naturally associated in their natural state. In some aspects, the cells are cultured in vitro. In other aspects, the cells are not cultured in vitro.

[00171] The term "therapeutic" as used herein means a treatment. A therapeutic effect is obtained by reduction, suppression, remission, or eradication of a disease state.

[00172] The term "prophylaxis" as used herein means the prevention of or protective treatment for a disease or disease state.

[00173] The terms "cancer associated antigen" or "tumor antigen" interchangeably refers to a molecule (typically a protein, carbohydrate or lipid) that is expressed on the surface of a cancer cell, either entirely or as a fragment (e.g., MHC/peptide), and which is useful for the preferential targeting of a pharmacological agent to the cancer cell. In some embodiments, a tumor antigen is a marker expressed by both normal cells and cancer cells, e.g., a lineage marker, e.g., CD19 on B cells. In some embodiments, a tumor antigen is a cell surface molecule that is overexpressed in a cancer cell in comparison to a normal cell, for instance, 1-fold over expression, 2-fold overexpression, 3-fold overexpression or more in comparison to a normal cell. In some enbodiments, a tumor antigen is a cell surface molecule that is inappropriately synthesized in the cancer cell, for instance, a molecule that contains deletions, additions or mutations in comparison to the molecule expressed on a normal cell. In some embodiments, a tumor antigen will be expressed exclusively on the cell surface of a cancer cell, entirely or as a fragment (e.g., MHC/peptide), and not synthesized or expressed on the surface of a normal cell. In some embodiments, the CARs of the present invention includes CARs comprising an antigen binding domain (e.g., antibody or antibody fragment) that binds to a MHC presented peptide. Normally, peptides derived from endogenous proteins fill the pockets of Major histocompatibility complex (MHC) class I molecules, and are recognized by T cell receptors (TCRs) on CD8 + T

lymphocytes. The MHC class I complexes are constitutively expressed by all nucleated cells. In cancer, virus-specific and/or tumor-specific peptide/MHC complexes represent a unique class of cell surface targets for immunotherapy. TCR-like antibodies targeting peptides derived from viral or tumor antigens in the context of human leukocyte antigen (HLA)-Al or HLA-A2 have been described (see, e.g., Sastry et al, J Virol. 2011 85(5):1935-1942; Sergeeva et al, Blood, 201 1 117(16):4262-4272; Verma et al, J Immunol 2010 184(4):2156-2165; Willemsen et al, Gene Ther 2001 8(21) :1601-1608 ; Dao et al, Sci Transl Med 2013 5(176) :176ra33 ; Tassev et al, Cancer Gene Ther 2012 19(2):84-100). For example, TCR-like antibody can be identified from screening a library, such as a human scFv phage displayed library.

[00174] The term "transfected" or "transformed" or "transduced" refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A "transfected" or "transformed" or "transduced" cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

[00175] The term "specifically binds," refers to an antibody, or a ligand, which recognizes and binds with a binding partner (e.g., tumor antigen) protein present in a sample, but which antibody or ligand does not substantially recognize or bind other molecules in the sample.

[00176] "Regulatable chimeric antigen receptor (RCAR),"as that term is used herein, refers to a set of polypeptides, typically two in the simplest embodiments, which when in a RCARX cell, provides the RCARX cell with specificity for a target cell, typically a cancer cell, and with regulatable intracellular signal generation or proliferation, which can optimize an immune effector property of the RCARX cell. An RCARX cell relies at least in part, on an antigen binding domain to provide specificity to a target cell that comprises the antigen bound by the antigen binding domain. In an embodiment, an RCAR includes a dimerization switch that, upon the presence of a dimerization molecule, can couple an intracellular signaling domain to the antigen binding domain.

[00177] "Membrane anchor" or "membrane tethering domain", as that term is used herein, refers to a polypeptide or moiety, e.g., a myristoyl group, sufficient to anchor an extracellular or intracellular domain to the plasma membrane.

[00178] "Switch domain," as that term is used herein, e.g., when referring to an RCAR, refers to an entity, typically a polypeptide-based entity, that, in the presence of a dimerization

molecule, associates with another switch domain. The association results in a functional coupling of a first entity linked to, e.g., fused to, a first switch domain, and a second entity linked to, e.g., fused to, a second switch domain. A first and second switch domain are collectively referred to as a dimerization switch. In embodiments, the first and second switch domains are the same as one another, e.g., they are polypeptides having the same primary amino acid sequence, and are referred to collectively as a homodimerization switch. In embodiments, the first and second switch domains are different from one another, e.g., they are polypeptides having different primary amino acid sequences, and are referred to collectively as a heterodimerization switch. In embodiments, the switch is intracellular. In embodiments, the switch is extracellular. In embodiments, the switch domain is a polypepti de-based entity, e.g., FKBP or FRB-based, and the dimerization molecule is small molecule, e.g., a rapalogue. In embodiments, the switch domain is a polypeptide-based entity, e.g., an scFv that binds a myc peptide, and the dimerization molecule is a polypeptide, a fragment thereof, or a multimer of a polypeptide, e.g., a myc ligand or multimers of a myc ligand that bind to one or more myc scFvs. In embodiments, the switch domain is a polypeptide-based entity, e.g., myc receptor, and the dimerization molecule is an antibody or fragments thereof, e.g., myc antibody.

[00179] "Dimerization molecule," as that term is used herein, e.g., when referring to an RCAR, refers to a molecule that promotes the association of a first switch domain with a second switch domain. In embodiments, the dimerization molecule does not naturally occur in the subject, or does not occur in concentrations that would result in significant dimerization. In embodiments, the dimerization molecule is a small molecule, e.g., rapamycin or a rapalogue, e.g, RAD001.

[001 80] The term "bioequivalent" refers to an amount of an agent other than the reference compound (e.g., RAD001), required to produce an effect equivalent to the effect produced by the reference dose or reference amount of the reference compound (e.g., RAD001). In an embodiment the effect is the level of mTOR inhibition, e.g., as measured by P70 S6 kinase inhibition, e.g., as evaluated in an in vivo or in vitro assay, e.g., as measured by an assay described herein, e.g., the Boulay assay, or measurement of phosphorylated S6 levels by western blot. In an embodiment, the effect is alteration of the ratio of PD-1 positive/PD-1 negative T

cells, as measured by cell sorting. In an embodiment a bioequivalent amount or dose of an mTOR inhibitor is the amount or dose that achieves the same level of P70 S6 kinase inhibition as does the reference dose or reference amount of a reference compound. In an embodiment, a bioequivalent amount or dose of an mTOR inhibitor is the amount or dose that achieves the same level of alteration in the ratio of PD-1 positive/PD-1 negative T cells as does the reference dose or reference amount of a reference compound.

[00181] The term 'low, immune enhancing, dose" when used in conjuction with an mTOR inhibitor, e.g., an allosteric mTOR inhibitor, e.g., RAD001 or rapamycin, or a catalytic mTOR inhibitor, refers to a dose of mTOR inhibitor that partially, but not fully, inhibits mTOR activity, e.g., as measured by the inhibition of P70 S6 kinase activity. Methods for evaluating mTOR activity, e.g., by inhibition of P70 S6 kinase, are discussed herein. The dose is insufficient to result in complete immune suppression but is sufficient to enhance the immune response. In an embodiment, the low, immune enhancing, dose of mTOR inhibitor results in a decrease in the number of PD-1 positive T cells and/or an increase in the number of PD-1 negative T cells, or an increase in the ratio of PD-1 negative T cells/PD-1 positive T cells. In an embodiment, the low, immune enhancing, dose of mTOR inhibitor results in an increase in the number of naive T cells. In an embodiment, the low, immune enhancing, dose of mTOR inhibitor results in one or more of the following:

an increase in the expression of one or more of the following markers: $CD62L^{hlgh}$, $CD127^{high}$, $CD27^{+}$, and BCL2, e.g., on memory T cells, e.g., memory T cell precursors;

a decrease in the expression of KLRG1, e.g., on memory T cells, e.g., memory T cell precursors; and

an increase in the number of memory T cell precursors, e.g., cells with any one or combination of the following characteristics: increased $CD62L^{hlgh}$, increased $CD127^{hlgh}$, increased $CD27^{+}$, decreased KLRG1, and increased BCL2;

wherein any of the changes described above occurs, e.g., at least transiently, e.g., as compared to a non-treated subject.

[00182] Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of

the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. As another example, a range such as 95-99% identity, includes something with 95%, 96%, 97%, 98%, or 99% identity, and includes subranges such as 96-99%, 96-98%, 96-97%, 97-99%, 97-98%, and 98-99% identity. This applies regardless of the breadth of the range.

Description

[00183] Provided herein are compositions of matter and methods of use for the treatment of a disease such as cancer using anti-mesothelin chimeric antigen receptors (CAR), e.g., human mesothelin CAR.

[00184] In one aspect, the invention provides a number of chimeric antigen receptors comprising an antibody or antibody fragment engineered for specific binding to a mesothelin protein. In one aspect, the invention provides a cell (e.g., T cell or NK cell) engineered to express a CAR, e.g., wherein the CAR T cell ("CART") exhibits an anticancer property. In one aspect a cell is transformed with the CAR and the CAR is expressed on the cell surface. In some embodiments, the cell (e.g., T cell or NK cell) is transduced with a viral vector encoding a CAR. In some embodiments, the viral vector is a retroviral vector. In some embodiments, the viral vector is a lentiviral vector. In some such embodiments, the cell may stably express the CAR. In another embodiment, the cell (e.g., T cell or NK cell) is transfected with a nucleic acid, e.g., mRNA, cDNA, DNA, encoding a CAR. In some such embodiments, the cell may transiently express the CAR.

[00185] In one aspect, the mesothelin protein binding portion of the CAR is a scFv antibody fragment. In one aspect such antibody fragments are functional in that they retain the equivalent binding affinity, i.e., they bind the same antigen with comparable affinity, as the IgG antibody from which it is derived. In one aspect such antibody fragments are functional in that they provide a biological response that can include, but is not limited to, activation of an immune response, inhibition of signal-transduction origination from its target antigen, inhibition of kinase

activity, and the like, as will be understood by a skilled artisan. In one aspect, the mesothelin antigen binding domain of the CAR is a scFv antibody fragment that is human or humanized compared to the murine sequence of the scFv from which it is derived. In one embodiment, the human anti-mesothelin scFv antibody fragment comprises a light chain variable region and/or a heavy chain variable region provided in Table 2, or a sequence with substantial identity thereto, e.g., 95-99% identity.

[00186] In some aspects, the antibodies of the invention are incorporated into a chimeric antigen receptor (CAR). In one aspect, the CAR comprises the polypeptide sequence provided herein as SEQ ID NO: 39; SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, and SEQ ID NO: 62, or a sequence with 95-99% identify thereof.

[001 87] In one aspect, the human scFv portion of a CAR is encoded by a transgene whose sequence has been codon optimized for expression in a mammalian cell. In one aspect, entire CAR construct of the invention is encoded by a transgene whose entire sequence has been codon optimized for expression in a mammalian cell. Codon optimization refers to the discovery that the frequency of occurrence of synonymous codons (i.e., codons that code for the same amino acid) in coding DNA is biased in different species. Such codon degeneracy allows an identical polypeptide to be encoded by a variety of nucleotide sequences. A variety of codon optimization methods is known in the art, and include, e.g., methods disclosed in at least US Patent Numbers 5,786,464 and 6,1 14,148.

[00188] In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 39. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 40. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 41. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 42. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 43. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 44. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ

ID NO: 45. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 46. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 47. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 48. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 49. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 50. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 51. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 52. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 53. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 54. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 55. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 56. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 57. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 58. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 59. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 60. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 61. In one aspect, the human mesothelin CAR molecule comprises the scFv portion provided in SEQ ID NO: 62.

[00189] In one aspect, the CAR disclosed herein combine an antigen binding domain of a specific antibody with an intracellular signaling molecule. For example, in some aspects, the intracellular signaling molecule includes, but is not limited to, CD3-zeta chain, 4-IBB and CD28 signaling modules and combinations thereof. In one aspect, the antigen binding domain binds to mesothelin. In one aspect, the -mesothelin CAR comprises the sequence provided in Table 2.

[00190] In one aspect, the mesothelin CAR comprises a CAR selected from the sequence provided in one or more of SEQ ID NOS: 63-86. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 63. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 64. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 65. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 66. In one aspect, themesothelin CAR comprises the

sequence provided in SEQ ID NO: 67. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 68. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 69. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 70. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 71. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 72. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 73. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 74. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 75. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 76. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 77. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 78. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 79. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 80. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 81. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 82. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 83. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 84. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 85. In one aspect, themesothelin CAR comprises the sequence provided in SEQ ID NO: 86.

[00191] Furthermore, the present invention provides mesothelin CAR compositions and their use in medicaments or methods for treating, among other diseases, cancer or any malignancy or autoimmune diseases involving cells or tissues which express mesothelin.

[00192] In one aspect, the invention provides a cell (e.g., T cell or NK cell) engineered to express a chimeric antigen receptor (CAR), wherein the CAR T cell ("CART") exhibits an antitumor property. A preferred antigen is mesothelin. In one aspect, the antigen binding domain of the CAR comprises a human anti-mesothelin antibody fragment. In one aspect, the antigen binding domain of the CAR comprises a human anti-mesothelin antibody fragment comprising an scFv. Accordingly, the invention provides an mesothelin CAR that comprises a human anti-mesothelin binding domain and is engineered into a T cell or NK cell and methods of their use for adoptive therapy.

[00193] In one aspect, mesothelin CAR comprises at least one intracellular signaling domain selected from the group consisting of a CD137 (4-IBB) signaling domain, a CD28 signaling domain, a CD3zeta signal domain, and any combination thereof. In one aspect, the mesothelin CAR comprises at least one intracellular signaling domain of one or more costimulatory molecule(s) other than a CD137 (4-1BB) or CD28, a CD3zeta signal domain, and any combination thereof.

[00194] Furthermore, the present invention provides mesothelin CAR compositions and their use in medicaments or methods for treating, among other diseases, cancer or any malignancy or autoimmune diseases involving cells or tissues which express mesothelin.

Chimeric Antigen Receptor (CAR)

[00195] The present invention encompasses a recombinant nucleic acid construct comprising sequences encoding a CAR, wherein the CAR comprises an antibody that binds specifically to mesothelin, e.g., a human antibody fragment that specifically binds to mesothelin. In one aspect, the mesothelin is human mesothelin, and the sequence of the antibody fragment is contiguous with, and in the same reading frame as a nucleic acid sequence encoding an intracellular signaling domain. The intracellular signaling domain can comprise a costimulatory signaling domain and/or a primary signaling domain, e.g., a zeta chain. The costimulatory signaling domain refers to a portion of the CAR comprising at least a portion of the intracellular domain of a costimulatory molecule.

[00196] In specific aspects, a CAR construct of the invention comprises a scFv domain selected from the group consisting of SEQ ID NOS: 39-62, wherein the scFv may be preceded by an optional leader sequence such as provided in SEQ ID NO: 1, and followed by an optional hinge sequence such as provided in SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or SEQ ID NO: 5, a transmembrane region such as provided in SEQ ID NO: 6, an intracellular signalling domain that includes SEQ ID NO: 7 or SEQ ID NO: 8 and a CD3 zeta sequence that includes SEQ ID NO: 9 or SEQ ID NO: 10, wherein the domains are contiguous with and in the same reading frame to form a single fusion protein. Also included in the invention is a nucleotide sequence that encodes the polypeptide selected from the group consisting of SEQ ID NO: 87; SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID

NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 109, and SEQ ID NO: 110, or a sequence with 95-99% identify thereof. Also included in the invention is a nucleotide sequence that encodes the polypeptide of each of the scFv fragments selected from the group consisting of SEQ ID NO: 39; SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, and SEQ ID NO: 62, or a sequence with 95-99% identify thereof, and each of the domains of SEQ ID NOS: 1, 2, and 6-9, plus the encoded mesothelin CAR fusion protein of the invention. In one aspect an exemplary mesothelin CAR constructs comprisean optional leader sequence, an extracellular mesothelin binding domain, a hinge, a transmembrane domain, and an intracellular stimulatory domain. In one aspect, the mesothelin CAR construct comprises an optional leader sequence, a mesothelin binding domain, a hinge, a transmembrane domain, an intracellular costimulatory domain and an intracellular stimulatory domain. Specific mesothelin CAR constructs containing human scFv domains are provided as SEQ ID NOs: 87-110.

[00197] Full-length CAR sequences are also provided herein as SEQ ID NO: 63; SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, 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, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, or SEQ ID NO: 86. An exemplary leader sequence is provided as SEQ ID NO: 1. An exemplary hinge/spacer sequence is provided as SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or SEQ ID NO: 5. An exemplary transmembrane domain sequence is provided as SEQ ID NO:6. An exemplary sequence of the intracellular signaling domain of the 4-IBB protein is provided as SEQ ID NO: 7. An exemplary sequence of the intracellular signaling domain of CD27 is provided as SEQ ID NO: 8. An exemplary CD3zeta domain sequence is provided as SEQ ID NO: 9 or SEQ ID NO: 10.

[00198] In one aspect, the present invention encompasses a recombinant nucleic acid construct comprising a nucleic acid molecule encoding a CAR, wherein the nucleic acid molecule comprises the nucleic acid sequence encoding an anti-mesothelin binding domain, e.g., described herein, that is contiguous with and in the same reading frame as a nucleic acid sequence encoding an intracellular signaling domain. In one aspect, the anti-mesothlin binding domain is selected from one or more of SEQ ID NOS: 87-1 10. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 87. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 88. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 89. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 90. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 9 1. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 92. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 93. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 94. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 95. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 96. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 97. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 98. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 99. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 100. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 101. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 102. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 103. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 104. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 105. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 106. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 107. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 108. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 109. In one aspect, the anti-mesothelin binding domain comprises SEQ ID NO: 110. In one aspect, the present invention encompasses a recombinant DNA construct comprising a transgene encoding a CAR, wherein the transgene comprises the nucleic acid sequence encoding an anti-mesothelin binding domain described herein, e.g., a human anti-mesothelin binding domain selected from one or more of SEQ ID NOS: 87-1 10, wherein the sequence is contiguous with and in the same reading frame as the nucleic acid sequence encoding an intracellular signaling

domain. An exemplary intracellular signaling domain that can be used in the CAR includes, but is not limited to, one or more intracellular signaling domains of, e.g., CD3-zeta, CD28, 4-1BB, and the like. In some instances, the CAR can comprise any combination of CD3-zeta, CD28, 4-1BB, and the like. In one aspect the nucleic acid sequence of a CAR construct of the invention is selected from one or more of SEQ ID NOS: 111-134. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 111. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 112. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 113. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 114. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 115. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 116. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 117. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 118. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 119. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 120. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 121. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 122. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 123. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 124. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 125. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 126. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 127. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 128. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 129. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 130. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 131. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 132. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 133. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO: 134.

[00199] The nucleic acid sequences coding for the desired molecules can be obtained using recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the nucleic acid of interest can be produced synthetically, rather than cloned.

[00200] The present invention includes retroviral and lentiviral vector constructs expressing a CAR that can be directly transduced into a cell. The present invention also includes an RNA construct that can be directly transfected into a cell. A method for generating mRNA for use in transfection involves in vitro transcription (IVT) of a template with specially designed primers, followed by polyA addition, to produce a construct containing 3' and 5' untranslated sequence ("UTR"), a 5' cap and/or Internal Ribosome Entry Site (IRES), the nucleic acid to be expressed, and a polyA tail, typically 50-2000 bases in length (SEQ ID NO: 35). RNA so produced can efficiently transfect different kinds of cells. In one embodiment, the template includes sequences for the CAR. In an embodiment, an RNA CAR vector is transduced into a T cell by electroporation.

Antigen binding domain

[00201] In one aspect, the CAR of the invention comprises a target-specific binding element otherwise referred to as an antigen binding domain. The choice of antigen binding domain depends upon the type and number of antigens that define the surface of a target cell. For example, the antigen binding domain may be chosen to recognize a antigen that acts as a cell surface marker on target cells associated with a particular disease state.

[00202] In one aspect, the CAR-mediated immune effector cell response can be directed to cells that express an antigen of interest, where the CAR comprises an antigen binding domain that specifically binds to the antigen of interest. In one aspect, the portion of the CAR comprising the antigen binding domain comprises an antigen binding domain that targets mesothelin. In one aspect, the antigen binding domain targets human mesothelin.

[00203] The antigen binding domain can be any domain that binds to the antigen including but not limited to a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody, and a functional fragment thereof, including but not limited to a single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL) and a variable domain (VHH) of camelid derived nanobody, and to an alternative scaffold known in the art to function as an antigen binding domain, such as a recombinant fibronectin domain, and the like. In some instances, it is beneficial for the antigen binding domain to be derived from the same species in which the CAR will ultimately be used in. For example, for use in humans, it may be beneficial for the antigen binding domain of the CAR to

comprise human or humanized residues for the antigen binding domain of an antibody or antibody fragment. Thus, in one aspect, the antigen binding domain comprises a human antibody or an antibody fragment.

[00204] In one embodiment, the anti-mesothelin binding domain does not compete, or competes poorly, for binding to human mesothelin with an antigen binding domain comprising an amino acid sequence comprising SEQ ID NO: 279, e.g., murine SSI scFv, e.g., in a competition assay described herein.

[00205] The amino acid sequence of murine SSI scFv is provided below (SEQ ID NO: 279):

QVQLQQSGPELEKPGASVKISCKASGYS FTGYTMNWVKQSHGKSLEWIGLITPYNGASS YNQKFRGKATLT VDKSS STAYMDLLSLTSEDSAVYFCARGGYDGRGFDYWGQGTTVTVS SGGGGSGGGGSGGGGSDIELTQS P AIMSAS PGEKVTMTCSAS SSVSYMHWYQQKSGTS PKRWIYDTSKLASGVPGRFSGSGSGNSYS LTI SSVEA EDDATYYCQQWSGYPLTFGAGTKLEI

[00206] In one embodiment, the anti-mesothelin binding domain competes for binding to human mesothelin with an antigen binding domain comprising a LC CDRl , LC CDR2 and LC CDR3 of an anti-mesothelin light chain amino acid sequence selected from SEQ ID NO: 43 or SEQ ID NO: 49 and an HC CDRl, HC CDR2, and HC CDR3 of an anti-mesothelin heavy chain amino acid sequence selected from SEQ ID NO: 43 or SEQ ID NO: 49, e.g., in a competition assay described herein. In one embodiment, the anti-mesothelin binding domain competes for binding to human mesothelin with an antigen binding domain comprising a LC CDRl selected from SEQ ID NO: 203 or SEQ ID NO: 209, a LC CDR2 selected from SEQ ID NO: 227 or SEQ ID NO: 233, and a LC CDR3 selected from SEQ ID NO: 251 or SEQ ID NO: 257; and a HC CDRl selected from SEQ ID NO: 138 or SEQ ID NO: 144, a HC CDR2 selected from SEQ ID NO: 156 or SEQ ID NO: 162, and a HC CDR3 selected from SEQ ID NO: 179 or SEQ ID NO: 185, e.g., in a competition assay described herein.

[00207] In one embodiment, the anti-mesothelin binding domain competes for binding to human mesothelin with an antigen binding domain comprising a sequence selected from SEQ ID NO: 43 or SEQ ID NO: 49, e.g., in a competition assay described herein.

[00208] In embodiments, the competition assay is an SPR-based assay. Briefly, the antigen, e.g., human mesothelin, is immobilized on a surface. Through a microflow system, a reference antibody is injected over the antigen layer. Upon binding of the reference antibody to the

antigen, an increase in signal, typically expressed in response units (RU) is detected, e.g., reference signal. After a desired time, a test antibody is injected over the antigen layer. If the test antibody binds to a different region or epitope of the antigen, then an additional increase in signal is detected, e.g., a 5% or more, 10% or more, 15% or more, 20% or more, 25% or more, 30% or more, 35%, or more, 40% or more, 45% or more, 50% or more, 55% of more, 60% or more, 65% or more, 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, or 95% or more increase in signal, e.g., RU, as compared to the highest signal detected upon binding of the reference antibody, e.g., the reference signal. If the test antibody binds to the same region or epitope of the antigen, then little or no increase in signal, e.g., RU, will be detected, e.g., less than 20%, less than 15%, less than 10%, less than 5%, less than 4%, less than 3%, less than 2 %, or less than 1% increase in signal, e.g., RU, as compared to the highest signal detected upon binding of the reference antibody, e.g., the reference signal. When using this SPR-based competition assay, an antibody is said to compete with the reference antibody when less than 20%, less than 15%, less than 10%, less than 5%, less than 4%, less than 3%, less than 2 %, or less than 1% increase in signal, e.g., RU, is detected when compared to the reference signal detected upon binding of the reference antibody to the antigen. An antibody is said to not compete, or compete poorly, with a reference antibody when a 5% or more, 10% or more, 15% or more, 20% or more, 25% or more, 30% or more, 35%, or more, 40% or more, 45% or more, 50% or more, 55% of more, 60% or more, 65% or more, 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, or 95% or more increase in signal, e.g., RU, is detected when compared to the reference signal detected upon binding of the reference antibody to the antigen.

[00209] Identification of the epitope bound by the antigen binding domains described herein can be determined by various methods known in the art. For example, crystal structures can be generated containing the antigen binding domain bound to, or in complex with, the antigen. In another example, assays, e.g., a protection assay, can be performed to identify the regions of the antigen contribute to the epitope, or to identify the epitope. An exemplary protection assay, a hydrogen/deuterium exchange (HDX) mass spectrometry assay, is described further in Example 18. The HDX mass spectrometry was performed to identify the putative epitopes on human MSLN, e.g., $hMSLN_{296-588}$, e.g., SEQ ID NO: 278, for murine SSI, e.g., SEQ ID NO: 279, and the M5 scFv described herein, e.g., SEQ ID NO: 43. $hMSLN_{296-588}$, e.g., SEQ ID NO: 278, represents amino acids 296-588 of human mesothelin, e.g., the first amino acid of SEQ ID NO:

278 is amino acid 296 and the last amino acid of SEQ ID NO: 278 is amino acid 588. The amino acid sequence for human mesothelin, amino acids 296-588 is provided below: (SEQ ID NO: 278)

EVEKTACPSGKKAREIDESLI FYKKWELEACVDAALLATQMDRVNAIPFTYEQLDVLKHKLDELYPQG
YPESVIQHLGYLFLKMSPEDIRKWNVTSLETLKALLEVNKGHEMSPQAPRRPLPQVATLIDRFVKGRG
QLDKDTLDTLTAFYPGYLCSLSPEELSSVPPSSIWAVRPQDLDTCDPRQLDVLYPKARLAFQNMNGSE
YFVKIQSFLGGAPTEDLKALSQQNVSMDLATFMKLRTDAVLPLTVAEVQKLLGPHVEGLKAEERHRPV
RDWILRQRQDDLDTLGLGLQG

[00210] The results of the HDX mass spectrometry assay indicated that one or more amino acids of 314-315, 317-318, 346-349, and 369-375 of $hMSLN_{296-588,}$ e.g., SEQ ID NO: 278 contribute to the epitope recognized by SSI. The results of the HDX mass spectrometry assay indicated that one or more amino acids of 485-490, 498-507, 532-537, or 545-572 of $hMSLN_{296-588,}$ e.g., SEQ ID NO: 278, contribute to the epitope recognized by an anti-mesothelin antigen binding domain described herein, e.g., M5 scFv, e.g., SEQ ID NO: 43.

[0021 1] In one embodiment, the anti-mesothelin binding domain described herein binds to a different epitope of human mesothelin, e.g., SEQ ID NO: 278, than the epitope of human mesothelin targeted by the antigen binding domain comprising a sequence comprising SEQ ID NO: 279, e.g., murine SSI.

[00212] In one embodiment, the epitope recognized by SSI comprises a sequence selected from amino acids 314-315, 317-318, 346-349, or 369-375 of $hMSLN_{296-588,}$ e.g., SEQ ID NO: 278, or any combination thereof. In one embodiment, the epitope recognized by SSI comprises one or more amino acids selected from amino acids 314-315, 317-318, 346-349, or 369-375 of $hMSLN_{296-588,}$ e.g., SEQ ID NO: 278.

[00213] In one embodiment, the anti-mesothelin binding domain described herein binds to the C-terminus of human mesothelin. In one embodiment, the anti-mesothelin binding domain described herein binds an epitope within amino acids 450-588 of SEQ ID NO: 278, e.g., wherein the epitope, in part or in whole, can be found within amino acids 450-588, within amino acids 480-580, or within amino acids 485-572 of SEQ ID NO: 278. In one embodiment, the epitope recognized by an anti-mesothelin binding domain described herein comprises a sequence selected from amino acids 485-490, 498-507, 532-537, or 545-572 of $hMSLN_{296-588,}$ e.g., SEQ ID NO: 278, or any combination thereof. In one embodiment, the epitope recognized by an anti-

mesothelin binding domain described herein comprises one or more amino acids selected from 485-490, 498-507, 532-537, or 545-572 of $hMSLN_{296-588}$, e.g., SEQ ID NO: 278, or any combination thereof.

[00214] In one embodiment, the anti-mesothelin binding domain comprises one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a human anti-mesothelin binding domain selected from SEQ ID NOS: 39-62 and one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a human anti-mesothelin binding domain selected from SEQ ID NOS: 39-62 In one embodiment, the human anti-mesothelin binding domain comprises a light chain variable region described herein (e.g., in Table 2) and/or a heavy chain variable region described herein (e.g., in Table 2). In one embodiment, the anti-mesothelin binding domain is a scFv comprising a light chain variable region and a heavy chain variable region of an amino acid sequence of Table 2. In an embodiment, the anti-mesothelin binding domain (e.g., an scFV) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in Table 2, or a sequence with 95-99% identity to an amino acid sequence of Table 2; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in Table 2, or a sequence with 95-99% identity to an amino acid sequence of Table 2.

[00215] In one embodiment, the human anti-mesothelin binding domain comprises a sequence selected from a group consisting of SEQ ID NOS: 39-62, or a sequence with 95-99% identify thereof. In one embodiment, the nucleic acid sequence encoding the human anti-mesothelin binding domain comprises a sequence selected from a group consisting of SEQ ID NO: 87-110, or a sequence with 95-99% identify thereof. In one embodiment, the human anti-mesothelin binding domain is a scFv, and a light chain variable region comprising an amino acid sequence described herein, e.g., in Table 2 or 3, is attached to a heavy chain variable region comprising an amino acid sequence described herein, e.g., in Table 2 or 3, via a linker, e.g., a linker described

herein. In one embodiment, the humanized anti-mesothelin binding domain includes a (Gly4-Ser)n linker (SEQ ID NO: 26), wherein n is 1, 2, 3, 4, 5, or 6, preferably 3 or 4. The light chain variable region and heavy chain variable region of a scFv can be, e.g., in any of the following orientations: light chain variable region-linker-heavy chain variable region or heavy chain variable region-linker-light chain variable region.

[00216] In one aspect, the antigen binding domain portion comprises one or more sequence selected from SEQ ID NOS:39-62. In one aspect the CAR is selected from one or more sequence selected from SEQ ID NOS: 63-86.

[00217] In one aspect, the antibodies of the invention may exist in a variety of other forms including, for example, Fab, Fab', $F(ab')_2$, Fv fragments, scFv antibody fragments, disulfide-linked Fvs (sdFv), a Fd fragment consisting of the VH and CHI domains, linear antibodies, single domain antibodies such as sdAb (either VL or VH), camelid VHH domains, multi-specific antibodies formed from antibody fragments such as a bivalent fragment comprising two Fab fragments linked by a disulfide brudge at the hinge region, and an isolated CDR or other epitope binding fragments of an antibody. In one aspect, the antibody fragment provided herein is a scFv. In some instances, a human scFv may also be derived from a yeast display library.

[00218] A display library is a collection of entities; each entity includes an accessible polypeptide component and a recoverable component that encodes or identifies the polypeptide component. The polypeptide component is varied so that different amino acid sequences are represented. The polypeptide component can be of any length, e.g. from three amino acids to over 300 amino acids. A display library entity can include more than one polypeptide component, for example, the two polypeptide chains of a Fab. In one exemplary embodiment, a display library can be used to identify an anti-mesothelin binding domain. In a selection, the polypeptide component of each member of the library is probed with mesothelin, or a fragment there, and if the polypeptide component binds to the mesothelin, the display library member is identified, typically by retention on a support.

[00219] Retained display library members are recovered from the support and analyzed. The analysis can include amplification and a subsequent selection under similar or dissimilar conditions. For example, positive and negative selections can be alternated. The analysis can also include determining the amino acid sequence of the polypeptide component, i.e., the anti-

mesothelin binding domain, and purification of the polypeptide component for detailed characterization.

[00220] A variety of formats can be used for display libraries. Examples include the phage display. In phage display, the protein component is typically covalently linked to a bacteriophage coat protein. The linkage results from translation of a nucleic acid encoding the protein component fused to the coat protein. The linkage can include a flexible peptide linker, a protease site, or an amino acid incorporated as a result of suppression of a stop codon. Phage display is described, for example, in U.S. 5,223,409; Smith (1985) *Science* 228:1315-1317; WO 92/18619; WO 91/17271; WO 92/20791; WO 92/15679; WO 93/01288; WO 92/01047; WO 92/09690; WO 90/02809; de Haard *et al.* (1999) *J. Biol. Chem* 274:18218-30; Hoogenboom *et al.* (1998) *Immunotechnology* 4:1-20; Hoogenboom *et al.* (2000) *Immunol Today* 2:371-8 and Hoet et al. (2005) *Nat Biotechnol.* 23(3)344-8. Bacteriophage displaying the protein component can be grown and harvested using standard phage preparatory methods, e.g. PEG precipitation from growth media. After selection of individual display phages, the nucleic acid encoding the selected protein components can be isolated from cells infected with the selected phages or from the phage themselves, after amplification. Individual colonies or plaques can be picked, the nucleic acid isolated and sequenced.

[00221] Other display formats include cell based display (see, e.g., WO 03/029456), protein-nucleic acid fusions (see, e.g., US 6,207,446), ribosome display (See, e.g., Mattheakis *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:9022 and Hanes *et al.* (2000) *Nat Biotechnol.* 18:1287-92; Hanes *et al.* (2000) *Methods Enzymol.* 328:404-30; and Schaffitzel *et al.* (1999) *J Immunol Methods.* 231(1-2):119-35), *anAE. coli* periplasmic display *(J Immunol Methods.* 2005 Nov 22;PMK>: 16337958).

[00222] In addition to the use of display libraries, other methods can be used to obtain an anti-mesothelin binding domain. For example, mesothelin or a fragment thereof can be used as an antigen in a non-human animal, e.g., a rodent.

[00223] In one embodiment, the non-human animal includes at least a part of a human immunoglobulin gene. For example, it is possible to engineer mouse strains deficient in mouse antibody production with large fragments of the human Ig loci. Using the hybridoma technology, antigen-specific monoclonal antibodies (Mabs) derived from the genes with the desired

specificity may be produced and selected. See, e.g., XENOMOUSE™, Green et al., 1994, *Nat. Gen.* 7:13-21; U.S. 2003-0070185, WO 96/34096, published Oct. 31, 1996, and PCT Application No. PCT/US96/05928, filed Apr. 29, 1996.

[00224] In some instances, scFvs can be prepared according to method known in the art (see, for example, Bird *etal,* (1988) Science 242:423-426 and Huston *etal.,* (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). ScFv molecules can be produced by linking VH and VL regions together, e.g., using flexible polypeptide linkers. The scFv molecules can comprise a linker (e.g., a Ser-Gly linker) with an optimized length and/or amino acid composition. The linker length can greatly affect how the variable regions of an scFv fold and interact. In fact, if a short polypeptide linker is employed (e.g., between 5-10 amino acids, intrachain folding is prevented. Interchain folding is also required to bring the two variable regions together to form a functional epitope binding site. For examples of linker orientation and size see, e.g., Hollinger *et al.* 1993 Proc Natl Acad. Sci. U.S.A. 90:6444-6448, U.S. Patent Application Publication Nos. 2005/0100543, 2005/0175606, 2007/0014794, and PCT publication Nos. WO2006/020258 and WO2007/024715, is incorporated herein by reference.

[00225] An scFv can comprise a linker of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, or more amino acid residues between its VL and VH regions. The linker sequence may comprise any naturally occurring amino acid. In some embodiments, the linker sequence comprises amino acids glycine and serine. In another embodiment, the linker sequence comprises sets of glycine and serine repeats such as $(Gly_4Ser)_n$, where n is a positive integer equal to or greater than 1. (SEQ ID NO: 135) In one embodiment, the linker can be $(Gly_4Ser)_4$ (SEQ ID NO: 27) or $(Gly_4Ser)_3$ (SEQ ID NO: 28). Variation in the linker length may retain or enhance activity, giving rise to superior efficacy in activity studies.

Stability and Mutations

[00226] The stability of an anti-mesothelin binding domain, e.g., scFv molecules (e.g., soluble scFv), can be evaluated in reference to the biophysical properties (e.g., thermal stability) of a conventional control scFv molecule or a full length antibody. In one embodiment, the human scFv has a thermal stability that is greater than about 0.1, about 0.25, about 0.5, about 0.75, about 1, about 1.25, about 1.5, about 1.75, about 2, about 2.5, about 3, about 3.5, about 4, about 4.5,

about 5, about 5.5, about 6, about 6.5, about 7, about 7.5, about 8, about 8.5, about 9, about 9.5, about 10 degrees, about 11 degrees, about 12 degrees, about 13 degrees, about 14 degrees, or about 15 degrees Celsius than a control binding molecule (e.g. a conventional scFv molecule) in the described assays.

[00227] The improved thermal stability of the anti-mesothelin binding domain, e.g., scFv, is subsequently conferred to the entire mesothelin CAR construct, leading to improved therapeutic properties of the mesothelin CAR construct. The thermal stability of the anti-mesothelin binding domain, e.g., scFv, can be improved by at least about 2°C or 3°C as compared to a conventional antibody. In one embodiment, the anti-mesothelin binding domain, e.g., scFv, has a 1°C improved thermal stability as compared to a conventional antibody. In another embodiment, the anti-mesothelin binding domain, e.g., scFv, has a 2°C improved thermal stability as compared to a conventional antibody. In another embodiment, the anti-mesothelin binding domain, e.g., scFv, has a 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15°C improved thermal stability as compared to a conventional antibody. Comparisons can be made, for example, between the scFv molecules disclosed herein and scFv molecules or Fab fragments of an antibody from which the scFv VH and VL were derived. Thermal stability can be measured using methods known in the art. For example, in one embodiment, Tm can be measured. Methods for measuring Tm and other methods of determining protein stability are described in more detail below.

[00228] Mutations in scFv (arising through direct mutagenesis of the soluble scFv) alter the stability of the scFv and improve the overall stability of the scFv and the CART construct. Stability of the humanized scFv is compared against the murine scFv using measurements such as Tm, temperature denaturation and temperature aggregation.

[00229] In one embodiment, the anti-mesothelin binding domain, e.g., scFv, comprises at least one mutation such that the mutated anti-mesothelin binding domain, e.g., scFv, confers improved stability to the anti-mesothelin construct. In another embodiment, the anti-mesothelin binding domain, e.g., scFv, comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mutations such that the mutated anti-mesothelin binding domain, e.g., scFv, confers improved stability to the anti-mesothelin construct. The binding capacity of the mutant scFvs can be determined using assays described in the Examples.

BindingAffinity

[00230] A wide variety of methods for determining binding affinity are known in the art. An exemplary method for determining binding affinity employs surface plasmon resonance. Surface plasmon resonance is an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, N.J.). For further descriptions, see Jonsson, U , *et al.* (1993) Ann. Biol. Clin. 51:19-26; Jonsson, U , i (1991) Biotechniques 11:620-627; Johnsson, B., *et al.* (1995) J. Mol. Recognit. 8:125-131; and Johnnson, B., *et al.* (1991) Anal. Biochem. 198:268-277.

[0023 1] In one aspect, the portion of a CAR composition of the invention comprising an antibody or fragment thereof comprises amino acid sequences that are homologous to the amino acid sequences described herein, and wherein the antibody or fragment thereof retains the desired functional properties of the anti-mesothelin antibody fragments of the invention. In one specific aspect, the CAR composition of the invention comprises an antibody fragment. In a further aspect, that antibody fragment comprises an scFv.

[00232] In various aspects, the portion comprising an antibody or antibody fragment of the CAR composition of the invention is engineered by modifying one or more amino acids within one or both variable regions (i.e., VH and/or VL), for example within one or more CDR regions and/or within one or more framework regions. In one specific aspect, the CAR composition of the invention comprises an antibody fragment. In a further aspect, that antibody fragment comprises an scFv.

[00233] It will be understood by one of ordinary skill in the art that the antibody or antibody fragment of the invention may further be modified such that they vary in amino acid sequence (e.g., from wild-type), but not in desired activity. For example, additional nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues may be made to the protein For example, a nonessential amino acid residue in a molecule may be replaced with another amino acid residue from the same side chain family. In another embodiment, a string of amino acids can be replaced with a structurally similar string that differs in order and/or composition of side chain family members, i.e., a conservative substitution, in

which an amino acid residue is replaced with an amino acid residue having a similar side chain, may be made.

[00234] Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

[00235] Percent identity in the context of two or more nucleic acids or polypeptide sequences, refers to two or more sequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (i.e., 60% identity, optionally 70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%,81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length.

[00236] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and Waterman, (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, (1970) J. Mol.

Biol. 48:443, by the search for similarity method of Pearson and Lipman, (1988) Proc. Nat'l. Acad. Sci. USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., Brent et al, (2003) Current Protocols in Molecular Biology).

[00237] Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., (1977) Nuc. Acids Res. 25:3389-3402; and Altschul et al., (1990) J. Mol. Biol. 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

[00238] The percent identity between two amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller, (1988) Comput. Appl. Biosci. 4:11-17) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (1970) J. Mol. Biol. 48:444-453) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

[00239] In one aspect, the present invention contemplates modifications of the starting antibody or fragment (e.g., scFv) amino acid sequence that generate functionally equivalent molecules. For example, the VH or VL of an anti-mesothelin binding domain, e.g., scFv comprised in the CAR can be modified to retain at least about 70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%,81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity of the starting VH or VL framework region of the anti-mesothelin binding domain, e.g., scFv. The present invention contemplates modifications of the entire CAR construct, e.g., modifications in one or more amino acid sequences of the various domains of the CAR construct in order to generate functionally equivalent molecules. The CAR construct can be modified to retain at least about 70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%,

88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity of the starting CAR construct.

Transmembrane domain

[00240] With respect to the transmembrane domain, in various embodiments, a CAR can be designed to comprise a transmembrane domain that is attached to the extracellular domain of the CAR. A transmembrane domain can include one or more additional amino acids adjacent to the transmembrane region, e.g., one or more amino acid associated with the extracellular region of the protein from which the transmembrane was derived (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 up to 15 amino acids of the extracellular region) and/or one or more additional amino acids associated with the intracellular region of the protein from which the transmembrane protein is derived (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 up to 15 amino acids of the intracellular region). In one aspect, the transmembrane domain is one that is associated with one of the other domains of the CAR is used, e.g., in one embodiment, the transmembrane domain may be from the same protein that the signaling domain, costimulatory domain or the hinge domain is derived from. In another aspect, the transmembrane domain is not derived from the same protein that any other domain of the CAR is derived from. In some instances, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins, e.g., to minimize interactions with other members of the receptor complex. In one aspect, the transmembrane domain is capable of homodimerization with another CAR on the cell surface of a CAR-expressing cell. In a different aspect, the amino acid sequence of the transmembrane domain may be modified or substituted so as to minimize interactions with the binding domains of the native binding partner present in the same CAR-expressing cell.

[00241] The transmembrane domain may be derived either from a natural or from a recombinant source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. In one aspect, the transmembrane domain is capable of signaling to the intracellular domain(s) whenever the CAR has bound to a target. A transmembrane domain of particular use in this invention may include at least the transmembrane domain(s) of, e.g., the alpha, beta or zeta chain of the T-cell receptor, CD28,

CD3 epsilon, CD45, CD4, CD5, CD8 (e.g., CD8 alpha, CD8 beta), CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154. In some embodiments, a transmembrane domain may include at least the transmembrane region(s) of, e.g., KIRDS2, OX40, CD2, CD27, LFA-1 (CDlla, CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD160, CD19, IL2Rbeta, IL2R gamma, IL7R a, ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CDlld, ITGAE, CD103, ITGAL, CDl la, LFA-1, ITGAM, CDllb, ITGAX, CDl lc, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, PAG/Cbp, NKG2D, and NKG2C.

[00242] In some instances, the transmembrane domain can be attached to the extracellular region of the CAR, e.g., the antigen binding domain of the CAR, via a hinge, e.g., a hinge from a human protein. For example, in one embodiment, the hinge can be a human Ig (immunoglobulin) hinge (e.g., an IgG4 hinge, an IgD hinge), a GS linker (e.g., a GS linker described herein), a KIR2DS2 hinge or a CD8a hinge. In one embodiment, the hinge or spacer comprises (e.g., consists of) the amino acid sequence of SEQ ID NO:2. In one aspect, the transmembrane domain comprises (e.g., consists of) a transmembrane domain of SEQ ID NO: 6.

[00243] In one aspect, the hinge or spacer comprises an IgG4 hinge. For example, in one embodiment, the hinge or spacer comprises a hinge of the amino acid sequence as follows: ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCV\^VDVSQEDPEVQFNWY VDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTIS KAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGKM (SEQ ID NO:3).

[00244] In some embodiments, the hinge or spacer comprises a hinge encoded by a nucleotide sequence as follows:
GAGAGCAAGTACGGCCCTCCCTGCCCCCCTTGCCCTGCCCCCGAGTTCCTGGGCGGA CCCAGCGTGTTCCTGTTCCCCCCCAAGCCCAAGGACACCCTGATGATCAGCCGGACC CCCGAGGTGACCTGTGTGGTGGTGGACGTGTCCCAGGAGGACCCCGAGGTCCAGTT

CAACTGGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGACCAAGCCCCGGGAG
GAGCAGTTCAATAGCACCTACCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAGGA
CTGGCTGAACGGCAAGGAATACAAGTGTAAGGTGTCCAACAAGGGCCTGCCCAGCA
GCATCGAGAAAACCATCAGCAAGGCCAAGGGCCAGCCTCGGGAGCCCCAGGTGTAC
ACCCTGCCCCCTAGCCAAGAGGAGATGACCAAGAACCAGGTGTCCCTGACCTGCCT
GGTGAAGGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAACGGCCAGC
CCGAGAACAACTACAAGACCACCCCCCCTGTGCTGGACAGCGACGGCAGCTTCTTC
CTGTACAGCCGGCTGACCGTGGACAAGAGCCGGTGGCAGGAGGGCAACGTCTTTAG
CTGCTCCGTGATGCACGAGGCCCTGCACAACCACTACACCCAGAAGAGCCTGAGCC
TGTCCCTGGGCAAGATG (SEQ ID NO: 14).

[00245] In one aspect, the hinge or spacer comprises an IgD hinge. For example, in one embodiment, the hinge or spacer comprises a hinge of the amino acid sequence
RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKEKEKEEQEERETK
TPECPSHTQPLGVYLLTPAVQDLWLRDKATFTCFWGSDLKDAHLTWEVAGKVPTGGV
EEGLLERHSNGSQSQHSRLTLPRSLWNAGTSVTCTLNHPSLPPQRLMALREPAAQAPVK
LSLNLLASSDPPEAASWLLCEVSGFSPPMLLMWLEDQREVNTSGFAPARPPPQPGSTTF
WAWSVLRVPAPPSPQPATYTCWSHEDSRTLLNASRSLEVSYVTDH (SEQ ID NO:4).

[00246] In some embodiments, the hinge or spacer comprises a hinge encoded by a nucleotide sequence of
AGGTGGCCCGAAAGTCCCAAGGCCCAGGCATCTAGTGTTCCTACTGCACAGCCCCA
GGCAGAAGGCAGCCTAGCCAAAGCTACTACTGCACCTGCCACTACGCGCAATACTG
GCCGTGGCGGGGAGGAGAAGAAAAAGGAGAAAGAGAAAGAAGAACAGGAAGAGA
GGGAGACCAAGACCCCTGAATGTCCATCCCATACCCAGCCGCTGGGCGTCTATCTCT
TGACTCCCGCAGTACAGGACTTGTGGCTTAGAGATAAGGCCACCTTTACATGTTTCG
TCGTGGGCTCTGACCTGAAGGATGCCCATTTGACTTGGGAGGTTGCCGGAAAGGTAC
CCACAGGGGGGGTTGAGGAAGGGTTGCTGGAGCGCCATTCCAATGGCTCTCAGAGC
CAGCACTCAAGACTCACCCTTCCGAGATCCCTGTGGAACGCCGGGACCTCTGTCACA
TGTACTCTAAATCATCCTAGCCTGCCCCCACAGCGTCTGATGGCCCTTAGAGAGCCA
GCCGCCCAGGCACCAGTTAAGCTTAGCCTGAATCTGCTCGCCAGTAGTGATCCCCCA
GAGGCCGCCAGCTGGCTCTTATGCGAAGTGTCCGGCTTTAGCCCGCCCAACATCTTG
CTCATGTGGCTGGAGGACCAGCGAGAAGTGAACACCAGCGGCTTCGCTCCAGCCCG

GCCCCCACCCCAGCCGGGTTCTACCACATTCTGGGCCTGGAGTGTCTTAAGGGTCCC
AGCACCACCTAGCCCCCAGCCAGCCACATACACCTGTGTTGTGTCCCATGAAGATAG
CAGGACCCTGCTAAATGCTTCTAGGAGTCTGGAGGTTTCCTACGTGACTGACCATT
(SEQ ID NO:15).

[00247] In one aspect, the transmembrane domain may be recombinant, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. In one aspect a triplet of phenylalanine, tryptophan and valine can be found at each end of a recombinant transmembrane domain.

[00248] Optionally, a short oligo- or polypeptide linker, between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic signaling region of the CAR. A glycine-serine doublet provides a particularly suitable linker. For example, in one aspect, the linker comprises the amino acid sequence of GGGGSGGGGS (SEQ ID NO: 5). In some embodiments, the linker is encoded by a nucleotide sequence of GGTGGCGGAGGTTCTGGAGGTGGAGGTTCC (SEQ ID NO: 16).

[00249] In one aspect, the hinge or spacer comprises a KTR2DS2 hinge and portions thereof.

Cytoplasmic domain

[00250] The cytoplasmic domain or region of the CAR includes an intracellular signaling domain. An intracellular signaling domain is generally responsible for activation of at least one of the normal effector functions of the immune cell in which the CAR has been introducede. 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 "intracellular 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 intracellular 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 intracellular 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 intracellular signaling domain is thus meant to include any

truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal.

[00251] Examples of intracellular signaling domains for use in the CAR of the invention include the cytoplasmic sequences of the T cell receptor (TCR) and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or variant of these sequences and any recombinant sequence that has the same functional capability.

[00252] It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary and/or costimulatory signal is also required. Thus, T cell activation can be said to be mediated by two distinct classes of cytoplasmic signaling sequences: those that initiate antigen-dependent primary activation through the TCR (primary intracellular signaling domains) and those that act in an antigen-independent manner to provide a secondary or costimulatory signal (secondary cytoplasmic domain, e.g., a costimulatory domain).

[00253] A primary cytoplasmic signaling domain regulates primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary intracellular signaling domains that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs.

[00254] Examples of ITAM containing primary intracellular signaling domains that are of particular use in the invention include those of CD3 zeta, common FcR gamma (FCERIG), Fc gamma RIIa,, FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta , CD3 epsilon, , CD79a, CD79b, DAP10, and DAP12. In one embodiment, a CAR of the invention comprises an intracellular signaling domain, e.g., a primary signaling domain of CD3-zeta.

[00255] In one embodiment, a primary signaling domain comprises a modified ITAM domain, e.g., a mutated ITAM domain which has altered (e.g., increased or decreased) activity as compared to the native ITAM domain. In one embodiment, a primary signaling domain comprises a modified ITAM-containing primary intracellular signaling domain, e.g., an optimized and/or truncated ITAM-containing primary intracellular signaling domain. In an embodiment, a primary signaling domain comprises one, two, three, four or more ITAM motifs.

[00256] Further examples of molecules containing a primary intracellular signaling domain that are of particular use in the invention include those of DAP10, DAP12, and CD32.

[00257] The intracellular domain of the CAR can comprise the CD3-zeta signaling domain by itself or it can be combined with any other desired intracellular signaling domain(s) useful in the context of a CAR of the invention. For example, the intracellular signaling domain of the CAR can comprise a CD3 zeta chain portion and a costimulatory signaling domain. The costimulatory signaling domain refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. A costimulatory molecule is a cell surface molecule other than an antigen receptor or its ligands that is required for an efficient response of lymphocytes to an antigen. Examples of such molecules include CD27, CD28, 4-IBB (CD137), OX40, CD30, CD40, PD-1 (also known as PD1), ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83, and the like. For example, CD27 costimulation has been demonstrated to enhance expansion, effector function, and survival of human CART cells in vitro and augments human T cell persistence and antitumor activity in vivo (Song et al. Blood. 2012; 119(3):696-706). Further examples of such costimulatory molecules include CDS, ICAM-1, GITR, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRFl), NKp44, NKp30, NKp46, CD160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CDlld, ITGAE, CD103, ITGAL, CDl la, LFA-1, ITGAM, CDllb, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), NKG2D, CEACAMl, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, and PAG/Cbp.

[00258] The intracellular signaling domains within the cytoplasmic portion of the CAR of the invention may be linked to each other in a random or specified order. Optionally, a short oligo- or polypeptide linker, for example, between 2 and 10 amino acids (e.g., 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids) in length may form the linkage between intracellular signaling domains. In one embodiment, a glycine-serine doublet can be used as a suitable linker. In one embodiment, a single amino acid, e.g., an alanine, a glycine, can be used as a suitable linker.

[00259] In one aspect, the intracellular signaling domain is designed to comprise two or more, e.g., 2, 3, 4, 5, or more, costimulatory signaling domains. In an embodiment, the two or more, e.g., 2, 3, 4, 5, or more, costimulatory signaling domains, are separated by a linker molecule, e.g., a linker molecule described herein. In one embodiment, the intracellular signaling domain comprises two costimulatory signaling domains. In some embodiments, the linker molecule is a glycine residue. In some embodiments, the linker is an alanine residue.

[00260] In one aspect, the intracellular signaling domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of CD28. In one aspect, the intracellular signaling domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of 4-1BB. In one aspect, the signaling domain of 4-1BB is a signaling domain of SEQ ID NO: 16. In one aspect, the signaling domain of CD3-zeta is a signaling domain of SEQ ID NO: 17.

[00261] In one aspect, the intracellular signaling domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of CD27. In one aspect, the signaling domain of CD27 comprises an amino acid sequence of QRRKYRSNKGESPVEPAEPCRYSCPREEEGSTIPIQEDYRKPEPACSP (SEQ ID NO: 8). In one aspect, the signalling domain of CD27 is encoded by a nucleic acid sequence of AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCGCCG CCCCGGGCCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAGC CTATCGCTCC (SEQ ID NO: 19).

[00262] In one aspect, the CAR-expressing cell described herein can further comprise a second CAR, e.g., a second CAR that includes a different antigen binding domain, e.g., to the same target (mesothelin) or a different target (e.g., a target other than mesothelin on stroma cells, e.g., FAP; a target other than mesothelin on prostate cancer cells, e.g., androgen receptor, OR51E2, PSMA, PSCA, PDGRF-β, TARP, GloboH, MAD-CT-1, or MAD-CT-2; a target other than mesothelin on ovararian cancer cells, e.g., Tn, PRSS21, CD171, Lewis Y, folate receptor a, claudin6, GloboH, or sperm protein 17, e.g., a target other than mesothelin on lung cancer cells, e.g.,VEGF, HER3, IGF-IR, EGFR, DLL4, or Trop-2). In one embodiment, the CAR-expressing cell comprises a first CAR that targets a first antigen and includes an intracellular signaling domain having a costimulatory signaling domain but not a primary signaling domain, and a second CAR that targets a second, different, antigen and includes an intracellular signaling

domain having a primary signaling domain but not a costimulatory signaling domain. Placement of a costimulatory signaling domain, e.g., 4-1BB, CD28, CD27 or OX-40, onto the first CAR, and the primary signaling domain, e.g., CD3 zeta, on the second CAR can limit the CAR activity to cells where both targets are expressed. In one embodiment, the CAR expressing cell comprises a first mesothelin CAR that includes a mesothelin binding domain, a transmembrane domain and a costimulatory domain and a second CAR that targets an antigen other than mesothelin (e.g., a target other than mesothelin on stroma cells, e.g., FAP; a target other than mesothelin on prostate cancer cells, e.g., androgen receptor, OR51E2, PSMA, PSCA, PDGRF-β, TARP, GloboH, MAD-CT-1, or MAD-CT-2; a target other than mesothelin on ovararian cancer cells, e.g., Tn, PRSS21, CD171, Lewis Y, folate receptor a, claudin6, GloboH, or sperm protein 17, e.g., a target other than mesothelin on lung cancer cells, e.g.,VEGF, HER3, IGF-1R, EGFR, DLL4, or Trop-2) and includes an antigen binding domain, a transmembrane domain and a primary signaling domain. In another embodiment, the CAR expressing cell comprises a first mesothelin CAR that includes a mesothelin binding domain, a transmembrane domain and a primary signaling domain and a second CAR that targets an antigen other than mesothelin (e.g., a target other than mesothelin on stroma cells, e.g., FAP; a target other than mesothelin on prostate cancer cells, e.g., androgen receptor, OR51E2, PSMA, PSCA, PDGRF-β, TARP, GloboH, MAD-CT-1, or MAD-CT-2; a target other than mesothelin on ovararian cancer cells, e.g., Tn, PRSS21, CD171, Lewis Y, folate receptor a, claudin6, GloboH, or sperm protein 17, e.g., a target other than mesothelin on lung cancer cells, e.g.,VEGF, HER3, IGF-1R, EGFR, DLL4, or Trop-2) and includes an antigen binding domain to the antigen, a transmembrane domain and a costimulatory signaling domain.

[00263] In one embodiment, the CAR-expressing cell comprises a mesothelin CAR described herein and an inhibitory CAR. In one embodiment, the inhibitory CAR comprises an antigen binding domain that binds an antigen found on normal cells but not cancer cells, e.g., normal cells that also express mesothelin. In one embodiment, the inhibitory CAR comprises the antigen binding domain, a transmembrane domain and an intracellular domain of an inhibitory molecule. For example, the intracellular domain of the inhibitory CAR can be an intracellular domain of PD1, PD-L1, CTLA4, TFM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 and TGFRbeta.

[00264] In one embodiment, when the CAR-expressing cell comprises two or more different CARs, the antigen binding domains of the different CARs can be such that the antigen binding domains do not interact with one another. For example, a cell expressing a first and second CAR can have an antigen binding domain of the first CAR, e.g., as a fragment, e.g., an scFv, that does not form an association with the antigen binding domain of the second CAR, e.g., the antigen binding domain of the second CAR is a VHH.

[00265] In some embodiments, the antigen binding domain comprises a single domain antigen binding (SDAB) molecules include molecules whose complementary determining regions are part of a single domain polypeptide. Examples include, but are not limited to, heavy chain variable domains, binding molecules naturally devoid of light chains, single domains derived from conventional 4-chain antibodies, engineered domains and single domain scaffolds other than those derived from antibodies. SDAB molecules may be any of the art, or any future single domain molecules. SDAB molecules may be derived from any species including, but not limited to mouse, human, camel, llama, lamprey, fish, shark, goat, rabbit, and bovine. This term also includes naturally occurring single domain antibody molecules from species other than Camelidae and sharks.

[00266] In one aspect, an SDAB molecule can be derived from a variable region of the immunoglobulin found in fish, such as, for example, that which is derived from the immunoglobulin isotype known as Novel Antigen Receptor (NAR) found in the serum of shark. Methods of producing single domain molecules derived from a variable region of NAR ("IgNARs") are described in WO 03/014161 and Streltsov (2005) Protein Sci. 14:2901-2909.

[00267] According to another aspect, an SDAB molecule is a naturally occurring single domain antigen binding molecule known as heavy chain devoid of light chains. Such single domain molecules are disclosed in WO 9404678 and Hamers-Casterman, C. et al. (1993) Nature 363:446-448, for example. For clarity reasons, this variable domain derived from a heavy chain molecule naturally devoid of light chain is known herein as a VHH or nanobody to distinguish it from the conventional VH of four chain immunoglobulins. Such a VHH molecule can be derived from Camelidae species, for example in camel, llama, dromedary, alpaca and guanaco. Other species besides Camelidae may produce heavy chain molecules naturally devoid of light chain; such VHHs are within the scope of the invention.

[00268] The SDAB molecules can be recombinant, CDR-grafted, humanized, camelized, de-immunized and/or in vitro generated (e.g., selected by phage display).

[00269] It has also been discovered, that cells having a plurality of chimeric membrane embedded receptors comprising an antigen binding domain that interactions between the antigen binding domain of the receptors can be undesirable, e.g., because it inhibits the ability of one or more of the antigen binding domains to bind its cognate antigen. Accordingly, disclosed herein are cells having a first and a second non-naturally occurring chimeric membrane embedded receptor comprising antigen binding domains that minimize such interactions. Also disclosed herein are nucleic acids encoding a first and a second non-naturally occurring chimeric membrane embedded receptor comprising an antigen binding domains that minimize such interactions, as well as methods of making and using such cells and nucleic acids. In an embodiment the antigen binding domain of one of the first and the second non-naturally occurring chimeric membrane embedded receptor, comprises an scFv, and the other comprises a single VH domain, e.g., a camelid, shark, or lamprey single VH domain, or a single VH domain derived from a human or mouse sequence.

[00270] In some embodiments, the claimed invention comprises a first and second CAR, wherein the antigen binding domain of one of the first and the second CAR does not comprise a variable light domain and a variable heavy domain. In some embodiments, the antigen binding domain of one of the first and the second CAR is an scFv, and the other is not an scFv. In some embodiments, the antigen binding domain of one of the first and the second CAR comprises a single VH domain, e.g., a camelid, shark, or lamprey single VH domain, or a single VH domain derived from a human or mouse sequence. In some embodiments, the antigen binding domain of one of the first and the second CAR comprises a nanobody. In some embodiments, the antigen binding domain of one of the first and the second CAR comprises a camelid VHH domain.

[00271] In some embodiments, the antigen binding domain of one of the first and thesecond CAR comprises an scFv, and the other comprises a single VH domain, e.g., a camelid, shark, or lamprey single VH domain, or a single VH domain derived from a human or mouse sequence. In some embodiments, the antigen binding domain of one of the first and the second CAR comprises an scFv, and the other comprises a nanobody. In some embodiments, the antigen

binding domain of one of the first and the second CAR comprises comprises an scFv, and the other comprises a camelid VHH domain.

[00272] In some embodiments, when present on the surface of a cell, binding of the antigen binding domain of the first CAR to its cognate antigen is not substantially reduced by the presence of the second CAR. In some embodiments, binding of the antigen binding domain of the first CAR to its cognate antigen in the presence of the second CAR is 85%, 90%, 95%, 96%, 97%, 98% or 99% of binding of the antigen binding domain of the first CAR to its cognate antigen in the absence of the second CAR.

[00273] In some embodiments, when present on the surface of a cell, the antigen binding domains of the first and the second CAR, associate with one another less than if both were scFv antigen binding domains. In some embodiments, the antigen binding domains of the first and the second CAR, associate with one another 85%, 90%, 95%, 96%, 97%, 98% or 99% less than if both were scFv antigen binding domains.

[00274] In another aspect, the CAR-expressing cell described herein can further express another agent, e.g., an agent which enhances the activity or fitness of a CAR-expressing cell. For example, in one embodiment, the agent can be an agent which inhibits a molecule that modulates or regulates, e.g., inhibits, T cell function. In some embodiments, the molecule that modulates or regulates T cell function is an inhibitory molecule. Inhibitory molecules, e.g., PDl, can, in some embodiments, decrease the ability of a CAR-expressing cell to mount an immune effector response. Examples of inhibitory molecules include PDl, PD-L1, CTLA4, TEVI3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIRl, CD 160, 2B4 and TGFR beta. In embodiments, an agent, e.g., an inhibitory nucleic acid, e.g., a dsRNA, e.g., an siRNA or shRNA; or e.g., an inhibitory protein or system, e.g., a clustered regularly interspaced short palindromic repeats (CRISPR), a transcription-activator like effector nuclease (TALEN), or a zinc finger endonuclease (ZFN), e.g., as described herein, can be used to inhibit expression of a molecule that modulates or regulates, e.g., inhibits, T-cell function in the CAR-expressing cell. In an embodiment the agent is an shRNA, e.g., an shRNA described herein. In an embodiment, the agent that modulates or regulates, e.g., inhibits, T-cell function is inhibited within a CAR-expressing cell. For example, a dsRNA molecule that

inhibits expression of a molecule that modulates or regulates, e.g., inhibits, T-cell function is linked to the nucleic acid that encodes a component, e.g., all of the components, of the CAR.

[00275] In one embodiment, the agent which inhibits an inhibitory molecule comprises a first polypeptide, e.g., an inhibitory molecule, associated with a second polypeptide that provides a positive signal to the cell, e.g., an intracellular signaling domain described herein. In one embodiment, the agent comprises a first polypeptide, e.g., of an inhibitory molecule such as PDl, PD-Ll, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 and TGFR beta T, or a fragment of any of these (e.g., at least a portion of an extracellular domain of any of these), and a second polypeptide which is an intracellular signaling domain described herein (e.g., comprising a costimulatory domain (e.g., 41BB, CD27 or CD28, e.g., as described herein) and/or a primary signaling domain (e.g., a CD3 zeta signaling domain described herein). In one embodiment, the agent comprises a first polypeptide of PDl or a fragment thereof (e.g., at least a portion of an extracellular domain of PDl), and a second polypeptide of an intracellular signaling domain described herein (e.g., a CD28 signaling domain described herein and/or a CD3 zeta signaling domain described herein). PDl is an inhibitory member of the CD28 family of receptors that also includes CD28, CTLA-4, ICOS, and BTLA. PD-1 is expressed on activated B cells, T cells and myeloid cells (Agata et al. 1996 Int. Immunol 8:765-75). Two ligands for PDl, PD-Ll and PD-L2 have been shown to downregulate T cell activation upon binding to PDl (Freeman et a. 2000 J Exp Med 192:1027-34; Latchman et al. 2001 Nat Immunol 2:261-8; Carter et al. 2002 Eur J Immunol 32:634-43). PD-L1 is abundant in human cancers (Dong et al. 2003 J Mol Med 81:281-7; Blank et al. 2005 Cancer Immunol. Immunother 54:307-314; Konishi et al. 2004 Clin Cancer Res 10:5094). Immune suppression can be reversed by inhibiting the local interaction of PDl with PD-Ll.

[00276] In one embodiment, the agent comprises the extracellular domain (ECD) of an inhibitory molecule, e.g., Programmed Death 1 (PDl), can be fused to a transmembrane domain and intracellular signaling domains such as 4IBB and CD3 zeta (also referred to herein as a PDl CAR). In one embodiment, the PDl CAR, when used in combinations with a mesothelin CAR described herein, improves the persistence of the T cell. In one embodiment, the CAR is a PDl CAR comprising the extracellular domain of PDl indicated as underlined in SEQ ID NO: 24 and a signal sequence at amino acids 1-21 of SEQ ID NO:24. In one embodiment, the PDl CAR comprises the amino acid sequence of SEQ ID NO:24.

[00277] Malpvtalllplalllhaarppgwfldspdrpwnpptfspallwtegdnatftcsfsntsesfylnwyrmspsnqtdkl aafpedrsqpgqdcrfrvtqlpngrdfhmswrarrndsgtylcgaislapkaqi keslraelrvterraevptahpspsprpagqfqtlvt ttpaprpptpaptiasqplslrpeacrpaaggavhtrgldfacdiyiwaplagtcgvlllsl^ itlyckrgrkkllyifkqpfmrpvqttqeed gcscrfpeeeeggcelrvkfsrsadapaykqgqnqlynelnlgrreeydvldkrrgrdpemggkprrknpqeglynelqkdkmaea yseigmkgerrrgkghdglyqglstatkdtydalhmqalppr (SEQ ID NO:24).

[00278] In one embodiment, the PD1 CAR without the N-terminal signal sequence comprises the amino acid sequence provided below (SEQ ID NO:22).

[00279] pgwfldspdrpwnpptfspallvvtegdnatftcsfsntsesfylnwyrmspsnqtdklaafpedrsqpgqdcrfrvtq lpngrdmmswrarrndsgtylcgaislapkaqikeslraelrvterraevptahpspsprpagqfqtlvtttpaprpptpaptiasqplslr peacrpaaggavhtrgldfacdiyiwaplagtcgvlllslvitlyckrgrkkllyiftqpfmrpvqttqeedgcscrfpeeeeggcelrvkf srsadapaykqgqnqlynelnlgrreeydvldkrrgrdpemggkprrknpqeglynelqkdkmaeayseigmkgerrrgkghdgl yqglstatkdtydalhmqalppr (SEQ ID NO:22).

[00280] In one embodiment, the agent comprises a nucleic acid sequence encoding the PD1 CAR with the N-terminal signal sequence, e.g., the PD1 CAR described herein. In one embodiment, the nucleic acid sequence for the PD1 CAR is shown below, with the PD1 ECD underlined below in SEQ ID NO: 23

[0028 1] atggccctccctgtcactgccctgcttctcccccctcgcactcctgctccacgccgctagacca cccggatggtttctggactc tccggatcgcccgtggaatcccccaaccttctcaccggcactcttggttgtgactgagggcgataatgcgaccttcacgtgctcgttctccaa cacctccgaatcattcgtgctgaactggtaccgcatgagcccgtcaaaccagaccgacaagctcgccgcgtttccggaagatcggtcgcaa ccgggacaggattgtcggttccgcgtgactcaactgccgaatggcagagacttccacatgagcgtggtccgcgctaggcgaaacgactcc gggacctacctgtgcggagccatctcgctggcgcctaaggcccaaatcaaagagagcttgagggccgaactgagagtgaccgagcgca gagctgaggtgccaactgcacatccatccccatcgcctcggcctgcggggcagtttcagaccctggtcacgaccactccggcgccgcgc ccaccgactccggccccaactatcgcgagccagcccctgtcgctgaggccggaagcatgccgccctgccgccggaggtgctgtgcatac ccggggattggacttcgcatgcgacatctacatttgggctcctctcgccggaacttgtggcgtgctccttctgtccctggtcatcaccctgtact gcaagcggggtcggaaaaagcttctgtacattttcaagcagcccttcatgaggcccgtgcaaaccacccaggaggaggacggttgctcct gccggttccccgaagaggaagaaggaggttgcgagctgcgcgtgaagttctcccggagcgccgacgcccccgcctataagcagggcca gaaccagctgtacaacgaactgaacctgggacggcgggaagagtacgatgtgctggacaagcggcgcggccgggaccccgaaatggg cgggaagcctagaagaaagaaccctcaggaaggcctgtataacgagctgcagaaggacaagatggccgaggcctactccgaaattggg atgaagggagagcggcggaggggaaaggggcacgacggcctgtaccaaggactgtccaccgccaccaaggacacatacgatgccct gcacatgcaggcccttcccccctcgc (SEQ ID NO: 23).

[00282] In another aspect, the present invention provides a population of CAR-expressing cells, e.g., CART cells. In some embodiments, the population of CAR-expressing cells comprises a mixture of cells expressing different CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CAR having an anti-CD 19 binding domain described herein, and a second cell expressing a CAR having a different anti- CD 19 binding domain, e.g., an anti-mesothelin binding domain described herein that differs from the anti-mesothelin binding domain in the CAR expressed by the first cell. As another example, the population of CAR-expressing cells can include a first cell expressing a CAR that includes an anti- mesothelin binding domain, e.g., as described herein, and a second cell expressing a CAR that includes an antigen binding domain to a target other than mesothelin (e.g., a target other than mesothelin on stroma cells, e.g., FAP; a target other than mesothelin on prostate cancer cells, e.g., androgen receptor, OR51E2, PSMA, PSCA, PDGRF- β, TARP, GloboH, MAD-CT-1, or MAD-CT-2; a target other than mesothelin on ovararian cancer cells, e.g., Tn, PRSS21, CD171, Lewis Y, folate receptor a, claudin6, GloboH, or sperm protein 17, e.g., a target other than mesothelin on lung cancer cells, e.g.,VEGF, HER3, IGF-1R, EGFR, DLL4, or Trop-2). In one embodiment, the population of CAR-expressing cells includes, e.g., a first cell expressing a CAR that includes a primary intracellular signaling domain, and a second cell expressing a CAR that includes a secondary signaling domain.

[00283] In another aspect, the present invention provides a population of cells wherein at least one cell in the population expresses a CAR having an anti-mesothelin binding domain described herein, and a second cell expressing another agent, e.g., an agent which enhances the activity or function of a CAR-expressing cell. For example, in one embodiment, the agent can be an agent which modulates or regulates, e.g., inhibits, T cell function. In some embodiments, the molecule that modulates or regulates T cell function is an inhibitory molecule, e.g., an agent described herein. Inhibitory molecules, e.g., can, in some embodiments, decrease the ability of a CAR-expressing cell to mount an immune effector response. Examples of inhibitory molecules include PD1, PD-L1, CTLA4, TEVI3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 and TGFR beta. In one embodiment, the agent which inhibits an inhibitory molecule comprises a first polypeptide, e.g., an inhibitory molecule, associated with a second polypeptide that provides a positive signal to the cell, e.g., an intracellular signaling domain described herein. In one embodiment, the agent

comprises a first polypeptide, e.g., of an inhibitory molecule such as PD1, PD-L1, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 and TGFR beta, or a fragment of any of these (e.g., at least a portion of an extracellular domain of any of these), and a second polypeptide which is an intracellular signaling domain described herein (e.g., comprising a costimulatory domain (e.g., 41BB, CD27 or CD28, e.g., as described herein) and/or a primary signaling domain (e.g., a CD3 zeta signaling domain described herein). In one embodiment, the agent comprises a first polypeptide of PD1 or a fragment thereof (e.g., at least a portion of the extracellular domain of PD1), and a second polypeptide of an intracellular signaling domain described herein (e.g., a CD28 signaling domain described herein and/or a CD3 zeta signaling domain described herein).

[00284] In one aspect, the present invention provides methods comprising administering a population of CAR-expressing cells, e.g., CART cells, e.g., a mixture of cells expressing different CARs, in combination with another agent, e.g., a kinase inhibitor, such as a kinase inhibitor described herein. In another aspect, the present invention provides methods comprising administering a population of cells wherein at least one cell in the population expresses a CAR having an anti-mesoothelinbinding domain as described herein, and a second cell expressing another agent, e.g., an agent which enhances the activity or fitness of a CAR-expressing cell, in combination with another agent, e.g., a kinase inhibitor, such as a kinase inhibitor described herein.

Regulatable Chimeric Antigen Receptors

[00285] In some embodiments, a regulatable CAR (RCAR) where the CAR activity can be controlled is desirable to optimize the safety and efficacy of a CAR therapy. There are many ways CAR activities can be regulated. For example, inducible apoptosis using, e.g., a caspase fused to a dimerization domain (see, e.g., Di et al, N Egnl. J. Med. 201 1 Nov. 3; 365(18): 1673-1683), can be used as a safety switch in the CAR therapy of the instant invention. In an aspect, a RCAR comprises a set of polypeptides, typically two in the simplest embodiments, in which the components of a standard CAR described herein, e.g., an antigen binding domain and an intracellular signaling domain, are partitioned on separate polypeptides or members. In some embodiments, the set of polypeptides include a dimerization switch that, upon the presence of a

dimerization molecule, can couple the polypeptides to one another, e.g., can couple an antigen binding domain to an intracellular signaling domain.

[00286] In an aspect, an RCAR comprises two polypeptides or members: 1) an intracellular signaling member comprising an intracellular signaling domain, e.g., a primary intracellular signaling domain described herein, and a first switch domain; 2) an antigen binding member comprising an antigen binding domain, e.g., that targets mesothelin as described herein, and a second switch domain. Optionally, the RCAR comprises a transmembrane domain described herein. In an embodiment, a transmembrane domain can be disposed on the intracellular signaling member, on the antigen binding member, or on both. (Unless otherwise indicated, when members or elements of an RCAR are described herein, the order can be as provided, but other orders are included as well. In other words, in an embodiment, the order is as set out in the text, but in other embodiments, the order can be different. E.g., the order of elements on one side of a transmembrane region can be different from the example, e.g., the placement of a switch domain relative to a intracellular signaling domain can be different, e.g., reversed).

[00287] In an embodiment, the first and second switch domains can form an intracellular or an extracellular dimerization switch. In an embodiment, the dimerization switch can be a homodimerization switch, e.g., where the first and second switch domain are the same, or a heterodimerization switch, e.g., where the first and second switch domain are different from one another.

[00288] In embodiments, an RCAR can comprise a "multi switch." A multi switch can comprise heterodimerization switch domains or homodimerization switch domains. A multi switch comprises a plurality of, e.g., 2, 3, 4, 5, 6, 7, 8, 9, or 10, switch domains, independently, on a first member, e.g., an antigen binding member, and a second member, e.g., an intracellular signaling member. In an embodiment, the first member can comprise a plurality of first switch domains, e.g., FKBP-based switch domains, and the second member can comprise a plurality of second switch domains, e.g., FRB-based switch domains. In an embodiment, the first member can comprise a first and a second switch domain, e.g., a FKBP-based switch domain and a FRB-based switch domain, and the second member can comprise a first and a second switch domain, e.g., a FKBP-based switch domain and a FRB-based switch domain.

[00289] In an embodiment, the intracellular signaling member comprises one or more intracellular signaling domains, e.g., a primary intracellular signaling domain and one or more costimulatory signaling domains.

[00290] In an embodiment, the antigen binding member may comprise one or more intracellular signaling domains, e.g., one or more costimulatory signaling domains. In an embodiment, the antigen binding member comprises a plurality, e.g., 2 or 3 costimulatory signaling domains described herein, e.g., selected from 41BB, CD28, CD27, ICOS, and OX40, and in embodiments, no primary intracellular signaling domain. In an embodiment, the antigen binding member comprises the following costimulatory signaling domains, from the extracellular to intracellular direction: 41BB-CD27; 41BB-CD27; CD27-41BB; 41BB-CD28; CD28-41BB; OX40-CD28; CD28-OX40; CD28-41BB; or 41BB-CD28. In such embodiments, the intracellular binding member comprises a CD3zeta domain. In one such embodiment the RCAR comprises (1) an antigen binding member comprising, an antigen binding domain, e.g., described herein, a transmembrane domain, and two costimulatory domains and a first switch domain; and (2) an intracellular signaling domain comprising a transmembrane domain or membrane tethering domain and at least one primary intracellular signaling domain, and a second switch domain.

[00291] An embodiment provides RCARs wherein the antigen binding member is not tethered to the surface of the CAR cell. This allows a cell having an intracellular signaling member to be conveniently paired with one or more antigen binding domains, without transforming the cell with a sequence that encodes the antigen binding member. In such embodiments, the RCAR comprises: 1) an intracellular signaling member comprising: a first switch domain, a transmembrane domain, an intracellular signaling domain, e.g., a primary intracellular signaling domain, and a first switch domain; and 2) an antigen binding member comprising: an antigen binding domain, e.g., described herein, and a second switch domain, wherein the antigen binding member does not comprise a transmembrane domain or membrane tethering domain, and, optionally, does not comprise an intracellular signaling domain. In some embodiments, the RCAR may further comprise 3) a second antigen binding member comprising: a second antigen binding domain, e.g., a second antigen binding domain that binds a different antigen than is bound by the antigen binding domain; and a second switch domain.

[00292] Also provided herein are RCARs wherein the antigen binding member comprises bispecific activation and targeting capacity. In this embodiment, the antigen binding member can comprise a plurality, e.g., 2, 3, 4, or 5 antigen binding domains, e.g., scFvs, wherein each antigen binding domain binds to a target antigen, e.g. different antigens or the same antigen, e.g., the same or different epitopes on the same antigen. In an embodiment, the plurality of antigen binding domains are in tandem, and optionally, a linker or hinge region is disposed between each of the antigen binding domains. Suitable linkers and hinge regions are described herein.

[00293] An embodiment provides RCARs having a configuration that allows switching of proliferation. In this embodiment, the RCAR comprises: 1) an intracellular signaling member comprising: optionally, a transmembrane domain or membrane tethering domain; one or more co-stimulatory signaling domain, e.g., selected from 41BB, CD28, CD27, ICOS, and OX40, and a switch domain; and 2) an antigen binding member comprising: an antigen binding domain, e.g., described herein, a transmembrane domain, and a primary intracellular signaling domain, e.g., a CD3zeta domain, wherein the antigen binding member does not comprise a switch domain, or does not comprise a switch domain that dimerizes with a switch domain on the intracellular signaling member. In an embodiment, the antigen binding member does not comprise a co-stimulatory signaling domain. In an embodiment, the intracellular signaling member comprises a switch domain from a homodimerization switch. In an embodiment, the intracellular signaling member comprises a first switch domain of a heterodimerization switch and the RCAR comprises a second intracellular signaling member which comprises a second switch domain of the heterodimerization switch. In such embodiments, the second intracellular signaling member comprises the same intracellular signaling domains as the intracellular signaling member. In an embodiment, the dimerization switch is intracellular. In an embodiment, the dimerization switch is extracellular.

[00294] In any of the RCAR configurations described here, the first and second switch domains comprise a FKBP/FRB-based switch as described herein.

[00295] Also provided herein are cells comprising an RCAR described herein. Any cell that is engineered to express a RCAR can be used as a RCARX cell. In an embodiment the RCARX cell is a T cell, and is referred to as a RCART cell. In an embodiment the RCARX cell is an NK cell, and is referred to as a RCARN cell.

[00296] Also provided herein are nucleic acids and vectors comprising RCAR encoding sequences. Sequence encoding various elements of an RCAR can be disposed on the same nucleic acid molecule, e.g., the same plasmid or vector, e.g., viral vector, e.g., lentiviral vector. In an embodiment, (i) sequence encoding an antigen binding member and (ii) sequence encoding an intracellular signaling member, can be present on the same nucleic acid, e.g., vector. Production of the corresponding proteins can be achieved, e.g., by the use of separate promoters, or by the use of a bicistronic transcription product (which can result in the production of two proteins by cleavage of a single translation product or by the translation of two separate protein products). In an embodiment, a sequence encoding a cleavable peptide, e.g., a P2A or F2A sequence, is disposed between (i) and (ii). In an embodiment, a sequence encoding an IRES, e.g., an EMCV or EV71 IRES, is disposed between (i) and (ii). In these embodiments, (i) and (ii) are transcribed as a single RNA. In an embodiment, a first promoter is operably linked to (i) and a second promoter is operably linked to (ii), such that (i) and (ii) are transcribed as separate mRNAs.

[00297] Alternatively, the sequence encoding various elements of an RCAR can be disposed on the different nucleic acid molecules, e.g., different plasmids or vectors, e.g., viral vector, e.g., lentiviral vector. E.g., the (i) sequence encoding an antigen binding member can be present on a first nucleic acid, e.g., a first vector, and the (ii) sequence encoding an intracellular signaling member can be present on the second nucleic acid, e.g., the second vector.

Dimerization switches

[00298] Dimerization switches can be non-covalent or covalent. In a non-covalent dimerization switch, the dimerization molecule promotes a non-covalent interaction between the switch domains. In a covalent dimerization switch, the dimerization molecule promotes a covalent interaction between the switch domains.

[00299] In an embodiment, the RCAR comprises a FKBP/FRAP, or FKBP/FRB, -based dimerization switch. FKBP12 (FKBP, or FK506 binding protein) is an abundant cytoplasmic protein that serves as the initial intracellular target for the natural product immunosuppressive drug, rapamycin. Rapamycin binds to FKBP and to the large PI3K homolog FRAP (RAFT, mTOR). FRB is a 93 amino acid portion of FRAP, that is sufficient for binding the FKBP-rapamycin complex (Chen, J., Zheng, X. F., Brown, E. J. & Schreiber, S. L. (1995) *Identification*

of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. Proc Natl Acad Sci U S A 92: 4947-51.)

[00300] In embodiments, an FKBP/FRAP, e.g., an FKBP/FRB, based switch can use a dimerization molecule, e.g., rapamycin or a rapamycin analog.

[00301] The amino acid sequence of FKBP is as follows:

D V P D Y A S L G G P S S P K K K R K V S R G V Q V E T I S P G D G R T F P K R G Q T C V V H Y T G M L E D G K K F D S S R D R N K P F K F M L G K Q E V I R G W E E G V A Q M S V G Q R A K L T I S P D Y A Y G A T G H P G I I P P H A T L V F D V E L L K L E T S Y (SEQ ID NO: 382)

[00302] In embodiments, an FKBP switch domain can comprise a FRB binding fragment of FKBP, e.g., the underlined portion of SEQ ID NO: 382, which is:

V Q V E T I S P G D G R T F P K R G Q T C V V H Y T G M L E D G K K F D S S R D R N K P F K F M L G K Q E V I R G W E E G V A Q M S V G Q R A K L T I S P D Y A Y G A T G H P G I I P P H A T L V F D V E L L K L E T S (SEQ ID NO:383)

[00303] The amino acid sequence of FRB is as follows:

ILWHEMWHEG LEEASRLYFG ERNVKGMFEV LEPLHAMMER GPQTLKETS F NQAYGRDLME AQEWCRKYMK SGNVKDLTQA WDLYYHVFRR ISK (SEQ ID NO: 384)

[00304] "FKBP/FRAP, e.g., an FKPP/FRB, based switch" as that term is used herein, refers to a dimerization switch comprising: a first switch domain, comprises an FRB binding fragment or an FKBP analog, e.g., RAD001, and has at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identity with, or differs by no more than 30, 25, 20, 15, 10, 5, 4, 3, 2, or 1 amino acid residues from, the FKBP sequence of SEQ ID NO: 382 or 383; and a second switch domain, which comprises an FKBP binding fragment or an FRB analog, and has at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identity with, or differs by no more than 30, 25, 20, 15, 10, 5, 4, 3, 2, or 1 amino acid residues from, the FRB sequence of SEQ ID NO: 384. In an embodiment, a RCAR described herein comprises one switch domain comprises amino acid residues disclosed in SEQ ID NO: 382 (or SEQ ID NO:383), and one switch domain comprises amino acid residues disclosed in SEQ ID NO: 384.

[00305] In embodiments, the FKBP/FRB dimerization switch comprises a modified FRB switch domain that exhibits altered, e.g., increased, affinity for the dimerization molecule, e.g., rapamycin or a rapalogue, e.g., RAD001. In an embodiment, the modified FRB switch domain comprises one or more mutations, e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more, selected from mutations at amino acid position(s)L203 1, E2032, S2035, R2036, F2039, G2040, T2098, W2101, D2102, Y2105, and F2108, where the wild-type amino acid is mutated to any other naturally-occurring amino acid. In an embodiment, a mutant FRB comprises a mutation at E2032, where E2032 is mutated to phenylalanine (E2032F), methionine (E2032M), arginine (E2032R), valine (E2032V), tyrosíne (E2032Y), isoleucine (E2032I), e.g., SEQ ID NO: 385, or leucine (E2032L), e.g., SEQ ID NO: 386. In an embodiment, a mutant FRB comprises a mutation at T2098, where T2098 is mutated to phenylalanine (T2098F) or leucine (T2098L), e.g., SEQ ID NO: 387. In an embodiment, a mutant FRB comprises a mutation at E2032 and at T2098, where E2032 is mutated to any amino acid, and where T2098 is mutated to any amino acid, e.g., SEQ ID NO: 388. In an embodiment, a mutant FRB comprises an E2032I and a T2098L mutation, e.g., SEQ ID NO: 389. In an embodiment, a mutant FRB comprises an E2032L and a T2098L mutation, e.g., SEQ ID NO: 340.

[00306] Table 15. Exemplary mutant FRB having increased affinity for a dimerization molecule.

FRB mutant	Amino Acid Sequence	SEQ ID NO:
E2032I mutant	ILWHEMWHEGLIEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRISKTS	385
E2032L mutant	ILWHEMWHEGLLEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRISKTS	386
T2098L mutant	ILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLLQAWDLYYHVFRRISKTS	387
E2032, T2098 mutant	ILWHEMWHEGL**X**EASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDL**X**QAWDLYYHVFRRISKTS	388
E2032I, T2098L mutant	ILWHEMWHEGLIEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLLQAWDLYYHVFRRISKTS	389
E2032L, T2098L mutant	ILWHEMWHEGLLEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLLQAWDLYYHVFRRISKTS	390

[00307] Other suitable dimerization switches include a GyrB-GyrB based dimerization switch, a Gibberellin-based dimerization switch, a tag/binder dimerization switch, and a halo-tag/snap-

tag dimerization switch. Following the guidance provided herein, such switches and relevant dimerization molecules will be apparent to one of ordinary skill.

Dimerization molecule

[00308] Association between the switch domains is promoted by the dimerization molecule. In the presence of dimerization molecule interaction or association between switch domains allows for signal transduction between a polypeptide associated with, e.g., fused to, a first switch domain, and a polypeptide associated with, e.g., fused to, a second switch domain. In the presence of non-limiting levels of dimerization molecule signal transduction is increased by 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 5, 10, 50, 100 fold, e.g., as measured in a system described herein.

[00309] Rapamycin and rapamycin analogs (sometimes referred to as rapalogues), e.g., RAD001, can be used as dimerization molecules in a FKBP/FRB-based dimerization switch described herein. In an embodiment the dimerization molecule can be selected from rapamycin (sirolimus), RAD001 (everolimus), zotarolimus, temsirolimus, AP-23573 (ridaforolimus), biolimus and AP21967. Additional rapamycin analogs suitable for use with FKBP/FRB-based dimerization switches are further described in the section entitled "Combination Therapies", or in the subsection entitled "Exemplary mTOR inhibitors".

RNA Transfection

[00310] Disclosed herein are methods for producing an in vitro transcribed RNA CAR. The present invention also includes a CAR encoding RNA construct that can be directly transfected into a cell. A method for generating mRNA for use in transfection can involve in vitro transcription (IVT) of a template with specially designed primers, followed by polyA addition, to produce a construct containing 3' and 5' untranslated sequence ("UTR"), a 5' cap and/or Internal Ribosome Entry Site (IRES), the nucleic acid to be expressed, and a polyA tail, typically 50-2000 bases in length (SEQ ID NO:35). RNA so produced can efficiently transfect different kinds of cells. In one aspect, the template includes sequences for the CAR.

[00311] In one aspect the mesothelin CAR is encoded by a messenger RNA (mRNA). In one aspect the mRNA encoding the mesothelin CAR is introduced into a T cell for production of a CART cell.

[00312] In one embodiment, the in vitro transcribed RNA CAR can be introduced to a cell as a form of transient transfection. The RNA is produced by in vitro transcription using a polymerase chain reaction (PCR)-generated template. DNA of interest from any source can be directly converted by PCR into a template for in vitro mRNA synthesis using appropriate primers and RNA polymerase. The source of the DNA can be, for example, genomic DNA, plasmid DNA, phage DNA, cDNA, synthetic DNA sequence or any other appropriate source of DNA. The desired temple for in vitro transcription is a CAR of the present invention. For example, the template for the RNA CAR comprises an extracellular region comprising a single chain variable domain of an anti-tumor antibody; a hinge region, a transmembrane domain (e.g., a transmembrane domain of CD8a); and a cytoplasmic region that includes an intracellular signaling domain, e.g., comprising the signaling domain of CD3-zeta and the signaling domain of 4-1BB.

[00313] In one embodiment, the DNA to be used for PCR contains an open reading frame. The DNA can be from a naturally occurring DNA sequence from the genome of an organism. In one embodiment, the nucleic acid can include some or all of the 5' and/or 3' untranslated regions (UTRs). The nucleic acid can include exons and introns. In one embodiment, the DNA to be used for PCR is a human nucleic acid sequence. In another embodiment, the DNA to be used for PCR is a human nucleic acid sequence including the 5' and 3' UTRs. The DNA can alternatively be an artificial DNA sequence that is not normally expressed in a naturally occurring organism. An exemplary artificial DNA sequence is one that contains portions of genes that are ligated together to form an open reading frame that encodes a fusion protein. The portions of DNA that are ligated together can be from a single organism or from more than one organism.

[00314] PCR is used to generate a template for in vitro transcription of mRNA which is used for transfection. Methods for performing PCR are well known in the art. Primers for use in PCR are designed to have regions that are substantially complementary to regions of the DNA to be used as a template for the PCR. The term "substantially complementary" refers to sequences of nucleotides where a majority or all of the bases in the primer sequence are complementary, or one or more bases are non-complementary, or mismatched. Substantially complementary sequences are able to anneal or hybridize with the intended DNA target under annealing conditions used for PCR. The primers can be designed to be substantially complementary to any portion of the DNA template. For example, the primers can be designed to amplify the portion of

a nucleic acid that is normally transcribed in cells (the open reading frame), including 5' and 3' UTRs. The primers can also be designed to amplify a portion of a nucleic acid that encodes a particular domain of interest. In one embodiment, the primers are designed to amplify the coding region of a human cDNA, including all or portions of the 5' and 3' UTRs. Primers useful for PCR can be generated by synthetic methods that are well known in the art. "Forward primers" are primers that contain a region of nucleotides that are substantially complementary to nucleotides on the DNA template that are upstream of the DNA sequence that is to be amplified. The term "upstream" refers to a location 5' to the DNA sequence to be amplified relative to the coding strand. "Reverse primers" are primers that contain a region of nucleotides that are substantially complementary to a double-stranded DNA template that are downstream of the DNA sequence that is to be amplified. The term "downstream" refers to a location 3' to the DNA sequence to be amplified relative to the coding strand.

[003 15] Any DNA polymerase useful for PCR can be used in the methods disclosed herein. The reagents and polymerase are commercially available from a number of sources.

[003 16] Chemical structures with the ability to promote stability and/or translation efficiency may also be used. The RNA preferably has 5' and 3' UTRs. In one embodiment, the 5' UTR is between one and 3000 nucleotides in length. The length of 5' and 3' UTR sequences to be added to the coding region can be altered by different methods, including, but not limited to, designing primers for PCR that anneal to different regions of the UTRs. Using this approach, one of ordinary skill in the art can modify the 5' and 3' UTR lengths required to achieve optimal translation efficiency following transfection of the transcribed RNA.

[00317] The 5' and 3' UTRs can be the naturally occurring, endogenous 5' and 3' UTRs for the nucleic acid of interest. Alternatively, UTR sequences that are not endogenous to the nucleic acid of interest can be added by incorporating the UTR sequences into the forward and reverse primers or by any other modifications of the template. The use of UTR sequences that are not endogenous to the nucleic acid of interest can be useful for modifying the stability and/or translation efficiency of the RNA. For example, it is known that AU-rich elements in 3' UTR sequences can decrease the stability of mRNA. Therefore, 3' UTRs can be selected or designed to increase the stability of the transcribed RNA based on properties of UTRs that are well known in the art.

[003 18] In one embodiment, the 5' UTR can contain the Kozak sequence of the endogenous nucleic acid. Alternatively, when a 5' UTR that is not endogenous to the nucleic acid of interest is being added by PCR as described above, a consensus Kozak sequence can be redesigned by adding the 5' UTR sequence. Kozak sequences can increase the efficiency of translation of some RNA transcripts, but does not appear to be required for all RNAs to enable efficient translation. The requirement for Kozak sequences for many mRNAs is known in the art. In other embodiments the 5' UTR can be 5'UTR of an RNA virus whose RNA genome is stable in cells. In other embodiments various nucleotide analogues can be used in the 3' or 5' UTR to impede exonuclease degradation of the mRNA.

[003 19] To enable synthesis of RNA from a DNA template without the need for gene cloning, a promoter of transcription should be attached to the DNA template upstream of the sequence to be transcribed. When a sequence that functions as a promoter for an RNA polymerase is added to the 5' end of the forward primer, the RNA polymerase promoter becomes incorporated into the PCR product upstream of the open reading frame that is to be transcribed. In one preferred embodiment, the promoter is a T7 polymerase promoter, as described elsewhere herein. Other useful promoters include, but are not limited to, T3 and SP6 RNA polymerase promoters. Consensus nucleotide sequences for T7, T3 and SP6 promoters are known in the art.

[00320] In a preferred embodiment, the mRNA has both a cap on the 5' end and a 3' poly(A) tail which determine ribosome binding, initiation of translation and stability mRNA in the cell. On a circular DNA template, for instance, plasmid DNA, RNA polymerase produces a long concatameric product which is not suitable for expression in eukaryotic cells. The transcription of plasmid DNA linearized at the end of the 3' UTR results in normal sized mRNA which is not effective in eukaryotic transfection even if it is polyadenylated after transcription.

[00321] On a linear DNA template, phage T7 RNA polymerase can extend the 3' end of the transcript beyond the last base of the template (Schenborn and Mierendorf, Nuc Acids Res., 13:6223-36 (1985); Nacheva and Berzal-Herranz, Eur. J. Biochem., 270:1485-65 (2003).

[00322] The conventional method of integration of polyA/T stretches into a DNA template is molecular cloning. However polyA/T sequence integrated into plasmid DNA can cause plasmid instability, which is why plasmid DNA templates obtained from bacterial cells are often highly contaminated with deletions and other aberrations. This makes cloning procedures not only

laborious and time consuming but often not reliable. That is why a method which allows construction of DNA templates with polyA/T 3' stretch without cloning highly desirable.

[00323] The polyA/T segment of the transcriptional DNA template can be produced during PCR by using a reverse primer containing a polyT tail, such as 100T tail (SEQ ID NO: 31) (size can be 50-5000 T (SEQ ID NO: 32)), or after PCR by any other method, including, but not limited to, DNA ligation or in vitro recombination. Poly(A) tails also provide stability to RNAs and reduce their degradation. Generally, the length of a poly(A) tail positively correlates with the stability of the transcribed RNA. In one embodiment, the poly(A) tail is between 100 and 5000 adenosines (SEQ ID NO: 33).

[00324] Poly(A) tails of RNAs can be further extended following in vitro transcription with the use of a poly(A) polymerase, such as E. coli polyA polymerase (E-PAP). In one embodiment, increasing the length of a poly(A) tail from 100 nucleotides to between 300 and 400 nucleotides (SEQ ID NO: 34) results in about a two-fold increase in the translation efficiency of the RNA. Additionally, the attachment of different chemical groups to the 3' end can increase mRNA stability. Such attachment can contain modified/artificial nucleotides, aptamers and other compounds. For example, ATP analogs can be incorporated into the poly(A) tail using poly(A) polymerase. ATP analogs can further increase the stability of the RNA.

[00325] 5' caps on also provide stability to RNA molecules. In a preferred embodiment, RNAs produced by the methods disclosed herein include a 5' cap. The 5' cap is provided using techniques known in the art and described herein (Cougot, et al., Trends in Biochem. Sci., 29:436-444 (2001); Stepinski, et al, RNA, 7:1468-95 (2001); Elango, et al., Biochim. Biophys. Res. Commua, 330:958-966 (2005)).

[00326] The RNAs produced by the methods disclosed herein can also contain an internal ribosome entry site (IRES) sequence. The IRES sequence may be any viral, chromosomal or artificially designed sequence which initiates cap-independent ribosome binding to mRNA and facilitates the initiation of translation. Any solutes suitable for cell electroporation, which can contain factors facilitating cellular permeability and viability such as sugars, peptides, lipids, proteins, antioxidants, and surfactants can be included.

[00327] RNA can be introduced into target cells using any of a number of different methods, for instance, commercially available methods which include, but are not limited to,

electroporation (Amaxa Nucleofector-II (Amaxa Biosystems, Cologne, Germany)), (ECM 830 (BTX) (Harvard Instruments, Boston, Mass.) or the Gene Pulser II (BioRad, Denver, Colo.), Multiporator (Eppendort, Hamburg Germany), cationic liposome mediated transfection using lipofection, polymer encapsulation, peptide mediated transfection, or biolistic particle delivery systems such as "gene guns" (see, for example, Nishikawa, et al. Hum Gene Ther., 12(8): 861-70 (2001).

Nucleic Acid Constructs Encoding a CAR

[00328] The present invention provides CAR transgenes comprising nucleic acid sequences encoding one or more CAR constructs of the invention. In one aspect, the CAR transgene is provided as a messenger RNA transcript. In one aspect, the CAR transgene is provided as a DNA construct.

[00329] Accordingly, in one aspect, the invention pertains to an isolated nucleic acid molecule encoding a chimeric antigen receptor (CAR), wherein the CAR comprises an anti-mesothelin binding domain (e.g., a human anti-mesothelin binding domain), a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain. In one embodiment, the anti-mesothelin binding domain is an anti-mesothelin binding domain described herein, e.g., an anti-mesothelin binding domain which comprises a sequence selected from a group consisting of SEQ ID NO: 87-111, or a sequence with 95-99% identify thereof. In one embodiment, the isolated nucleic acid molecule further comprises a sequence encoding a costimulatory domain. In one embodiment, the transmembrane domain is a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154. In one embodiment, the transmembrane domain comprises a sequence of SEQ ID NO: 6, or a sequence with 95-99% identity thereof. In one embodiment, the anti-mesothelin binding domain is connected to the transmembrane domain by a hinge region, e.g., a hinge described herein. In one embodiment, the hinge region comprises SEQ ID NO: 2 or SEQ ID NO:3 or SEQ ID NO:4 or SEQ ID NO:5, or a sequence with 95-99% identity thereof. In one embodiment, the isolated nucleic acid molecule further comprises a sequence encoding a costimulatory domain. In one embodiment, the costimulatory domain is a functional signaling domain of a protein selected from the group consisting of OX40, CD27, CD28, CDS, ICAM-1,

LFA-1 (CDlla/CD18), ICOS (CD278), and 4-1BB (CD137). Further examples of such costimulatory molecules include CDS, ICAM-1, GITR, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CDlld, ITGAE, CD103, ITGAL, CDl la, LFA-1, ITGAM, CDllb, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAMl (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAMl, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, and PAG/Cbp. In one embodiment, the costimulatory domain comprises a sequence of SEQ ID NO: 7, or a sequence with 95-99% identity thereof. In one embodiment, the intracellular signaling domain comprises a functional signaling domain of 4-1BB and a functional signaling domain of CD3 zeta. In one embodiment, the intracellular signaling domain comprises the sequence of SEQ ID NO: 7 or SEQ ID NO: 8, or a sequence with 95-99% identity thereof, and the sequence of SEQ ID NO: 9 or SEQ ID NO: 10, or a sequence with 95-99% identity thereof, wherein the sequences comprising the intracellular signaling domain are expressed in the same frame and as a single polypeptide chain. In another aspect, the invention pertains to an isolated nucleic acid molecule encoding a CAR construct comprising a leader sequence of SEQ ID NO: 1, a scFv domain having a sequence selected from the group consisting of SEQ ID NO: 39; SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, and SEQ ID NO: 62, (or a sequence with 95-99% identify thereof), a hinge region of SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or SEQ ID NO: 5 (or a sequence with 95-99% identity thereof), a transmembrane domain having a sequence of SEQ ID NO: 6 (or a sequence with 95-99% identity thereof), a 4-1BB costimulatory domain having a sequence of SEQ ID NO: 7 (or a sequence with 95-99% identity thereof) or a CD27 costimulatory domain having a sequence of SEQ ID NO: 8 (or a sequence with 95-99% identity thereof), and a CD3 zeta stimulatory domain having a sequence of SEQ ID NO: 9 or SEQ ID NO: 10 (or a sequence with 95-99% identity thereof).

[00330] In another aspect, the invention pertains to an isolated polypeptide molecule encoded by the nucleic acid molecule. In one embodiment, the isolated polypeptide molecule comprises a sequence selected from the group consisting of SEQ ID NO: 63; SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, 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, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, and SEQ ID NO: 86, or a sequence with 95-99% identity thereof.

[00331] In another aspect, the invention pertains to an isolated nucleic acid molecule encoding a chimeric antigen receptor (CAR) molecule that comprises an anti-mesothelin binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein the nucleic acid encoding the anti-mesothelin binding domain comprises a sequence selected from the group consisting of SEQ ID NO: 111; SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 120, SEQ ID NO: 121, SEQ ID NO: 122, SEQ ID NO: 123, SEQ ID NO: 124, SEQ ID NO: 125, SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, SEQ ID NO: 130, SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 133, SEQ ID NO:134, or a sequence with 95-99% identify thereof.

[00332] In one embodiment, the encoded CAR molecule further comprises a sequence encoding a costimulatory domain. In one embodiment, the costimulatory domain is a functional signaling domain of a protein selected from the group consisting of OX40, CD27, CD28, CDS, ICAM-1, LFA-1 (CDl la/CD18) and 4-1BB (CD137). In one embodiment, the costimulatory domain comprises a sequence of SEQ ID NO: 7. In one embodiment, the transmembrane domain is a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154. In one embodiment, the transmembrane domain comprises a sequence of SEQ ID NO: 6. In one embodiment, the intracellular signaling domain comprises a functional signaling domain of 4-IBB and a functional signaling domain of zeta. In one embodiment, the intracellular signaling domain comprises the sequence of SEQ ID NO: 7 and the sequence of SEQ ID NO: 9, wherein the sequences comprising the intracellular signaling domain are expressed in the same frame and as

a single polypeptide chain. In one embodiment, the anti-mesothelin binding domain is connected to the transmembrane domain by a hinge region. In one embodiment, the hinge region comprises SEQ ID NO:2. In one embodiment, the hinge region comprises SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 5.

[00333] In another aspect, the invention pertains to an isolated CAR molecule comprising a leader sequence of SEQ ID NO: 1, a scFv domain having a sequence selected from the group consisting of SEQ ID NOS: 39-62, or a sequence with 95-99% identify thereof, a hinge region of SEQ ID NO:2 or SEQ ID NO: 3 or SEQ ID NO: 4 or SEQ ID NO: 5, a transmembrane domain having a sequence of SEQ ID NO: 6, a 4-IBB costimulatory domain having a sequence of SEQ ID NO: 7 or a CD27 costimulatory domain having a sequence of SEQ ID NO: 8, and a CD3 zeta stimulatory domain having a sequence of SEQ ID NO: 9 or SEQ ID NO: 10. In one embodiment, the encoded CAR molecule comprises a sequence selected from the group consisting of SEQ ID NOS: 63-86, or a sequence with 95-99% identify thereof.

[00334] The present invention further provides vectors comprising CAR transgenes. In one aspect, a CAR vectors can be directly transduced into a cell, *e.g.,* a T cell or NK cell. In one aspect, the vector is a cloning or expression vector, *e.g.,* a vector including, but not limited to, one or more plasmids *(e.g.,* expression plasmids, cloning vectors, minicircles, minivectors, double minute chromosomes), retroviral and lentiviral vector constructs. In one aspect, the vector is capable of expressing the CAR construct in mammalian T cells or NK cells. In one aspect, the mammalian T cell is a human T cell or a human NK cell.

[00335] The present invention also includes a CAR encoding RNA construct that can be directly transfected into a cell, e.g., a T cell or a NK cell. A method for generating mRNA for use in transfection involves in vitro transcription (IVT) of a template with specially designed primers, followed by polyA addition, to produce a construct containing 3' and 5' untranslated sequence ("UTR"), a 5' cap and/or Internal Ribosome Entry Site (IRES), the gene to be expressed, and a polyA tail, typically 50-2000 bases in length. RNA so produced can efficiently transfect different kinds of cells. In one aspect, the template includes sequences for the CAR.

[00336] In one aspect the mesothelin CAR transgene is encoded by a messenger RNA (mRNA). In one aspect the mRNA encoding the mesothelin CAR transgene is introduced into a T cell for production of a CART cell, or a NK cell.

Vectors

[00337] The present invention also provides vectors in which a DNA of the present invention is inserted. Vectors derived from retroviruses such as the lentivirus are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors have the added advantage over vectors derived from onco-retroviruses such as murine leukemia viruses in that they can transduce non-proliferating cells, such as hepatocytes. They also have the added advantage of low immunogenicity.

[00338] In one embodiment, the vector comprising the nucleic acid encoding the desired CAR of the invention is a DNA, a RNA, a plasmid, an adenoviral vector, a lentivirus vector, or a retrovirus vector.

[00339] In another embodiment, the vector comprising the nucleic acid encoding the desired CAR of the invention is an adenoviral vector (A5/35). In another embodiment, the expression of nucleic acids encoding CARs can be accomplished using of transposons such as sleeping beauty, CRISPR, CAS9, and zinc finger nucleases. See, e.g., June *et al.* 2009 *Nature Reviews Immunology* 9.10: 704-716, incorporated herein by reference in its entirety.

[00340] In brief summary, the expression of natural or synthetic nucleic acids encoding CARs is typically achieved by operably linking a nucleic acid encoding the CAR polypeptide or portions thereof to a promoter, and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration eukaryotes. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

[00341] The expression constructs of the present invention may also be used for nucleic acid immunization and gene therapy, using standard gene delivery protocols. Methods for gene delivery are known in the art. See, e.g., U.S. Pat. Nos. 5,399,346, 5,580,859, 5,589,466, incorporated by reference herein in their entireties. In another embodiment, the invention provides a gene therapy vector.

[00342] The nucleic acid can be cloned into a number of types of vectors. For example, the nucleic acid can be cloned into a vector including, but not limited to a plasmid, a phagemid, a

phage derivative, an animal virus, and a cosmid. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

[00343] Further, the expression vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook *et al,* 2012, MOLECULAR CLONING: A LABORATORY MANUAL, volumes 1-4, Cold Spring Harbor Press, NY), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno- associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers, (e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193).

[00344] A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either in vivo or ex vivo. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

[00345] Additional promoter elements, e.g., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. Exemplary promoters include the CMV IE gene, EF-1α, ubiquitin C, or phosphoglycerokinase (PGK) promoters.

[00346] An example of a promoter that is capable of expressing a CAR transgene in a mammalian T cell is the EFlalpha promoter (EFla or EFla). The native EFla promoter drives

expression of the alpha subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. The EFla promoter has been extensively used in mammalian expression plasmids and has been shown to be effective in driving CAR expression from transgenes cloned into a lentiviral vector. See, e.g., Milone *et ah,* Mol. Ther. 17(8): 1453-1464 (2009). In one aspect, the EFla promoter comprises the sequence provided as SEQ ID NO: 11.

[00347] Another example of a promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (FflV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the elongation factor-la promoter, the hemoglobin promoter, and the creatine kinase promoter. Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are also contemplated as part of the invention. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

[00348] In order to assess the expression of a CAR polypeptide or portions thereof, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co- transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

[00349] Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei *et al*., 2000 FEBS Letters 479: 79-82). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter- driven transcription.

[00350] In one embodiment, the vector can further comprise a nucleic acid encoding a second CAR. In one embodiment, the second CAR includes an antigen binding domain to, e.g., a target other than mesothelin on stroma cells, e.g., FAP; a target other than mesothelin on prostate cancer cells, e.g., androgen receptor, OR51E2, PSMA, PSCA, PDGRF-β, TARP, GloboH, MAD-CT-1, or MAD-CT-2; a target other than mesothelin on ovararian cancer cells, e.g., Tn, PRSS21, CD171, Lewis Y, folate receptor a, claudin6, GloboH, or sperm protein 17; e.g., a target other than mesothelin on lung cancer cells, e.g.,VEGF, HER3, IGF-1R, EGFR, DLL4, or Trop-2. In one embodiment, the vector comprises a nucleic acid sequence encoding a first CAR that targets a first antigen and includes an intracellular signaling domain having a costimulatory signaling domain but not a primary signaling domain, and a nucleic acid encoding a second CAR that targets a second, different, antigen and includes an intracellular signaling domain having a primary signaling domain but not a costimulatory signaling domain. In one embodiment, the vector comprises a nucleic acid encoding a first mesothelin CAR that includes a mesothelin binding domain, a transmembrane domain and a costimulatory domain and a nucleic acid encoding a second CAR that targets an antigen other than mesothelin (e.g., a target other than mesothelin on stroma cells, e.g., FAP; a target other than mesothelin on prostate cancer cells, e.g., androgen receptor, OR51E2, PSMA, PSCA, PDGRF-β, TARP, GloboH, MAD-CT-1, or MAD-CT-2; a target other than mesothelin on ovararian cancer cells, e.g., Tn, PRSS21, CD171, Lewis Y, folate receptor a, claudin6, GloboH, or sperm protein 17;

e.g., a target other than mesothelin on lung cancer cells, e.g.,VEGF, HER3, IGF-1R, EGFR, DLL4, or Trop-2) and includes an antigen binding domain, a transmembrane domain and a primary signaling domain. In another embodiment, the vector comprises a nucleic acid encoding a first mesothelin CAR that includes a mesothelin binding domain, a transmembrane domain and a primary signaling domain and a nucleic acid encoding a second CAR that targets an antigen other than mesothelin (e.g., a target other than mesothelin on stroma cells, e.g., FAP; a target other than mesothelin on prostate cancer cells, e.g., androgen receptor, OR51E2, PSMA, PSCA, PDGRF- β, TARP, GloboH, MAD-CT-1, or MAD-CT-2; a target other than mesothelin on ovararian cancer cells, e.g., Tn, PRSS21, CD171, Lewis Y, folate receptor a, claudin6, GloboH, or sperm protein 17; e.g., a target other than mesothelin on lung cancer cells, e.g.,VEGF, HER3, IGF-1R, EGFR, DLL4, or Trop-2) and includes an antigen binding domain to the antigen, a transmembrane domain and a costimulatory signaling domain.

[00351] In one embodiment, the vector comprises a nucleic acid encoding a mesothelin CAR described herein and a nucleic acid encoding an inhibitory CAR. In one embodiment, the inhibitory CAR comprises an antigen binding domain that binds an antigen found on normal cells but not cancer cells, e.g., normal cells that also express CLL. In one embodiment, the inhibitory CAR comprises the antigen binding domain, a transmembrane domain and an intracellular domain of an inhibitory molecule. For example, the intracellular domain of the inhibitory CAR can be an intracellular domain of PDl, PD-Ll, CTLA4, TFM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIRl, CD160, 2B4 and TGFR beta.

[00352] In one embodiment, the vector comprises a nucleic acid encoding a mesothelin CAR described herein and an inhibitory nucleic acid, e.g., a dsRNA, e.g., an siRNA or shRNA, e.g., as described herein.

[00353] Methods of introducing and expressing genes into a cell are known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

[00354] Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and

the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook *et al,* 2012, MOLECULAR CLONING: A LABORATORY MANUAL, volumes 1 -4, Cold Spring Harbor Press, NY). A preferred method for the introduction of a polynucleotide into a host cell is lipofection, e.g., using Lipofectamine (Life Technologies).

[00355] Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

[00356] Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle). Other methods of state-of-the-art targeted delivery of nucleic acids are available, such as delivery of polynucleotides with targeted nanoparticles or other suitable sub-micron sized delivery system.

[00357] In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (in vitro, ex vivo or in vivo). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming

aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

[00358] Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma, St. Louis, MO; dicetyl phosphate ("DCP") can be obtained from K & K Laboratories (Plainview, NY); cholesterol ("Choi") can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL.). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh *et ah,* 1991 Glycobiology 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

[00359] Regardless of the method used to introduce exogenous nucleic acids into a host cell or otherwise expose a cell to the inhibitor of the present invention, in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

[00360] The present invention further provides a vector comprising a CAR encoding nucleic acid molecule. In one aspect, a CAR vector can be directly transduced into a cell, *e.g.,* a T cell or a NK cell. In one aspect, the vector is a cloning or expression vector, *e.g.,* a vector including, but not limited to, one or more plasmids *(e.g.,* expression plasmids, cloning vectors, mini circles, minivectors, double minute chromosomes), retroviral and lentiviral vector constructs. In one aspect, the vector is capable of expressing the CAR construct in mammalian T cells. In one aspect, the mammalian T cell is a human T cell. In one aspect, the mammalian cell is a human NK cell.

Sources of Cells

[00361] Prior to expansion and genetic modification, a source of cells (e.g., T cells or NK cells) is obtained from a subject. The term "subject" is intended to include living organisms in which an immune response can be elicited (e.g., mammals). Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain aspects of the present invention, any number of T cell lines available in the art, may be used. In certain aspects of the present invention, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll™ separation. In one preferred aspect, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one aspect, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In one aspect of the invention, the cells are washed with phosphate buffered saline (PBS). In an alternative aspect, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Initial activation steps in the absence of calcium can lead to magnified activation. As those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor, the Baxter CytoMate, or the Haemonetics Cell Saver 5) according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers,

such as, for example, Ca-free, Mg-free PBS, PlasmaLyte A, or other saline solution with or without buffer. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

[00362] In one aspect, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLLTM gradient or by counterflow centrifugal elutriation. A specific subpopulation of T cells, such as CD3+, CD28+, CD4+, CD8+, CD45RA+, and CD45RO+T cells, can be further isolated by positive or negative selection techniques. For example, in one aspect, T cells are isolated by incubation with anti-CD3/anti-CD28 (e.g., 3x28)-conjugated beads, such as DYNABEADS® M-450 CD3/CD28 T, for a time period sufficient for positive selection of the desired T cells. In one aspect, the time period is about 30 minutes. In a further aspect, the time period ranges from 30 minutes to 36 hours or longer and all integer values there between. In a further aspect, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In yet another preferred aspect, the time period is 10 to 24 hours. In one aspect, the incubation time period is 24 hours. For isolation of T cells from patients with leukemia, use of longer incubation times, such as 24 hours, can increase cell yield. Longer incubation times may be used to isolate T cells in any situation where there are few T cells as compared to other cell types, such in isolating tumor infiltrating lymphocytes (TIL) from tumor tissue or from immunocompromised individuals. Further, use of longer incubation times can increase the efficiency of capture of CD8+ T cells. Thus, by simply shortening or lengthening the time T cells are allowed to bind to the CD3/CD28 beads and/or by increasing or decreasing the ratio of beads to T cells (as described further herein), subpopulations of T cells can be preferentially selected for or against at culture initiation or at other time points during the process. Additionally, by increasing or decreasing the ratio of anti-CD3 and/or anti-CD28 antibodies on the beads or other surface, subpopulations of T cells can be preferentially selected for or against at culture initiation or at other desired time points. The skilled artisan would recognize that multiple rounds of selection can also be used in the context of this invention. In certain aspects, it may be desirable to perform the selection procedure and use the "unselected" cells in the activation and expansion process. "Unselected" cells can also be subjected to further rounds of selection.

[00363] Enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells.

One method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4+ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CDlib, CD16, HLA-DR, and CD8. In certain aspects, it may be desirable to enrich for or positively select for regulatory T cells which typically express CD4+, CD25+, CD62Lhi, GITR+, and FoxP3+. Alternatively, in certain aspects, T regulatory cells are depleted by anti-C25 conjugated beads or other similar method of selection.

[00364] In one embodiment, a T cell population can be selected that expresses one or more of IFN-D , TNFa, IL17A, IL-2, IL-3, IL-4, GM-CSF, IL-10, IL-13, granzyme B, and perforin, or other appropriate molecules, e.g., other cytokines. Methods for screening for cell expression can be determined, e.g., by the methods described in PCT Publication No.: WO 2013/126712.

[00365] In one embodiment, a T cell population is diaglycerol kinase (DGK)-deficient. DGK-deficient cells include cells that do not express DGK RNA or protein, or have reduced or inhibited DGK activity. DGK-deficient cells can be generated by genetic approaches, e.g., administering RNA-interfering agents, e.g., siRNA, shRNA, miRNA, to reduce or prevent DGK expression. Alternatively, DGK-deficient cells can be generated by treatment with DGK inhibitors described herein.

[00366] In one embodiment, a T cell population is Ikaros-deficient. Ikaros-deficient cells include cells that do not express Ikaros RNA or protein, or have reduced or inhibited Ikaros activity, Ikaros-deficient cells can be generated by genetic approaches, e.g., administering RNA-interfering agents, e.g., siRNA, shRNA, miRNA, to reduce or prevent Ikaros expression. Alternatively, Ikaros-deficient cells can be generated by treatment with Ikaros inhibitors, e.g., lenalidomide.

[00367] In embodiments, a T cell population is DGK-deficient and Ikaros-deficient, e.g., does not express DGK and Ikaros, or has reduced or inhibited DGK and Ikaros activity. Such DGK and Ikaros-deficient cells can be generated by any of the methods described herein.

[00368] For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (e.g., particles such as beads) can be varied. In certain aspects, it may be desirable to significantly decrease the volume in which beads and cells are mixed

together (e.g., increase the concentration of cells), to ensure maximum contact of cells and beads. For example, in one aspect, a concentration of 2 billion cells/ml is used. In one aspect, a concentration of 1 billion cells/ml is used. In a further aspect, greater than 100 million cells/ml is used. In a further aspect, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet one aspect, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further aspects, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells, or from samples where there are many tumor cells present (e.g., leukemic blood, tumor tissue, etc.). Such populations of cells may have therapeutic value and would be desirable to obtain. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

[00369] In a related aspect, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and surface (e.g., particles such as beads), interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4+ T cells express higher levels of CD28 and are more efficiently captured than CD8+ T cells in dilute concentrations. In one aspect, the concentration of cells used is 5 X $10e^6$/ml. In other aspects, the concentration used can be from about 1 X 10^5/ml to 1 X 10^6/ml, and any integer value in between.

[00370] In other aspects, the cells may be incubated on a rotator for varying lengths of time at varying speeds at either 2-10°C or at room temperature.

[00371] T cells for stimulation can also be frozen after a washing step. Wishing not to be bound by theory, the freeze and subsequent thaw step provides a more uniform product by removing granulocytes and to some extent monocytes in the cell population. After the washing step that removes plasma and platelets, the cells may be suspended in a freezing solution. While many freezing solutions and parameters are known in the art and will be useful in this context, one method involves using PBS containing 20% DMSO and 8% human serum albumin, or culture media containing 10% Dextran 40 and 5% Dextrose, 20% Human Serum Albumin and

7.5% DMSO, or 31.25% Plasmalyte-A, 31.25% Dextrose 5%, 0.45% NaCl, 10% Dextran 40 and 5% Dextrose, 20% Human Serum Albumin, and 7.5% DMSO or other suitable cell freezing media containing for example, Hespan and PlasmaLyte A, the cells then are frozen to -80°C at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank. Other methods of controlled freezing may be used as well as uncontrolled freezing immediately at -20° C or in liquid nitrogen.

[00372] In certain aspects, cryopreserved cells are thawed and washed as described herein and allowed to rest for one hour at room temperature prior to activation using the methods of the present invention.

[00373] Also contemplated in the context of the invention is the collection of blood samples or apheresis product from a subject at a time period prior to when the expanded cells as described herein might be needed. As such, the source of the cells to be expanded can be collected at any time point necessary, and desired cells, such as T cells, isolated and frozen for later use in T cell therapy for any number of diseases or conditions that would benefit from T cell therapy, such as those described herein. In one aspect a blood sample or an apheresis is taken from a generally healthy subject. In certain aspects, a blood sample or an apheresis is taken from a generally healthy subject who is at risk of developing a disease, but who has not yet developed a disease, and the cells of interest are isolated and frozen for later use. In certain aspects, the T cells may be expanded, frozen, and used at a later time. In certain aspects, samples are collected from a patient shortly after diagnosis of a particular disease as described herein but prior to any treatments. In a further aspect, the cells are isolated from a blood sample or an apheresis from a subject prior to any number of relevant treatment modalities, including but not limited to treatment with agents such as natalizumab, efalizumab, antiviral agents, chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies, Cytoxan, fludarabine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin). (Liu *et al.*, Cell 66:807-815, 1991; Henderson *et al.*, Immun. 73:316-321, 1991; Bierer *et al.*, Curr. Opin. Immun. 5:763-773, 1993). In a further aspect, the cells are isolated for a patient and frozen for later use in conjunction with (e.g., before, simultaneously or

following) bone marrow or stem cell transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In one aspect, the cells are isolated prior to and can be frozen for later use for treatment following B-cell ablative therapy such as agents that react with CD20, e.g., Rituxan.

[00374] In a further aspect of the present invention, T cells are obtained from a patient directly following treatment that leaves the subject with functional T cells. In this regard, it has been observed that following certain cancer treatments, in particular treatments with drugs that damage the immune system, shortly after treatment during the period when patients would normally be recovering from the treatment, the quality of T cells obtained may be optimal or improved for their ability to expand ex vivo. Likewise, following ex vivo manipulation using the methods described herein, these cells may be in a preferred state for enhanced engraftment and in vivo expansion. Thus, it is contemplated within the context of the invention to collect blood cells, including T cells, dendritic cells, or other cells of the hematopoietic lineage, during this recovery phase. Further, in certain aspects, mobilization (for example, mobilization with GM-CSF) and conditioning regimens can be used to create a condition in a subject wherein repopulation, recirculation, regeneration, and/or expansion of particular cell types is favored, especially during a defined window of time following therapy. Illustrative cell types include T cells, B cells, dendritic cells, and other cells of the immune system.

[00375] In an embodiment, the NK cells are obtained from the subject. In another embodiment, the NK cells are an NK cell line, e.g., NK-92 cell line (Conkwest).

Allogeneic CAR

[00376] In embodiments described herein, the immune effector cell can be an allogeneic immune effector cell, e.g., T cell or NK cell. For example, the cell can be an allogeneic T cell, e.g., an allogeneic T cell lacking expression of a functional T cell receptor (TCR) and/or human leukocyte antigen (HLA), e.g., HLA class I and/or HLA class II.

[00377] A T cell lacking a functional TCR can be, e.g., engineered such that it does not express any functional TCR on its surface, engineered such that it does not express one or more subunits that comprise a functional TCR or engineered such that it produces very little functional TCR on its surface. Alternatively, the T cell can express a substantially impaired TCR, e.g., by

expression of mutated or truncated forms of one or more of the subunits of the TCR. The term "substantially impaired TCR" means that this TCR will not elicit an adverse immune reaction in a host.

[00378] A T cell described herein can be, e.g., engineered such that it does not express a functional HLA on its surface. For example, a T cell described herein, can be engineered such that cell surface expression HLA, e.g., HLA class 1 and/or HLA class II, is downregulated.

[00379] In some embodiments, the T cell can lack a functional TCR and a functional HLA, e.g., HLA class I and/or HLA class II.

[00380] Modified T cells that lack expression of a functional TCR and/or HLA can be obtained by any suitable means, including a knock out or knock down of one or more subunit of TCR or HLA. For example, the T cell can include a knock down of TCR and/or HLA using siRNA, shRNA, clustered regularly interspaced short palindromic repeats (CRISPR) transcription-activator like effector nuclease (TALEN), or zinc finger endonuclease (ZFN).

[00381] In some embodiments, the allogeneic cell can be a cell which does not expresses or expresses at low levels an inhibitory molecule, e.g. by any mehod described herein. For example, the cell can be a cell that does not express or expresses at low levels an inhibitory molecule, e.g., that can decrease the ability of a CAR-expressing cell to mount an immune effector response. Examples of inhibitory molecules include PD1, PD-L1, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIRl, CD160, 2B4 and TGFR beta. Inhibition of an inhibitory molecule, e.g., by inhibition at the DNA, RNA or protein level, can optimize a CAR-expressing cell performance. In embodiments, an inhibitory nucleic acid, e.g., an inhibitory nucleic acid, e.g., a dsRNA, e.g., an siRNA or shRNA, a clustered regularly interspaced short palindromic repeats (CRISPR), a transcription-activator like effector nuclease (TALEN), or a zinc finger endonuclease (ZFN), e.g., as described herein, can be used.

siRNA and shRNA to inhibit TCR or HLA

[00382] In some embodiments, TCR expression and/or HLA expression can be inhibited using siRNA or shRNA that targets a nucleic acid encoding a TCR and/or HLA in a T cell.

[00383] Expression of siRNA and shRNAs in T cells can be achieved using any conventional expression system, e.g., such as a lentiviral expression system.

[00384] Exemplary shRNAs that downregulate expression of components of the TCR are described, e.g., in US Publication No.: 2012/0321667. Exemplary siRNA and shRNA that downregulate expression of HLA class I and/or HLA class II genes are described, e.g., in U.S. publication No.: US 2007/0036773.

CRISPR to inhibit TCR or HLA

[00385] "CRISPR" or "CRISPR to TCR and/or HLA" or "CRISPR to inhibit TCR and/or HLA" as used herein refers to a set of clustered regularly interspaced short palindromic repeats, or a system comprising such a set of repeats. "Cas", as used herein, refers to a CRISPR-associated protein. A "CRISPR/Cas" system refers to a system derived from CRISPR and Cas which can be used to silence or mutate a TCR and/or HLA gene.

[00386] Naturally-occurring CRISPR/Cas systems are found in approximately 40% of sequenced eubacteria genomes and 90% of sequenced archaea. Grissa *et al.* (2007) *BMC Bioinformatics* 8: 172. This system is a type of prokaryotic immune system that confers resistance to foreign genetic elements such as plasmids and phages and provides a form of acquired immunity. Barrangou *et al.* (2007) *Science* 315: 1709-1712; Marragini *et al.* (2008) *Science* 322: 1843-1845.

[00387] The CRISPR/Cas system has been modified for use in gene editing (silencing, enhancing or changing specific genes) in eukaryotes such as mice or primates. Wiedenheft *et al.* (2012) *Nature* 482: 331-8. This is accomplished by introducing into the eukaryotic cell a plasmid containing a specifically designed CRISPR and one or more appropriate Cas.

[00388] The CRISPR sequence, sometimes called a CRISPR locus, comprises alternating repeats and spacers. In a naturally-occurring CRISPR, the spacers usually comprise sequences foreign to the bacterium such as a plasmid or phage sequence; in the TCR and/or HLA CRISPR/Cas system, the spacers are derived from the TCR or HLA gene sequence.

[00389] RNA from the CRISPR locus is constitutively expressed and processed by Cas proteins into small RNAs. These comprise a spacer flanked by a repeat sequence. The RNAs guide other Cas proteins to silence exogenous genetic elements at the RNA or DNA level.

Horvath *ei a/*. (2010) *Science* 327: 167-170; Makar ova *et al.* (2006) *Biology Direct* 1: 7. The spacers thus serve as templates for RNA molecules, analogously to siRNAs. Pennisi (2013) *Science* 341: 833-836.

[00390] As these naturally occur in many different types of bacteria, the exact arrangements of the CRISPR and structure, function and number of Cas genes and their product differ somewhat from species to species. Haft *et al.* (2005) *PLoS Comput. Biol.* 1: e60; Kunin *et al.* (2007) *Genome Biol.* 8: R61; Mojica *et al.* (2005) *J. Mol. Evol.* 60: 174-182; Bolotin *et al.* (2005) *Microbiol.* 151: 2551-2561; Pourcel et al. (2005) *Microbiol.* 151: 653-663; and Stern *et al.* (2010) *Trends. Genet.* 28: 335-340. For example, the Cse (Cas subtype, E. coli) proteins (e.g., CasA) form a functional complex, Cascade, that processes CRISPR RNA transcripts into spacer-repeat units that Cascade retains. Brouns et al. (2008) *Science* 321: 960-964. In other prokaryotes, Cas6 processes the CRISPR transcript. The CRISPR-based phage inactivation in E. coli requires Cascade and Cas3, but not Casl or Cas2. The Cmr (Cas RAMP module) proteins in Pyrococcus furiosus and other prokaryotes form a functional complex with small CRISPR RNAs that recognizes and cleaves complementary target RNAs. A simpler CRISPR system relies on the protein Cas9, which is a nuclease with two active cutting sites, one for each strand of the double helix. Combining Cas9 and modified CRISPR locus RNA can be used in a system for gene editing. Pennisi (2013) *Science* 341: 833-836.

[00391] The CRISPR/Cas system can thus be used to edit a TCR and/or HLA gene (adding or deleting a basepair), or introducing a premature stop which thus decreases expression of a TCR and/or HLA. The CRISPR/Cas system can alternatively be used like RNA interference, turning off TCR and/or HLA gene in a reversible fashion. In a mammalian cell, for example, the RNA can guide the Cas protein to a TCR and/or HLA promoter, sterically blocking RNA polymerases.

[00392] Artificial CRISPR/Cas systems can be generated which inhibit TCR and/or HLA, using technology known in the art, e.g., that described in U.S. Publication No. 20140068797, and Cong (2013) *Science* 339: 819-823. Other artificial CRISPR/Cas systems that are known in the art may also be generated which inhibit TCR and/or HLA, e.g., that described in Tsai (2014) *Nature Biotechnol.,* 32:6 569-576, U.S. Patent No.: 8,871,445; 8,865,406; 8,795,965; 8,771,945; and 8,697,359.

TALEN to inhibit TCR and/or HLA

[00393] "TALEN" or "TALEN to HLA and/or TCR" or "TALEN to inhibit HLA and/or TCR" refers to a transcription activator-like effector nuclease, an artificial nuclease which can be used to edit the HLA and/or TCR gene.

[00394] TALENs are produced artificially by fusing a TAL effector DNA binding domain to a DNA cleavage domain. Transcription activator-like effects (TALEs) can be engineered to bind any desired DNA sequence, including a portion of the HLA or TCR gene. By combining an engineered TALE with a DNA cleavage domain, a restriction enzyme can be produced which is specific to any desired DNA sequence, including a HLA or TCR sequence. These can then be introduced into a cell, wherein they can be used for genome editing. Boch (201 1) *Nature Biotech.* 29: 135-6; and Boch et al. (2009) *Science* 326: 1509-12; Moscou et al. (2009) *Science* 326: 3501.

[00395] TALEs are proteins secreted by Xanthomonas bacteria. The DNA binding domain contains a repeated, highly conserved 33-34 amino acid sequence, with the exception of the 12th and 13th amino acids. These two positions are highly variable, showing a strong correlation with specific nucleotide recognition. They can thus be engineered to bind to a desired DNA sequence.

[00396] To produce a TALEN, a TALE protein is fused to a nuclease (N), which is a wild-type or mutated Fokl endonuclease. Several mutations to Fokl have been made for its use in TALENs; these, for example, improve cleavage specificity or activity. Cermak et al. (201 1) *Nucl. Acids Res.* 39: e82; Miller et al. (201 1) *Nature Biotech.* 29: 143-8; Hockemeyer et al. (201 1) *Nature Biotech.* 29: 731-734; Wood et al. (201 1) *Science* 333: 307; Doyon et al. (2010) *Nature Methods* 8: 74-79; Szczepek et al. (2007) *Nature Biotech.* 25: 786-793; and Guo et al. (2010) *J. Mol. Biol.* 200: 96.

[00397] The Fokl domain functions as a dimer, requiring two constructs with unique DNA binding domains for sites in the target genome with proper orientation and spacing. Both the number of amino acid residues between the TALE DNA binding domain and the Fokl cleavage domain and the number of bases between the two individual TALEN binding sites appear to be important parameters for achieving high levels of activity. Miller et al. (201 1) *Nature Biotech.* 29: 143-8.

[00398] A HLA or TCR TALEN can be used inside a cell to produce a double-stranded break (DSB). A mutation can be introduced at the break site if the repair mechanisms improperly

repair the break via non-homologous end joining. For example, improper repair may introduce a frame shift mutation. Alternatively, foreign DNA can be introduced into the cell along with the TALEN; depending on the sequences of the foreign DNA and chromosomal sequence, this process can be used to correct a defect in the HLA or TCR gene or introduce such a defect into a wt HLA or TCR gene, thus decreasing expression of HLA or TCR.

[00399] TALENs specific to sequences in HLA or TCR can be constructed using any method known in the art, including various schemes using modular components. Zhang et al. (2011) *Nature Biotech.* 29: 149-53; Geibler et al. *(2*011) *PLoS ONE 6:* e19509.

Zincfinger nuclease to inhibit HLA and/or TCR

[00400] "ZFN" or "Zinc Finger Nuclease" or "ZFN to HLA and/or TCR" or "ZFN to inhibit HLA and/or TCR" refer to a zinc finger nuclease, an artificial nuclease which can be used to edit the HLA and/or TCR gene.

[00401] Like a TALEN, a ZFN comprises a Fokl nuclease domain (or derivative thereof) fused to a DNA-binding domain. In the case of a ZFN, the DNA-binding domain comprises one or more zinc fingers. Carroll et al. (2011) *Genetics Society of America* 188: 773-782; and Kim et al. (1996) *Proc. Natl. Acad. Sci. USA* 93: 1156-1160.

[00402] A zinc finger is a small protein structural motif stabilized by one or more zinc ions. A zinc finger can comprise, for example, Cys2His2, and can recognize an approximately 3-bp sequence. Various zinc fingers of known specificity can be combined to produce multi-finger polypeptides which recognize about 6, 9, 12, 15 or 18-bp sequences. Various selection and modular assembly techniques are available to generate zinc fingers (and combinations thereof) recognizing specific sequences, including phage display, yeast one-hybrid systems, bacterial one-hybrid and two-hybrid systems, and mammalian cells.

[00403] Like a TALEN, a ZFN must dimenze to cleave DNA. Thus, a pair of ZFNs are required to target non-palindromic DNA sites. The two individual ZFNs must bind opposite strands of the DNA with their nucleases properly spaced apart. Bitinaite et al. (1998) *Proc. Natl. Acad. Sci. USA* 95: 10570-5.

[00404] Also like a TALEN, a ZFN can create a double-stranded break in the DNA, which can create a frame-shift mutation if improperly repaired, leading to a decrease in the expression

and amount of HLA and/or TCR in a cell. ZFNs can also be used with homologous recombination to mutate in the HLA or TCR gene.

[00405] ZFNs specific to sequences in HLA AND/OR TCR can be constructed using any method known in the art. See, e.g., Provasi (201 1) *Nature Med.* 18: 807-815; Torikai (2013) *Blood* 122: 1341-1349; Cathomen et al. (2008) Mo/. *Ther.* 16: 1200-7; and Guo et al. (2010) *J. Mol. Biol.* 400: 96; U.S. Patent Publication 201 1/0158957; U.S. Patent Publication 2012/0060230.

Activation and Expansion of Cells

[00406] Cells may be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005.

[00407] Generally, the T cells of the invention may be expanded by contact with a surface having attached thereto an agent that stimulates a CD3/TCR complex associated signal and a ligand that stimulates a costimulatory molecule on the surface of the T cells. In particular, T cell populations may be stimulated as described herein, such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. To stimulate proliferation of either CD4+ T cells or CD8+ T cells, an anti-CD3 antibody and an anti-CD28 antibody. Examples of an anti-CD28 antibody include 9.3, B-T3, XR-CD28 (Diaclone, Besancon, France) can be used as can other methods commonly known in the art (Berg *et al.*, Transplant Proc. 30(8):3975-3977, 1998; Haanen *ei a/.*, J. Exp. Med. 190(9):13191328, 1999; Garland *et al*, J. Immunol Meth. 227(1-2):53-63, 1999).

[00408] In certain aspects, the primary stimulatory signal and the costimulatory signal for the T cell may be provided by different protocols. For example, the agents providing each signal may be in solution or coupled to a surface. When coupled to a surface, the agents may be coupled to the same surface (i.e., in "cis" formation) or to separate surfaces (i.e., in "trans" formation). Alternatively, one agent may be coupled to a surface and the other agent in solution. In one aspect, the agent providing the costimulatory signal is bound to a cell surface and the agent providing the primary activation signal is in solution or coupled to a surface. In certain aspects, both agents can be in solution. In one aspect, the agents may be in soluble form, and then cross-linked to a surface, such as a cell expressing Fc receptors or an antibody or other binding agent which will bind to the agents. In this regard, see for example, U.S. Patent Application Publication Nos. 20040101519 and 20060034810 for artificial antigen presenting cells (aAPCs) that are contemplated for use in activating and expanding T cells in the present invention.

[00409] In one aspect, the two agents are immobilized on beads, either on the same bead, i.e., "cis," or to separate beads, i.e., "trans." By way of example, the agent providing the primary activation signal is an anti-CD3 antibody or an antigen-binding fragment thereof and the agent providing the costimulatory signal is an anti-CD28 antibody or antigen-binding fragment thereof; and both agents are co-immobilized to the same bead in equivalent molecular amounts. In one aspect, a 1:1 ratio of each antibody bound to the beads for CD4+ T cell expansion and T cell growth is used. In certain aspects of the present invention, a ratio of anti CD3:CD28 antibodies bound to the beads is used such that an increase in T cell expansion is observed as compared to the expansion observed using a ratio of 1:1. In one particular aspect an increase of from about 1 to about 3 fold is observed as compared to the expansion observed using a ratio of 1:1. In one aspect, the ratio of CD3:CD28 antibody bound to the beads ranges from 100: 1 to 1:100 and all integer values there between. In one aspect of the present invention, more anti-CD28 antibody is bound to the particles than anti-CD3 antibody, i.e., the ratio of CD3:CD28 is less than one. In certain aspects of the invention, the ratio of anti CD28 antibody to anti CD3 antibody bound to the beads is greater than 2:1. In one particular aspect, a 1:100 CD3 :CD28 ratio of antibody bound to beads is used. In one aspect, a 1:75 CD3:CD28 ratio of antibody bound to beads is used. In a further aspect, a 1:50 CD3:CD28 ratio of antibody bound to beads is used. In one aspect, a 1:30 CD3:CD28 ratio of antibody bound to beads is used. In one preferred aspect, a 1:10 CD3:CD28

ratio of antibody bound to beads is used. In one aspect, a 1:3 CD3:CD28 ratio of antibody bound to the beads is used. In yet one aspect, a 3:1 CD3:CD28 ratio of antibody bound to the beads is used.

[0041 0] Ratios of particles to cells from 1:500 to 500: 1 and any integer values in between may be used to stimulate T cells or other target cells. As those of ordinary skill in the art can readily appreciate, the ratio of particles to cells may depend on particle size relative to the target cell. For example, small sized beads could only bind a few cells, while larger beads could bind many. In certain aspects the ratio of cells to particles ranges from 1:100 to 100: 1 and any integer values in-between and in further aspects the ratio comprises 1:9 to 9:1 and any integer values in between, can also be used to stimulate T cells. The ratio of anti-CD3- and anti-CD28-coupled particles to T cells that result in T cell stimulation can vary as noted above, however certain preferred values include 1:100, 1:50, 1:40, 1:30, 1:20, 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, and 15:1 with one preferred ratio being at least 1:1 particles per T cell. In one aspect, a ratio of particles to cells of 1:1 or less is used. In one particular aspect, a preferred particle: cell ratio is 1:5. In further aspects, the ratio of particles to cells can be varied depending on the day of stimulation. For example, in one aspect, the ratio of particles to cells is from 1:1 to 10:1 on the first day and additional particles are added to the cells every day or every other day thereafter for up to 10 days, at final ratios of from 1:1 to 1:10 (based on cell counts on the day of addition). In one particular aspect, the ratio of particles to cells is 1:1 on the first day of stimulation and adjusted to 1:5 on the third and fifth days of stimulation. In one aspect, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:5 on the third and fifth days of stimulation. In one aspect, the ratio of particles to cells is 2:1 on the first day of stimulation and adjusted to 1:10 on the third and fifth days of stimulation. In one aspect, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:10 on the third and fifth days of stimulation. One of skill in the art will appreciate that a variety of other ratios may be suitable for use in the present invention. In particular, ratios will vary depending on particle size and on cell size and type. In one aspect, the most typical ratios for use are in the neighborhood of 1:1, 2:1 and 3:1 on the first day.

[0041 1] In further aspects of the present invention, the cells, such as T cells, are combined with agent-coated beads, the beads and the cells are subsequently separated, and then the cells

are cultured. In an alternative aspect, prior to culture, the agent-coated beads and cells are not separated but are cultured together. In a further aspect, the beads and cells are first concentrated by application of a force, such as a magnetic force, resulting in increased ligation of cell surface markers, thereby inducing cell stimulation.

[00412] By way of example, cell surface proteins may be ligated by allowing paramagnetic beads to which anti-CD3 and anti-CD28 are attached (3x28 beads) to contact the T cells. In one aspect the cells (for example, 10^4 to 10^9 T cells) and beads (for example, DYNABEADS® M-450 CD3/CD28 T paramagnetic beads at a ratio of 1:1) are combined in a buffer, for example PBS (without divalent cations such as, calcium and magnesium). Again, those of ordinary skill in the art can readily appreciate any cell concentration may be used. For example, the target cell may be very rare in the sample and comprise only 0.01% of the sample or the entire sample (i.e., 100%) may comprise the target cell of interest. Accordingly, any cell number is within the context of the present invention. In certain aspects, it may be desirable to significantly decrease the volume in which particles and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and particles. For example, in one aspect, a concentration of about 2 billion cells/ml is used. In one aspect, greater than 100 million cells/ml is used. In a further aspect, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet one aspect, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further aspects, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells. Such populations of cells may have therapeutic value and would be desirable to obtain in certain aspects. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

[00413] In one aspect of the present invention, the mixture may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. In one aspect, the mixture may be cultured for 21 days. In one aspect of the invention the beads and the T cells are cultured together for about eight days. In one aspect, the beads and T cells are cultured together for 2-3 days. Several cycles of stimulation may also be desired such that culture time of T cells can be 60 days or more. Conditions appropriate for T cell culture include an appropriate media

(e.g., Minimal Essential Media or RPMI Media 1640 or, X-vivo 15, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-γ, IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGFP, and TNF-a or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, ATM-V,DMEM, MEM, a-MEM, F-12, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, e.g., penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (e.g., 37° C) and atmosphere (e.g., air plus 5% $C0_2$).

[00414] T cells that have been exposed to varied stimulation times may exhibit different characteristics. For example, typical blood or apheresed peripheral blood mononuclear cell products have a helper T cell population (TH, CD4+) that is greater than the cytotoxic or suppressor T cell population (TC, CD8+). Ex vivo expansion of T cells by stimulating CD3 and CD28 receptors produces a population of T cells that prior to about days 8-9 consists predominately of TH cells, while after about days 8-9, the population of T cells comprises an increasingly greater population of TC cells. Accordingly, depending on the purpose of treatment, infusing a subject with a T cell population comprising predominately of TH cells may be advantageous. Similarly, if an antigen-specific subset of TC cells has been isolated it may be beneficial to expand this subset to a greater degree.

[0041 5] Further, in addition to CD4 and CD8 markers, other phenotypic markers vary significantly, but in large part, reproducibly during the course of the cell expansion process. Thus, such reproducibility enables the ability to tailor an activated T cell product for specific purposes.

[00416] Once a mesothelin CAR is constructed, various assays can be used to evaluate the activity of the molecule, such as but not limited to, the ability to expand T cells following antigen stimulation, sustain T cell expansion in the absence of re-stimulation, and anti-cancer

activities in appropriate in vitro and animal models. Assays to evaluate the effects of a mesothelin CAR are described in further detail below

[00417] Western blot analysis of CAR expression in primary T cells can be used to detect the presence of monomers and dimers. See, *e.g.,* Milone *et ah,* Molecular Therapy 17(8): 1453-1464 (2009). Very briefly, T cells (1:1 mixture of $CD4^+$ and $CD8^+$ T cells) expressing the CARs are expanded *in vitro* for more than 10 days followed by lysis and SDS-PAGE under reducing conditions. CARs containing the full length TCR-ζ cytoplasmic domain and the endogenous TCR-ζ chain are detected by western blotting using an antibody to the TCR-ζ chain. The same T cell subsets are used for SDS-PAGE analysis under non-reducing conditions to permit evaluation of covalent dimer formation.

[00418] *In vitro* expansion of CAR^+ T cells following antigen stimulation can be measured by flow cytometry. For example, a mixture of $CD4^+$ and $CD8^+$ T cells are stimulated with aCD3/aCD28 aAPCs followed by transduction with lentiviral vectors expressing GFP under the control of the promoters to be analyzed. Exemplary promoters include the CMV IE gene, EF-1α, ubiquitin C, or phosphoglycerokinase (PGK) promoters. GFP fluorescence is evaluated on day 6 of culture in the $CD4^+$ and/or $CD8^+$ T cell subsets by flow cytometry. See, *e.g.,* Milone *et ah,* Molecular Therapy 17(8): 1453-1464 (2009). Alternatively, a mixture of $CD4^+$ and $CD8^+$ T cells are stimulated with aCD3/aCD28 coated magnetic beads on day 0, and transduced with CAR on day 1 using a bicistronic lentiviral vector expressing CAR along with eGFP using a 2A ribosomal skipping sequence. Cultures are re-stimulated, e.g., with K562 cells expressing hCD32 and 4-1BBL in the presence of anti-CD3 and anti-CD28 antibody (K562-BBL-3/28) following washing. Exogenous IL-2 is added to the cultures every other day at 100 IU/ml. GFP^+ T cells are enumerated by flow cytometry using bead-based counting. See, *e.g.,* Milone *et ah,* Molecular Therapy 17(8): 1453-1464 (2009).

[00419] Sustained CAR^+ T cell expansion in the absence of re-stimulation can also be measured. See, *e.g.,* Milone *et al.,* Molecular Therapy 17(8): 1453-1464 (2009). Briefly, mean T cell volume (fl) is measured on day 8 of culture using a Coulter Multisizer III particle counter following stimulation with aCD3/aCD28 coated magnetic beads on day 0, and transduction with the indicated CAR on day 1.

[00420] Assessment of cell proliferation and cytokine production has been previously described, *e.g.,* at Milone *et al.,* Molecular Therapy 17(8): 1453-1464 (2009). Briefly, assessment of CAR-mediated proliferation is performed in microtiter plates by mixing washed T cells with target cells, such as K562-Meso, Ovcar3, Ovcar8, SW1990, Panc02.03 cells expressing mesothelin or CD32 and CD137 (KT32-BBL) for a final T-cell:target cell ratio of 1:1. Anti-CD3 (clone OKT3) and anti-CD28 (clone 9.3) monoclonal antibodies are added to cultures with KT32-BBL cells to serve as a positive control for stimulating T-cell proliferation since these signals support long-term $CD8^+$ T cell expansion *ex vivo.* T cells are enumerated in cultures using CountBright™ fluorescent beads (Invitrogen, Carlsbad, CA) and flow cytometry as described by the manufacturer. CAR^+ T cells are identified by GFP expression using T cells that are engineered with eGFP-2A linked CAR-expressing lentiviral vectors. For CAR+ T cells not expressing GFP, the CAR+ T cells are detected with biotinylated recombinant mesothelin protein and a secondary avidin-PE conjugate. CD4+ and $CD8^+$ expression on T cells are also simultaneously detected with specific monoclonal antibodies (BD Biosciences). Cytokine measurements are performed on supernatants collected 24 hours following re-stimulation using the human TH1/TH2 cytokine cytometric bead array kit (BD Biosciences, San Diego, CA) according the manufacturer's instructions. Fluorescence is assessed using a FACScalibur flow cytometer, and data is analyzed according to the manufacturer's instructions.

[00421] Cytotoxicity can be assessed by methods described herein, e.g., in the examples, or by a standard 51Cr-release assay. See, *e.g.,* Milone *et al.,* Molecular Therapy 17(8): 1453-1464 (2009). Briefly, target cells (e.g., BHK or CHO cells expressing mesothelin) are loaded with 51Cr (as NaCr04, New England Nuclear, Boston, MA) at 37°C for 2 hours with frequent agitation, washed twice in complete RPMI and plated into microtiter plates. Effector T cells are mixed with target cells in the wells in complete RPMI at varying ratios of effector celktarget cell (E:T). Additional wells containing media only (spontaneous release, SR) or a 1% solution of triton-X 100 detergent (total release, TR) are also prepared. After 4 hours of incubation at 37°C, supernatant from each well is harvested. Released ^{51}Cr is then measured using a gamma particle counter (Packard Instrument Co., Waltham, MA). Each condition is performed in at least triplicate, and the percentage of lysis is calculated using the formula: % Lysis = (ER- SR) / (TR - SR), where ER represents the average 51Cr released for each experimental condition. Alternative cytotoxicity assays may also be used, such as flow based cytotoxicity assays.

[00422] Click beetle red and click beetle green luciferase can be used to simultaneously follow tumor progression and T cell trafficking, as each use the same luciferin substrate but emit light at the opposite ends of the visible light spectrum.

[00423] Other assays, including those described in the Example section herein as well as those that are known in the art can also be used to evaluate the mesothelin CAR constructs of the invention.

Therapeutic Application for Mesothelin Expressing Diseases and Disorders

[00424] The present invention provides compositions and methods for treating diseases and disorders associated with mesothelin. An example of a disease or disorder associated with mesothelin is mesothelioma.

[00425] Malignant mesothelioma is a type of cancer that occurs in the thin layer of cells lining the body's internal organs, known as the mesothelium. There are three recognized types of mesothelioma. Pleural mesothelioma (e.g., malignant pleural mesothelioma, or MPM) is the most common form of the disease, accounting for roughly 70% of cases, and occurs in the lining of the lung known as the pleura. Peritoneal mesothelioma occurs in the lining of the abdominal cavity, known as the peritoneum. Pericardial mesothelioma originates in the pericardium, which lines the heart.

[00426] A subject may be at risk to develop mesothelioma if the subject was exposed to asbestos. Exposure to asbestos and the inhalation of asbestos particles can cause mesothelioma. In most cases, mesothelioma symptoms will not appear in a subject exposed to asbestos until many years after the exposure has occurred.

[00427] Symptoms of pleural mesothelioma include, e.g., lower back pain or side chest pain, and shortness of breath. Other symptoms include difficulty swallowing, persistent cough, fever, weight loss or fatigue. Additional symptoms that some patients experience are muscle weakness, loss of sensory capability, coughing up blood, facial and arm swelling, and hoarseness. In the early stages of the disease, such as stage 1 mesothelioma, symptoms may be mild. Patients usually report pain in one area of the chest that never seems to go away, weight loss and fever.

[00428] Peritoneal mesothelioma originates in the abdomen and as a result, symptoms often include abdominal pain, weight loss, nausea, and vomiting. Fluid buildup may occur in the

abdomen as well as a result of the cancer. Peritoneal mesothelioma originates in the abdomen and will frequently spread to other organs in area including the liver, spleen or bowel. Severe abdominal pain is the most common complaint that patients first experience. There may also be a discomfort level with fluid buildup in the abdomen as well. Other symptoms of peritoneal mesothelioma may include difficult bowel movements, nausea and vomiting, fever and swollen feet.

[00429] Pericardial mesothelioma is the least common form of mesothelioma. Pericardial mesothelioma, as the name suggests, involves the heart. This rare type of mesothelioma cancer invades the pericardium, the sac that surrounds the heart. As the cancer progresses, the heart is not able to deliver oxygen as efficiently to the body causing further decline in health at an increasingly rapid rate. The symptoms most commonly associated with pericardial mesothelioma mimic those of a heart attack: nausea, pain in the chest and shortness of breath.

[00430] Subjects benefiting from treatment according to the invention include subjects with a mesothelioma, or subjects suspected of having mesothelioma, e.g., as evidenced by the presence of one or more of the symptoms described herein and/or exposure to asbestos. In particular embodiments, the mesothelioma is pleural mesothelioma (e.g., malignant pleural mesothelioma). In other aspects, the subject may be treated that has a precancerous condition such as, e.g., pleural plaques, benign mesothelioma or mesothelial hyperplasia.

[0043 1] Another example of a disease or disorder associated with mesothelin is pancreatic cancer. Pancreatic cancers that can be treated with methods described herein include, but are not limited to, exocrine pancreatic cancers and endocrine pancreatic cancers. Exocrine pancreatic cancers include, but are not limited to, adenocarcinomas, acinar cell carcinomas, adenosquamous carcinomas, colloid carcinomas, undifferentiated carcinomas with osteoclast-like giant cells, hepatoid carcinomas, intraductal papillary-mucinous neoplasms, mucinous cystic neoplasms, pancreatoblastomas, serous cystadenomas, signet ring cell carcinomas, solid and pseuodpapiUary tumors, pancreatic ductal carcinomas, and undifferentiated carcinomas. In some embodiments, the exocrine pancreatic cancer is pancreatic ductal carcinoma. Endocrine pancreatic cancers include, but are not limited to, insulinomas and glucagonomas.

[00432] In some embodiments, the pancreatic cancer is any of early stage pancreatic cancer, non-metastatic pancreatic cancer, primary pancreatic cancer, resected pancreatic cancer,

advanced pancreatic cancer, locally advanced pancreatic cancer, metastatic pancreatic cancer, unresectable pancreatic cancer, pancreatic cancer in remission, recurrent pancreatic cancer, pancreatic cancer in an adjuvant setting, or pancreatic cancer in a neoadjuvant setting. In some embodiments, the pancreatic cancer is locally advanced pancreatic cancer, unresectable pancreatic cancer, or metastatic pancreatic ductal carcinoma. In some embodiments, the pancreatic cancer is resistant to the gemcitabine-based therapy. In some embodiments, the pancreatic cancer is refractory to the gemcitabine-based therapy.

[00433] In other aspects, the disorder associated with mesothelin expression is ovarian cancer. Ovarian cancer is classified according to the histology of the tumor. Surface epithelial-stromal tumor, also known as ovarian epithelial carcinoma, is the most common type of ovarian cancer. It includes serous tumor (including serous papillary cystadenocarcinoma), endometrioid tumor and mucinous cystadenocarcinoma.

[00434] The methods described herein can be used to treat various stages of ovarian cancer, e.g., stage I, stage II, stage III or stage IV. Staging can be performed, e.g., when the ovarian cancer is removed. Ovarian cancer is staged as follows:

[00435] Stage I cancer is confined to one or both ovaries. The cancer is stage II if either one or both of the ovaries is involved and has spread to the uterus and/or the fallopian tubes or other sites in the pelvis. The cancer is stage III cancer if one or both of the ovaries is involved and has spread to lymph nodes or other sites outside of the pelvis but is still within the abdominal cavity, such as the surface of the intestine or liver. The cancer is stage IV cancer if one or both ovaries are involved and the cancer has spread outside the abdomen or to the inside of the liver.

[00436] In some embodiments, the ovarian cancer is resistant to one or more chemotherapeutic agent. In some embodiments, the ovarian cancer is refractory to the one or more chemotherapeutic agent.

[00437] Other cancers that can be treated with the CAR compositions described herein include, e.g., brain cancer, bladder cancer, breast cancer, cervical cancer, colorectal cancer, liver cancer, kidney cancer, lymphoma, leukemia, lung cancer (e.g., lung adenocarcinoma), melanoma, metastatic melanoma, mesothelioma, neuroblastoma, ovarian cancer, prostate cancer, pancreatic cancer, renal cancer, skin cancer, thymoma, sarcoma, non-Hodgkin's lymphoma, Hodgkin's lymphoma, uterine cancer, and any combination thereof.

[00438] The present invention provides methods for inhibiting the proliferation or reducing a mesothelin-expressing cell population, the methods comprising contacting a population of cells comprising a mesothelin expressing cell with a mesothelin CAR-expressing cell of the invention that binds to the mesothelin-expressing cell. In a specific embodiment, the invention provides methods for inhibiting the proliferation or reducing the population of cancer cells expressing mesothelin, the methods comprising contacting the mesothelin -expressing cancer cell population with a mesothelin CAR-expressing cell of the invention that binds to the mesothelin-expressing cell. In another embodiment, the invention provides methods for inhibiting the proliferation or reducing the population of cancer cells expressing mesothelin, the methods comprising contacting the mesothelin -expressing cancer cell population with a mesothelin CAR-expressing cell of the invention that binds to the mesothelin-expressing cell. In certain embodiments, the mesothelin CAR-expressing cell of the invention reduces the quantity, number, amount or percentage of cells and/or cancer cells by at least 25%, at least 30%, at least 40%, at least 50%, at least 65%, at least 75%, at least 85%, at least 95%, or at least 99% in a subject with or animal model of mesothelioma or another cancer associated with mesothelin -expressing cells relative to a negative control. In one aspect, the subject is a human.

[00439] The invention also provides methods for preventing, treating and/or managing a disorder associated with mesothelin -expressing cells (e.g., mesothelioma), the methods comprising administering to a subject in need a mesothelioma CAR-expressing cell of the invention that binds to the mesothelin -expressing cell. In one aspect, the subject is a human.

[00440] The invention provides methods for preventing relapse of cancer associated with mesothelin-expressing cells, the methods comprising administering to a subject in need thereof a mesothelin CAR-expressing cell of the invention that binds to the mesothelin-expressing cell. In another embodiment, the methods comprise administering to the subject in need thereof an effective amount of a mesothelin CAR-expressing cell of the invention that binds to the mesothelin-expressing cell in combination with an effective amount of another therapy.

[00441] In one aspect, the invention pertains to a vector comprising a sequence encoding a mesothelin CAR operably linked to promoter for expression in mammalian immune effector cells. In one aspect, the invention provides a recombinant immune effector cell expressing the mesothelin CAR for use in treating mesothelin -expressing tumors. In one aspect, the mesothelin

CAR-expressing cell of the invention is capable of contacting a tumor cell with at least one mesothelin CAR of the invention expressed on its surface such that the mesothelin CAR-expressing cell is activated in response to the antigen and the CAR-expressing cell targets the cancer cell and growth of the cancer is inhibited.

[00442] In one aspect, the invention pertains to a method of inhibiting growth of a mesothelin -expressing cancer cell, comprising contacting the tumor cell with a-mesothelin CAR-expressing cell such that the CAR-expressing cell is activated in response to the antigen and targets the cancer cell, wherein the growth of the cancer is inhibited. In one aspect, the activated CART targets and kills the cancer cell.

[00443] In one aspect, the invention pertains to a method of treating cancer in a subject. The method comprises administering to the subject a mesothelin CAR-expressing cell such that the cancer is treated in the subject. An example of a cancer that is treatable by the mesothelin CAR-expressing cell of the invention is a cancer associated with expression of mesothelin. In one aspect, the cancer associated with expression of mesothelin is selected from mesothelioma, pancreatic cancer, ovarian cancer and lung cancer.

[00444] The invention includes a type of cellular therapy where immune effector cells, e.g., T cells or NK cells, are genetically modified to express a chimeric antigen receptor (CAR) and the CAR-expressing cell is infused to a recipient in need thereof. The infused cell is able to kill tumor cells in the recipient. Unlike antibody therapies, CAR-modified immune effector cells are able to replicate in vivo resulting in long-term persistence that can lead to sustained tumor control. In various aspects, the cells administered to the patient, or their progeny, persist in the patient for at least four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, twelve months, thirteen months, fourteen month, fifteen months, sixteen months, seventeen months, eighteen months, nineteen months, twenty months, twenty-one months, twenty-two months, twenty-three months, two years, three years, four years, or five years after administration of the cell to the patient.

[00445] The invention also includes a type of cellular therapy where immune effector cells are modified, e.g., by in vitro transcribed RNA, to transiently express a chimeric antigen receptor (CAR) and the CAR-expressing cell is infused to a recipient in need thereof. The infused cell is able to kill cancer cells in the recipient. Thus, in various aspects, the cells administered to the

patient, is present for less than one month, e.g., three weeks, two weeks, one week, after administration of the cell to the patient.

[00446] Without wishing to be bound by any particular theory, the anti-cancer immunity response elicited by the CAR-modified immune effector cells may be an active or a passive immune response, or alternatively may be due to a direct vs indirect immune response. In one aspect, the CAR transduced T cells exhibit specific proinflammatory cytokine secretion and potent cytolytic activity in response to human cancer cells expressing mesothelin, and mediate bystander killing and mediate regression of an established human tumor. For example, antigen-less tumor cells within a heterogeneous field of mesothelin-expressing tumor may be susceptible to indirect destruction by mesothelin-redirected T cells that has previously reacted against adjacent antigen-positive cancer cells.

[00447] In one aspect, the fully-human scFv bearing CAR-modified immune effector cells of the invention may be a type of vaccine for ex vivo immunization and/or in vivo therapy in a mammal. In one aspect, the mammal is a human.

[00448] With respect to ex vivo immunization, at least one of the following occurs in vitro prior to administering the cell into a mammal: i) expansion of the cells, ii) introducing a nucleic acid encoding a CAR to the cells or iii) cryopreservation of the cells.

[00449] Ex vivo procedures are well known in the art and are discussed more fully below. Briefly, cells are isolated from a mammal (e.g., a human) and genetically modified (i.e., transduced or transfected in vitro) with a vector expressing a CAR disclosed herein. The CAR-modified cell can be administered to a mammalian recipient to provide a therapeutic benefit. The mammalian recipient may be a human and the CAR-modified cell can be autologous with respect to the recipient. Alternatively, the cells can be allogeneic, syngeneic or xenogeneic with respect to the recipient.

[00450] The procedure for ex vivo expansion of hematopoietic stem and progenitor cells is described in U.S. Pat. No. 5,199,942, incorporated herein by reference, can be applied to the cells of the present invention. Other suitable methods are known in the art therefore the present invention is not limited to any particular method of ex vivo expansion of the cells. Briefly, ex vivo culture and expansion of T cells comprises: (1) collecting CD34+ hematopoietic stem and progenitor cells from a mammal from peripheral blood harvest or bone marrow explants; and (2)

expanding such cells ex vivo. In addition to the cellular growth factors described in U.S. Pat. No. 5,199,942, other factors such as flt3-L, IL-1, IL-3 and c-kit ligand, can be used for culturing and expansion of the cells.

[00451] In addition to using a cell-based vaccine in terms of ex vivo immunization, the present invention also provides compositions and methods for in vivo immunization to elicit an immune response directed against an antigen in a patient.

[00452] Generally, the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised. In particular, the CAR-modified immune effector cells of the invention are used in the treatment of diseases, disorders and conditions associated with expression of mesothelin. In certain aspects, the cells of the invention are used in the treatment of patients at risk for developing diseases, disorders and conditions associated with expression of mesothelin. Thus, the invention provides methods for the treatment or prevention of diseases, disorders and conditions associated with expression of mesothelin comprising administering to a subject in need thereof, a therapeutically effective amount of the CAR-modified T cells of the invention.

[00453] The CAR-modified T cells of the present invention may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components such as IL-2 or other cytokines or cell populations.

[00454] The present invention also provides methods for inhibiting the proliferation or reducing a mesothelin-expressing cell population, the methods comprising contacting a population of cells comprising a mesothelin-expressing cell with a mesothelin CAR-expressing cell (, e.g., a mesothelin CART also referred to as "CART-MSLN") of the invention that binds to the mesothelin-expressing cell. In a specific aspect, the invention provides methods for inhibiting the proliferation or reducing the population of cancer cells expressing mesothelin, the methods comprising contacting the mesothelin-expressing cancer cell population with a mesothelin CAR-expressing cell of the invention that binds to the mesothelin-expressing cell. In one aspect, the present invention provides methods for inhibiting the proliferation or reducing the population of cancer cells expressing mesothelin, the methods comprising contacting the mesothelin-expressing cancer cell population with a mesothelin CAR-expressing cell of the invention that binds to the mesothelin-expressing cell. In certain aspects, the mesothelin CAR-expressing cell

of the invention reduces the quantity, number, amount or percentage of cells and/or cancer cells by at least 25%, at least 30%, at least 40%, at least 50%, at least 65%, at least 75%, at least 85%, at least 95%, or at least 99% in a subject with or animal model for mesothelioma or another cancer associated with mesothelin-expressing cells relative to a negative control. In one aspect, the subject is a human.

[00455] The present invention also provides methods for preventing, treating and/or managing a disease associated with mesothelin-expressing cells (e.g., mesothelioma), the methods comprising administering to a subject in need a mesothelin CAR-expressing cell of the invention that binds to the mesothelin-expressing cell. In one aspect, the subject is a human.

[00456] The present invention provides methods for preventing relapse of cancer associated with mesothelin-expressing cells, the methods comprising administering to a subject in need thereof a mesothelin CAR-expressing cell of the invention that binds to the mesothelin-expressing cell. In one aspect, the methods comprise administering to the subject in need thereof an effective amount of a mesothelin CAR-expressing cell of the invention that binds to the mesothelin-expressing cell in combination with an effective amount of another therapy.

Combination Therapies

[00457] A CAR-expressing cell described herein may be used in combination with other known agents and therapies. Administered "in combination", as used herein, means that two (or more) different treatments are delivered to the subject during the course of the subject's affliction with the disorder, e.g., the two or more treatments are delivered after the subject has been diagnosed with the disorder and before the disorder has been cured or eliminated or treatment has ceased for other reasons. In some embodiments, the delivery of one treatment is still occurring when the delivery of the second begins, so that there is overlap in terms of administration. This is sometimes referred to herein as "simultaneous" or "concurrent delivery". In other embodiments, the delivery of one treatment ends before the delivery of the other treatment begins. In some embodiments of either case, the treatment is more effective because of combined administration. For example, the second treatment is more effective, e.g., an equivalent effect is seen with less of the second treatment, or the second treatment reduces symptoms to a greater extent, than would be seen if the second treatment were administered in the absence of the first treatment, or the analogous situation is seen with the first treatment. In

some embodiments, delivery is such that the reduction in a symptom, or other parameter related to the disorder is greater than what would be observed with one treatment delivered in the absence of the other. The effect of the two treatments can be partially additive, wholly additive, or greater than additive. The delivery can be such that an effect of the first treatment delivered is still detectable when the second is delivered.

[00458] A CAR-expressing cell described herein and the at least one additional therapeutic agent can be administered simultaneously, in the same or in separate compositions, or sequentially. For sequential administration, the CAR-expressing cell described herein can be administered first, and the additional agent can be administered second, or the order of administration can be reversed.

[00459] In further aspects, a CAR-expressing cell described herein may be used in a treatment regimen in combination with surgery, chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludarabine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, and irradiation. peptide vaccine, such as that described in Izumoto et al. 2008 J Neurosurg 108:963-971.

[00460] In one embodiment, a CAR-expressing cell described herein can be used in combination with a chemotherapeutic agent. Exemplary chemotherapeutic agents include an anthracycline (e.g., doxorubicin (e.g., liposomal doxorubicin)). a vinca alkaloid (e.g., vinblastine, vincristine, vindesine, vinorelbine), an alkylating agent (e.g., cyclophosphamide, decarbazine, melphalan, ifosfamide, temozolomide), an immune cell antibody (e.g., alemtuzamab, gemtuzumab, rituximab, tositumomab), an antimetabolite (including, e.g., folic acid antagonists, pyrimidine analogs, purine analogs and adenosine deaminase inhibitors (e.g., fludarabine)), an mTOR inhibitor, a TNFR glucocorticoid induced TNFR related protein (GITR) agonist, a proteasome inhibitor (e.g., aclacinomycin A, gliotoxin or bortezomib), an immunomodulator such as thalidomide or a thalidomide derivative (e.g., lenalidomide).

[00461] General Chemotherapeutic agents considered for use in combination therapies include anastrozole (Arimidex®), bicalutamide (Casodex®), bleomycin sulfate (Blenoxane®), busulfan (Myleran®), busulfan injection (Busulfex®), capecitabine (Xeloda®), N4-

pentoxycarbonyl-5-deoxy-5-fluorocytidine, carboplatin (Paraplatin®), carmustine (BiCNU®), chlorambucil (Leukeran®), cisplatin (Platinol®), cladribine (Leustatin®), cyclophosphamide (Cytoxan® or Neosar®), cytarabine, cytosine arabinoside (Cytosar-U®), cytarabine liposome injection (DepoCyt®), dacarbazine (DTIC-Dome®), dactinomycin (Actinomycin D, Cosmegan), daunorubicin hydrochloride (Cerubidine®), daunorubicin citrate liposome injection (DaunoXome®), dexamethasone, docetaxel (Taxotere®), doxorubicin hydrochloride (Adriamycin®, Rubex®), etoposide (Vepesid®), fludarabine phosphate (Fludara®), 5-fluorouracil (Adrucil®, Efudex®), flutamide (Eulexin®), tezacitibine, Gemcitabine (difluorodeoxycitidine), hydroxyurea (Hydrea®), Idarubicin (Idamycin®), ifosfamide (IFEX®), irinotecan (Camptosar®), L-asparaginase (ELSPAR®), leucovorin calcium, melphalan (Alkeran®), 6-mercaptopurine (Purinethol®), methotrexate (Folex®), mitoxantrone (Novantrone®), mylotarg, paclitaxel (Taxol®), phoenix (Yttrium90/MX-DTPA), pentostatin, polifeprosan 20 with carmustine implant (Gliadel®), tamoxifen citrate (Nolvadex®), teniposide (Vumon®), 6-thioguanine, thiotepa, tirapazamine (Tirazone®), topotecan hydrochloride for injection (Hycamptin®), vinblastine (Velban®), vincristine (Oncovin®), and vinorelbine (Navelbine®).

[00462] Exemplary alkylating agents include, without limitation, nitrogen mustards, ethylenimine derivatives, alkyl sulfonates, nitrosoureas and triazenes): uracil mustard (Aminouracil Mustard®, Chlorethaminacil®, Demethyldopan®, Desmethyldopan®, Haemanthamine®, Nordopan®, Uracil nitrogen mustard®, Uracillost®, Uracilmostaza®, Uramustin®, Uramustine®), chlormethine (Mustargen®), cyclophosphamide (Cytoxan®, Neosar®, Clafen®, Endoxan®, Procytox®, Revimmune™), ifosfamide (Mitoxana®), melphalan (Alkeran®), Chlorambucil (Leukeran®), pipobroman (Amedel®, Vercyte®), triethylenemelamine (Hemel®, Hexalen®, Hexastat®), triethylenethiophosphoramine, Temozolomide (Temodar®), thiotepa (Thioplex®), busulfan (Busilvex®, Myleran®), carmustine (BiCNU®), lomustine (CeeNU®), streptozocin (Zanosar®), and Dacarbazine (DTIC-Dome®). Additional exemplary alkylating agents include, without limitation, Oxaliplatin (Eloxatin®); Temozolomide (Temodar® and Temodal®); Dactinomycin (also known as actinomycin-D, Cosmegen®); Melphalan (also known as L-PAM, L-sarcolysin, and phenylalanine mustard, Alkeran®); Altretamine (also known as hexamethylmelamine (HMM), Hexalen®); Carmustine (BiCNU®); Bendamustine (Treanda®); Busulfan (Busulfex® and

Myleran®); Carboplatin (Paraplatin®); Lomustine (also known as CCNU, CeeNU®); Cisplatin (also known as CDDP, Platinol® and Platinol®-AQ); Chlorambucil (Leukeran®); Cyclophosphamide (Cytoxan® and Neosar®); Dacarbazine (also known as DTIC, DIC and imidazole carboxamide, DTIC-Dome®); Altretamine (also known as hexamethylmelamine (HMM), Hexalen®); Ifosfamide (Ifex®); Prednumustine; Procarbazine (Matulane®); Mechlorethamine (also known as nitrogen mustard, mustine and mechloroethamine hydrochloride, Mustargen®); Streptozocin (Zanosar®); Thiotepa (also known as thiophosphoamide, TESPA and TSPA, Thioplex®); Cyclophosphamide (Endoxan®, Cytoxan®, Neosar®, Procytox®, Revimmune®); and Bendamustine HC1 (Treanda®).

[00463] Exemplary mTOR inhibitors include, e.g., temsirolimus; ridaforolimus (formally known as deferolimus, (1*R*,2*R*,4*S*)-4-[(2K)-2 *[(\R,9S,\2S,\5R,\6E,\8R,\9R,2\R,* 23^,24£,26£,28Z,30^,325',35i?)-l,18-dihydroxy-19,30-dimethoxy-15,17,21,23, 29,35-hexamethyl-2,3,10,14,20-pentaoxo-l l,36-dioxa-4-azatricyclo[30.3. $1.0^{4,9}$] hexatriaconta-16,24,26,28-tetraen-12-yl]propyl]-2-methoxycyclohexyl dimethylphosphinate, also known as AP23573 and MK8669, and described in PCT Publication No. WO 03/064383); everolimus (Afinitor® or RADOOl); rapamycin (AY22989, Sirolimus®); simapimod (CAS 164301-51-3); emsirolimus, (5-{2,4-Bis[(3*S*)-3-methylmorpholin-4-yl]pyrido[2,3-i*I*]pyrimidin-7-yl}-2-methoxyphenyl)methanol (AZD8055); 2-Amino-8-[/ram-4-(2-hydroxyethoxy)cyclohexyl]-6-(6-methoxy-3-pyridinyl)-4-methyl-pyrido[2,3-i/]pyrimidin-7(8 *H*)-one (PF04691502, CAS 1013101-36-4); and N^2-[l ,4-dioxo-4-[[4-(4-oxo-8-phenyl-4 *H*-l -benzopyran-2-yl)morpholinium-4-yl]methoxy]butyl]-L-arginylglycyl-L-a-aspartylL-serine-, inner salt (SF1126, CAS 936487-67-1), and XL765.

[00464] Exemplary immunomodulators include, e.g., afutuzumab (available from Roche®); pegfilgrastim (Neulasta®); lenalidomide (CC-5013, Revlimid®); thalidomide (Thalomid®), actimid (CC4047); and IRX-2 (mixture of human cytokines including interleukin 1, interleukin 2, and interferon γ, CAS 951209-71-5, available from IRX Therapeutics).

[00465] Exemplary anthracyclines include, e.g., doxorubicin (Adriamycin® and Rubex®); bleomycin (lenoxane®); daunorubicin (dauorubicin hydrochloride, daunomycin, and rubidomycin hydrochloride, Cerubidine®); daunorubicin liposomal (daunorubicin citrate liposome, DaunoXome®); mitoxantrone (DHAD, Novantrone®); epirubicin (Ellence™);

idarubicin (Idamycin®, Idamycin PFS®); mitomycin C (Mutamycin®); geldanamycin; herbimycin; ravidomycin; and desacetylravidomycin.

[00466] Exemplary vinca alkaloids include, e.g., vinorelbine tartrate (Navelbine®), Vincristine (Oncovin®), and Vindesine (Eldisine®)); vinblastine (also known as vinblastine sulfate, vincaleukoblastine and VLB, Alkaban-AQ® and Velban®); and vinorelbine (Navelbine®).

[00467] Exemplary proteosome inhibitors include bortezomib (Velcade®); carfilzomib (PX-171-007, (^)-4-Methyl-*N*-((^)-l-(((^)-4-methyl-l-((i?)-2-methyloxiran-2-yl)-l-oxopentan-2-yl)amino)-l-oxo-3-phenylpropan-2-yl)-2-((*S*)-2-(2-morpholinoacetamido)-4-phenylbutanamido)-pentanamide); marizomib (NPI-0052); ixazomib citrate (MLN-9708); delanzomib (CEP-18770); and 0-Methyl-*N*-[(2-methyl-5-thiazolyl)carbonyl]-L-seryl-0-methyl-*N*-[(l *S*)-2-[(2i?)-2-methyl-2-oxiranyl]-2-oxo-1-(phenylmethyl)ethyl]-L-serinamide (ONX-0912).

[00468] In one embodiment, a CAR expressing cell described herein is administered to a subject in combination with a molecule targeting GITR and/or modulating GITR functions, such as a GITR agonist and/or a GITR antibody that depletes regulatory T cells (Tregs). In one embodiment, the GITR binding molecules and/or molecules modulating GITR functions (e.g., GITR agonist and/or Treg depleting GITR antibodies) are administered prior to the CAR-expressing cell. For example, in one embodiment, the GITR agonist can be administered prior to apheresis of the cells. Exemplary GITR agonists include, e.g., GITR fusion proteins and anti-GITR antibodies (e.g., bivalent anti-GITR antibodies) such as, e.g., a GITR fusion protein described in U.S. Patent No.: 6,111,090, European Patent No.: 090505B1, U.S Patent No.: 8,586,023, PCT Publication Nos.: WO 2010/003118 and 2011/090754, or an anti-GITR antibody described, e.g., in U.S. Patent No.: 7,025,962, European Patent No.: 1947183B1, U.S. Patent No.: 7,812,135, U.S. Patent No.: 8,388,967, U.S. Patent No.: 8,591,886, European Patent No.: EP 1866339, PCT Publication No.: WO 2011/028683, PCT Publication No.:WO 2013/039954, PCT Publication No.: WO2005/007190, PCT Publication No.: WO 2007/133822, PCT Publication No.: WO2005/055808, PCT Publication No.: WO 99/40196, PCT Publication No.: WO 2001/03720, PCT Publication No.: WO99/20758, PCT Publication No.: WO2006/083289, PCT Publication No.: WO 2005/115451, U.S. Patent No.: 7,618,632, and PCT Publication No.: WO 2011/051726.

[00469] In one embodiment, a CAR expressing cell described herein is administered to a subject in combination with an mTOR inhibitor, e.g., an mTOR inhibitor described herein, e.g., a rapalog such as everolimus. In one embodiment, the mTOR inhibitor is administered prior to the CAR-expressing cell. For example, in one embodiment, the mTOR inhibitor can be administered prior to apheresis of the cells.

[00470] In one embodiment, a CAR expressing cell described herein is administered to a subject in combination with a GITR agonist, e.g., a GITR agonist described herein. In one embodiment, the GITR agonist is administered prior to the CAR-expressing cell. For example, in one embodiment, the GITR agonist can be administered prior to apheresis of the cells.

[00471] In one embodiment, a CAR expressing cell described herein is administered to a subject in combination with a protein tyrosine phosphatase inhibitor, e.g., a protein tyrosine phosphatase inhibitor described herein. In one embodiment, the protein tyrosine phosphatase inhibitor is an SHP-1 inhibitor, e.g., an SHP-1 inhibitor described herein, such as, e.g., sodium stibogluconate. In one embodiment, the protein tyrosine phosphatase inhibitor is an SHP-2 inhibitor, e.g., an SHP-2 inhibitor described herein.

[00472] In one embodiment, a CAR-expressing cell described herein can be used in combination with a kinase inhibitor. In one embodiment, the kinase inhibitor is a CDK4 inhibitor, e.g., a CDK4 inhibitor described herein, e.g., a CDK4/6 inhibitor, such as, e.g., 6-Acetyl-8-cyclopentyl-5-methyl-2-(5-piperazin-l-yl-pyridin-2-ylamino)-8 *H*-pyrido[2,3-</Jpyrimidin-7-one, hydrochloride (also referred to as palbociclib or PD0332991). In one embodiment, the kinase inhibitor is a BTK inhibitor, e.g., a BTK inhibitor described herein, such as, e.g., ibrutinib. In one embodiment, the kinase inhibitor is an mTOR inhibitor, e.g., an mTOR inhibitor described herein, such as, e.g., rapamycin, a rapamycin analog, OSI-027. The mTOR inhibitor can be, e.g., an mTORCl inhibitor and/or an mTORC2 inhibitor, e.g., an mTORCl inhibitor and/or mTORC2 inhibitor described herein. In one embodiment, the kinase inhibitor is a MNK inhibitor, e.g., a MNK inhibitor described herein, such as, e.g., 4-amino-5-(4-fluoroanilino)-pyrazolo [3,4-*d*J pyrimidine. The MNK inhibitor can be, e.g., a MNKla, MNKlb, MNK2a and/or MNK2b inhibitor. In one embodiment, the kinase inhibitor is a dual PBK/mTOR inhibitor described herein, such as, e.g., PF-04695102. In one embodiment, the

kinase inhibitor is a DGK inhibitor, e.g., a DGK inhibitor described herein, such as, e.g., DGKinhl (D5919) or DGKinh2 (D5794).

[00473] In one embodiment, the kinase inhibitor is a CDK4 inhibitor selected from aloisine A; flavopiridol or HMR-1275, 2-(2-chlorophenyl)-5,7-dihydroxy-8-[(3S,4R)-3-hydroxy-l-methyl-4-piperidinyl]-4-chromenone; crizotinib (PF-02341066; 2-(2-Chlorophenyl)-5,7-dihydroxy-8-[(2i?,3<S)-2-(hydroxymethyl)-l-methyl-3-pyrrolidinyl]- 4*H*-l-benzopyran-4-one, hydrochloride (P276-00); l-methyl-5-[[2-[5-(trifluoromethyl)-l *H*-imidazol-2-yl]-4-pyridinyl]oxy *]-N*-[4-(trifluoromethyl)phenyl]-l *H*-benzimidazol-2-amine (RAF265); indisulam (E7070); roscovitine (CYC202); palbociclib (PD0332991); dinaciclib (SCH727965); N-[5-[[(5-terf-butyloxazol-2-yl)methyl]thio]thiazol-2-yl]piperidine-4-carboxamide (BMS 387032); 4-[[9-chloro-7-(2,6-difluorophenyl)-5 *H*-pyrimido[5,4-i/][2]benzazepin-2-yl]amino]-benzoic acid (MLN8054); 5-[3-(4,6-difluoro-lH-benzimidazol-2-yl)-lH-indazol-5-yl]-N-ethyl-4-methyl-3-pyridinemethanamine (AG-024322); 4-(2,6-dichlorobenzoylamino)-lH-pyrazole-3-carboxylic acid N-(piperidin-4-yl)amide (AT7519); 4-[2-methyl-l-(l-methylethyl)-l *H*-imidazol-5-yl *]-N*-[4-(methylsulfonyl)phenyl]- 2-pyrimidinamine (AZD5438); and XL281 (BMS908662).

[00474] In one embodiment, the kinase inhibitor is a CDK4 inhibitor, e.g., palbociclib (PD0332991), and the palbociclib is administered at a dose of about 50 mg, 60 mg, 70 mg, 75 mg, 80 mg, 90 mg, 100 mg, 105 mg, 110 mg, 115 mg, 120 mg, 125 mg, 130 mg, 135 mg (e.g., 75 mg, 100 mg or 125 mg) daily for a period of time, e.g., daily for 14-21 days of a 28 day cycle, or daily for 7-12 days of a 21 day cycle. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more cycles of palbociclib are administered.

[00475] In one embodiment, the kinase inhibitor is a BTK inhibitor selected from ibrutinib (PCI-32765); GDC-0834; RN-486; CGI-560; CGI-1764; HM-71224; CC-292; ONO-4059; CNX-774; and LFM-A13. In a preferred embodiment, the BTK inhibitor does not reduce or inhibit the kinase activity of interleukin-2-inducible kinase (ITK), and is selected from GDC-0834; RN-486; CGI-560; CGI-1764; HM-71224; CC-292; ONO-4059; CNX-774; and LFM-A13.

[00476] In one embodiment, the kinase inhibitor is a BTK inhibitor, e.g., ibrutinib (PCI-32765), and the ibrutinib is administered at a dose of about 250 mg, 300 mg, 350 mg, 400 mg, 420 mg, 440 mg, 460 mg, 480 mg, 500 mg, 520 mg, 540 mg, 560 mg, 580 mg, 600 mg (e.g., 250 mg, 420 mg or 560 mg) daily for a period of time, e.g., daily for 21 day cycle cycle, or daily for

28 day cycle. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more cycles of ibrutinib are administered.

[00477] In one embodiment, the kinase inhibitor is an mTOR inhibitor selected from temsirolimus; ndaforolimus *{\R,2R,AS)-A-[{2R)-2 [(\R,9S,\2S,\5R,\6E,\8R,\9R,2\R,* 23^,24£,26£,28Z,30^,325',35i?)-l,18-dihydroxy-19,30-dimethoxy-15,17,21,23, 29,35-hexamethyl-2,3,10,14,20-pentaoxo-l l,36-dioxa-4-azatricyclo[30.3. $1.0^{4'9}$] hexatriaconta-16,24,26,28-tetraen-12-yl]propyl]-2-methoxycyclohexyl dimethylphosphinate, also known as AP23573 and MK8669; everolimus (RAD001); rapamycin (AY22989); simapimod; (5-{2,4-bis[(3<S)-3-methylmorpholin-4-yl]pyrido[2,3-i/]pyrimidin-7-yl}-2-methoxyphenyl)methanol (AZD8055); 2-amino-8-[iram-4-(2-hydroxyethoxy)cyclohexyl]-6-(6-methoxy-3-pyridinyl)-4-methyl-pyrido[2,3-i/]pyrimidin-7(8 *H*)-one (PF04691502); and N^2-[l,4-dioxo-4-[[4-(4-oxo-8-phenyl-4 *H*-l-benzopyran-2-yl)morpholinium-4-yl]methoxy]butyl]-L-arginylglycyl-L-a-aspartylL-senne- (SEQ ID NO: 272), inner salt (SF1 126); and XL765.

[00478] In one embodiment, the kinase inhibitor is an mTOR inhibitor, e.g., rapamycin, and the rapamycin is administered at a dose of about 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg (e.g., 6 mg) daily for a period of time, e.g., daily for 21 day cycle cycle, or daily for 28 day cycle. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more cycles of rapamycin are administered. In one embodiment, the kinase inhibitor is an mTOR inhibitor, e.g., everolimus and the everolimus is administered at a dose of about 2 mg, 2.5 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 11 mg, 12 mg, 13 mg, 14 mg, 15 mg (e.g., 10 mg) daily for a period of time, e.g., daily for 28 day cycle. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more cycles of everolimus are administered.

[00479] In one embodiment, the kinase inhibitor is an MNK inhibitor selected from CGP052088; 4-amino-3-(p-fluorophenylamino)-pyrazolo [3,4-*d*] pyrimidine (CGP57380); cercosporamide; ETC-1780445-2; and 4-amino-5-(4-fluoroanilino)-pyrazolo [3,4-*d*] pyrimidine.

[00480] In one embodiment, the kinase inhibitor is a dual phosphatidylinositol 3-kinase (PI3K) and mTOR inhibitor selected from 2-Amino-8-[iram-4-(2-hydroxyethoxy)cyclohexyl]-6-(6-methoxy-3-pyridinyl)-4-methyl-pyrido[2,3-i/]pyrimidin-7(8 *H*)-one (PF-04691 502); *N*-[4-[[4-(Dimethylamino)-l-piperidinyl]carbonyl]phenyl *]-N*-[4-(4,6-di-4-morpholinyl-l,3,5-triazin-2-yl)phenyl]urea (PF-05212384, PKI-587); 2-Methyl-2-{4-[3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-

dihydro-l *H*-imidazo[4,5-c]quinolin-l-yl]phenyl}propanenitrile (BEZ-235); apitolisib (GDC-0980, RG7422); 2,4-Difluoro-N- {2-(methyloxy)-5-[4-(4-pyndazinyl)-6-quinolinyl]-3-pyridinyl }benzenesulfonamide (GSK2 126458); 8-(6-methoxypyridin-3 -yl)-3 -methyl- 1-(4-(piperazin- 1-yl)-3 -(trifluoromethyl)phenyl)- 1H-imidazo[4, 5-c] quinolin-2(3H)-one Maleic acid (NVP-BGT226); 3-[4-(4-Morpholinylpyndo[3',2':4,5]furo[3,2-d]pyrimidin-2-yl]phenol (PI-103); 5-(9-isopropyl-8-methyl-2-morpholino-9H-purin-6-yl)pyrimidin-2-amine (VS-5584, SB2343); and N-[2-[(3,5-Dimethoxyphenyl)amino] quinoxalin-3 -yl] -4-[(4-methyl-3 -methoxyphenyl)carbonyl]aminophenylsulfonamide (XL765).

[00481] Drugs that inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin). (Liu *etal,* Cell 66:807-815, 1991; Henderson *etal.,* Immun. 73:316-321, 1991; Bierer *et al.,* Curr. Opin. Immun. 5:763-773, 1993) can also be used. In a further aspect, the cell compositions of the present invention may be administered to a patient in conjunction with (e.g., before, simultaneously or following) bone marrow transplantation, T cell ablative therapy using chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, and/or antibodies such as OKT3 or CAMPATH. In one aspect, the cell compositions of the present invention are administered following B-cell ablative therapy such as agents that react with CD20, e.g., Rituxan. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present invention. In an additional embodiment, expanded cells are administered before or following surgery.

[00482] In one embodiment, the subject can be administered an agent which reduces or ameliorates a side effect associated with the administration of a CAR-expressing cell. Side effects associated with the administration of a CAR-expressing cell include, but are not limited to CRS, and hemophagocytic lymphohistiocytosis (HLH), also termed Macrophage Activation Syndrome (MAS). Symptoms of CRS include high fevers, nausea, transient hypotension, hypoxia, and the like. CRS may include clinical constitutional signs and symptoms such as fever, fatigue, anorexia, myalgias, arthalgias, nausea, vomiting, and headache. CRS may include clinical skin signs and symptoms such as rash. CRS may include clinical gastrointestinal signs and symsptoms such as nausea, vomiting and diarrhea. CRS may include clinical respiratory

signs and symptoms such as tachypnea and hypoxemia. CRS may include clinical cardiovascular signs and symptoms such as tachycardia, widened pulse pressure, hypotension, increased cardac output (early) and potentially diminished cardiac output (late). CRS may include clinical coagulation signs and symptoms such as elevated d-dimer, hypofibrinogenemia with or without bleeding. CRS may include clinical renal signs and symptoms such as azotemia. CRS may include clinical hepatic signs and symptoms such as transaminitis and hyperbilirubinemia. CRS may include clinical neurologic signs and symptoms such as headache, mental status changes, confusion, delirium, word finding difficulty or frank aphasia, hallucinations, tremor, dymetria, altered gait, and seizures.

[00483] Accordingly, the methods described herein can comprise administering a CAR-expressing cell described herein to a subject and further administering one or more agents to manage elevated levels of a soluble factor resulting from treatment with a CAR-expressing cell. In one embodiment, the soluble factor elevated in the subject is one or more of IFN-γ, TNFα, IL-2 and IL-6. In an embodiment, the factor elevated in the subject is one or more of IL-1, GM-CSF, IL-10, IL-8, IL-5 and fraktalkine. Therefore, an agent administered to treat this side effect can be an agent that neutralizes one or more of these soluble factors. In one embodiment, the agent that neutralizes one or more of these soluble forms is an antibody or antigen binding fragment thereof. Examples of such agents include, but are not limited to a steroid (e.g., corticosteroid), an inhibitor of TNFa, and an inhibitor of IL-6. An example of a TNFa inhibitor is an anti-TNFa antibody molecule such as, infliximab, adalimumab, certolizumab pegol, and golimumab. Another example of a TNFa inhibitor is a fusion protein such as entanercept. Small molecule inhibitor of TNFa include, but are not limited to, xanthine derivatives (e.g. pentoxifylline) and bupropion. An example of an IL-6 inhibitor is an anti-IL-6 antibody molecule or an anti-IL-6 receptor antibody molecule such as tocilizumab (toe), sarilumab, elsilimomab, CNTO 328, ALD518/BMS-945429, CNTO 136, CPSI-2364, CDP6038, VX30, ARGX-109, FE301, and FM101. In one embodiment, the anti-IL-6 antibody molecule is tocilizumab. An example of an IL-1R based inhibitor is anakinra.

[00484] In some embodiment, the subject is administered a corticosteroid, such as, e.g., methylprednisolone, hydrocortisone, among others.

[00485] In some embodiments, the subject is administered a vasopressor, such as, e.g., norepinephrine, dopamine, phenylephrine, epinephrine, vasopressin, or a combination thereof.

[00486] In an embodiment, the subject can be administered an antipyretic agent. In an embodiment, the subject can be administered an analgesic agent.

[00487] In one embodiment, the subject can be administered an agent which enhances the activity or fitness of a CAR-expressing cell. For example, in one embodiment, the agent can be an agent which inhibits a molecule that modulates or regulates, e.g., inhibits, T cell function. In some embodiments, the molecule that modulates or regulates T cell function is an inhibitory molecule. Inhibitory molecules, e.g., Programmed Death 1 (PD1), can, in some embodiments, decrease the ability of a CAR-expressing cell to mount an immune effector response. Examples of inhibitory molecules include PD1, PD-L1, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 and TGFR beta. Inhibition of a molecule that modulates or regulates, e.g., inhibits, T cell function, e.g., by inhibition at the DNA, RNA or protein level, can optimize a CAR-expressing cell performance. . In embodiments, an agent, e.g., an inhibitory nucleic acid, e.g., a dsRNA, e.g., an siRNA or shRNA; or e.g., an inhibitory protein or system, e.g., a clustered regularly interspaced short palindromic repeats (CRISPR), a transcription-activator like effector nuclease (TALEN), or a zinc finger endonuclease (ZFN), e.g., as described herein, can be used to inhibit expression of a molecule that modulates or regulates, e.g., inhibits, T-cell function in the CAR-expressing cell. In an embodiment the agent is an shRNA. In an embodiment, the agent that modulates or regulates, e.g., inhibits, T-cell function is inhibited within a CAR-expressing cell. In these embodiments, a dsRNA molecule that inhibits expression of a molecule that modulates or regulates, e.g., inhibits, T-cell function is linked to the nucleic acid that encodes a component, e.g., all of the components, of the CAR. In an embodiment, a nucleic acid molecule that encodes a dsRNA molecule that inhibits expression of the molecule that modulates or regulates, e.g., inhibits, T-cell function is operably linked to a promoter, e.g., a H1- or a U6-derived promoter such that the dsRNA molecule that inhibits expression of the molecule that modulates or regulates, e.g., inhibits, T-cell function is expressed, e.g., is expressed within a CAR-expressing cell. See e.g., Tiscornia G , "Development of Lentiviral Vectors Expressing siRNA," Chapter 3, in *Gene Transfer: Delivery and Expression of DNA and RNA* (eds. Friedmann and Rossi). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2007; Brummelkamp TR, *et al.*

(2002) *Science* 296: 550-553; Miyagishi M, *etal.* (2002) *Nat. Biotechnol.* 19: 497-500. In an embodiment the nucleic acid molecule that encodes a dsRNA molecule that inhibits expression of the molecule that modulates or regulates, e.g., inhibits, T-cell function is present on the same vector, e.g., a lentiviral vector, that comprises a nucleic acid molecule that encodes a component, e.g., all of the components, of the CAR. In such an embodiment, the nucleic acid molecule that encodes a dsRNA molecule that inhibits expression of the molecule that modulates or regulates, e.g., inhibits, T-cell function is located on the vector, e.g., the lentiviral vector, 5'- or 3'- to the nucleic acid that encodes a component, e.g., all of the components, of the CAR. The nucleic acid molecule that encodes a dsRNA molecule that inhibits expression of the molecule that modulates or regulates, e.g., inhibits, T-cell function can be transcribed in the same or different direction as the nucleic acid that encodes a component, e.g., all of the components, of the CAR. In an embodiment the nucleic acid molecule that encodes a dsRNA molecule that inhibits expression of the molecule that modulates or regulates, e.g., inhibits, T-cell function is present on a vector other than the vector that comprises a nucleic acid molecule that encodes a component, e.g., all of the components, of the CAR. In an embodiment, the nucleic acid molecule that encodes a dsRNA molecule that inhibits expression of the molecule that modulates or regulates, e.g., inhibits, T-cell function it transiently expressed within a CAR-expressing cell. In an embodiment, the nucleic acid molecule that encodes a dsRNA molecule that inhibits expression of the molecule that modulates or regulates, e.g., inhibits, T-cell function is stably integrated into the genome of a CAR-expressing cell. Figure 47 depicts examples of vectors for expressing a component, e.g., all of the components, of the CAR with a dsRNA molecule that inhibits expression of the molecule that modulates or regulates, e.g., inhibits, T-cell function.

[00488] Examples of dsRNA molecules useful for inhibiting expression of a molecule that modulates or regulates, e.g., inhibits, T-cell function, wherein the molecule that modulates or regulates, e.g., inhibits, T-cell function is PD-1 are provided below.

[00489] Provided in Table 16 below are the names of PDCD 1 (PD 1) RNAi agents (derived from their position in the mouse PDCD1 gene sequence NM_008798.2), along with the SEQ ID NOs: 280-327 representing the DNA sequence. Both sense (S) and antisense (AS) sequences are presented as 19mer and 21mer sequences are in this table. Also note that the position (PoS, e.g., 176) is derived from the position number in the mouse PDCD1 gene sequence NM_008798.2. SEQ ID NOs are indicated in groups of 12 that correspond with "sense 19" SEQ ID NOs: 280-

291; "sense 21" SEQ ID NOs: 292-303; "asense 21" SEQ ID NOs: 304-315; "asense 19" SEQ ID NOs: 316-327.

Table 16. Mouse PDCDl (PDl) shRNA sequences

Position on NM_008798.2	Target region	Sense19	Sense21	Asense21	Asense19
176	CDS	GGAGGTCCCTCACCTTCTA (SEQ ID NO: 280)	CTGGAGGTCCCTCACCTTCTA (SEQ ID NO: 292)	TAGAAGGTGAGGGACCTCCAG (SEQ ID NO: 304)	TAGAAGGTGAGGGACCTCC (SEQ ID NO: 316)
260	CDS	CGGAGGATCTTATGCTGAA (SEQ ID NO: 281)	GTCGGAGGATCTTATGCTGAA (SEQ ID NO: 293)	TTCAGCATAAGATCCTCCGAC (SEQ ID NO: 305)	TTCAGCATAAGATCCTCCG (SEQ ID NO: 317)
359	CDS	CCCGCTTCCAGATCATACA (SEQ ID NO: 282)	TGCCCGCTTCCAGATCATACA (SEQ ID NO: 294)	TGTATGATCTGGAAGCGGGCA (SEQ ID NO: 306)	TGTATGATCTGGAAGCGGG (SEQ ID NO: 318)
528	CDS	GGAGACCTCAACAAGATAT (SEQ ID NO: 283)	CTGGAGACCTCAACAAGATAT (SEQ ID NO: 295)	ATATCTTGTTGAGGTCTCCAG (SEQ ID NO: 307)	ATATCTTGTTGAGGTCTCC (SEQ ID NO: 319)
581	CDS	AAGGCATGGTCATTGGTAT (SEQ ID NO: 284)	TCAAGGCATGGTCATTGGTAT (SEQ ID NO: 296)	ATACCAATGACCATGCCTTGA (SEQ ID NO: 308)	ATACCAATGACCATGCCTT (SEQ ID NO: 320)
584	CDS	GCATGGTCATTGGTATCAT (SEQ ID NO: 285)	AGGCATGGTCATTGGTATCAT (SEQ ID NO: 297)	ATGATACCAATGACCATGCCT (SEQ ID NO: 309)	ATGATACCAATGACCATGC (SEQ ID NO: 321)
588	CDS	GGTCATTGGTATCATGAGT (SEQ ID NO: 286)	ATGGTCATTGGTATCATGAGT (SEQ ID NO: 298)	ATGGTCATTGGTATCATGAGT (SEQ ID NO: 310)	ATGGTCATTGGTATCATGA (SEQ ID NO: 322)
609	CDS	CCTAGTGGGTATCCCTGTA (SEQ ID NO: 287)	GCCCTAGTGGGTATCCCTGTA (SEQ ID NO: 299)	GCCCTAGTGGGTATCCCTGTA (SEQ ID NO: 311)	GCCCTAGTGGGTATCCCTG (SEQ ID NO: 323)
919	CDS	GAGGATGGACATTGTTCTT (SEQ ID NO: 288)	ATGAGGATGGACATTGTTCTT (SEQ ID NO: 300)	ATGAGGATGGACATTGTTCTT (SEQ ID NO: 312)	ATGAGGATGGACATTGTTC (SEQ ID NO: 324)
1021	3'UTR	GCATGCAGGCTACAGTTCA (SEQ ID NO: 289)	GAGCATGCAGGCTACAGTTCA (SEQ ID NO: 301)	GAGCATGCAGGCTACAGTTCA (SEQ ID NO: 313)	GAGCATGCAGGCTACAGTT (SEQ ID NO: 325)
1097	3'UTR	CCAGCACATGCACTGTTGA (SEQ ID NO: 290)	TTCCAGCACATGCACTGTTGA (SEQ ID NO: 302)	TTCCAGCACATGCACTGTTGA (SEQ ID NO: 314)	TTCCAGCACATGCACTGTT (SEQ ID NO: 326)
1101	3'UTR	CACATGCACTG	AGCACATGCAC	AGCACATGCAC	AGCACATGCAC

		TTGAGTGA (SEQ ID NO: 291)	TGTTGAGTGA (SEQ ID NO: 303)	TGTTGAGTGA (SEQ ID NO: 315)	TGTTGAGT (SEQ ID NO: 327)

Provided in Table 17 below are the names of PDCDl (PDl) RNAi agents (derived from their position in the human PDCDl gene sequence, along with the SEQ ID NOs. 323-370 representing the DNA sequence. Both sense (S) and antisense (AS) sequences are presented as 19mer and 21mer sequences. SEQ ID NOs are indicated in groups of 12 that correspond with "sense 19" SEQ ID NOs: 328-339; "sense 21" SEQ ID NOs: 340-351; "asense 21" SEQ ID NOs: 352-363; "asense 19" SEQ ID NOs: 364-375.

Table 17. Human PDCDl (PDl) shRNA sequences

Position on NM_005018.2	Target region	Sense19	Asense19	Sense21	Asense21
145	CDS	GGCCAGGATG GTTCTTAGA (SEQ ID NO: 328)	TCTAAGAACCA TCCTGGCC (SEQ ID NO: 340)	GCGGCCAGGA TGGTTCTTAGA (SEQ ID NO: 352)	TCTAAGAACCA TCCTGGCCGC (SEQ ID NO: 364)
271	CDS	GCTTCGTGCTA AACTGGTA (SEQ ID NO: 329)	TACCAGTTTAG CACGAAGC (SEQ ID NO: 341)	GAGCTTCGTGC TAAACTGGTA (SEQ ID NO: 353)	TACCAGTTTAG CACGAAGCTC (SEQ ID NO: 365)
393	CDS	GGGCGTGACTT CCACATGA (SEQ ID NO: 330)	TCATGTGGAAG TCACGCCC (SEQ ID NO: 342)	ACGGGCGTGA CTTCCACATGA (SEQ ID NO: 354)	TCATGTGGAAG TCACGCCCGT (SEQ ID NO: 366)
1497	3'UTR	CAGGCCTAGAG AAGTTTCA (SEQ ID NO: 331)	TGAAACTTCTC TAGGCCTG (SEQ ID NO: 343)	TGCAGGCCTAG AGAAGTTTCA (SEQ ID NO: 355)	TGAAACTTCTC TAGGCCTGCA (SEQ ID NO: 367)
1863	3'UTR	CTTGGAACCCA TTCCTGAA (SEQ ID NO: 332)	TTCAGGAATGG GTTCCAAG (SEQ ID NO: 344)	TCCTTGGAACC CATTCCTGAA (SEQ ID NO: 356)	TTCAGGAATGG GTTCCAAGGA (SEQ ID NO: 368)
1866	3'UTR	GGAACCCATTC CTGAAATT (SEQ ID NO: 333)	AATTTCAGGAA TGGGTTCC (SEQ ID NO: 345)	TTGGAACCCAT TCCTGAAATT (SEQ ID NO: 357)	AATTTCAGGAA TGGGTTCCAA (SEQ ID NO: 369)
1867	3'UTR	GAACCCATTCC TGAAATTA (SEQ ID NO: 334)	TAATTTCAGGA ATGGGTTC (SEQ ID NO: 346)	TGGAACCCATT CCTGAAATTA (SEQ ID NO: 358)	TAATTTCAGGA ATGGGTTCCA (SEQ ID NO: 370)
1868	3'UTR	AACCCATTCCT GAAATTAT (SEQ ID NO: 335)	ATAATTTCAGG AATGGGTT (SEQ ID NO: 347)	GGAACCCATTC CTGAAATTAT (SEQ ID NO: 359)	ATAATTTCAGG AATGGGTTCC (SEQ ID NO:371)
1869	3'UTR	ACCCATTCCTG	AATAATTTCAG	GAACCCATTCC	AATAATTTCAG

		AAATTATT (SEQ ID NO: 336)	GAATGGGT (SEQ ID NO: 348)	TGAAATTATT (SEQ ID NO: 360)	GAATGGGTTC (SEQ ID NO: 372)
1870	3'UTR	CCCATTCCTGA AATTATTT (SEQ ID NO: 337)	AAATAATTTCA GGAATGGG (SEQ ID NO: 349)	AACCCATTCCT GAAATTATTT (SEQ ID NO: 361)	AAATAATTTCA GGAATGGGTT (SEQ ID NO: 373)
2079	3'UTR	CTGTGGTTCTA TTATATTA (SEQ ID NO: 338)	TAATATAATAGA ACCACAG (SEQ ID NO: 350	CCCTGTGGTTC TATTATATTA (SEQ ID NO: 362)	TAATATAATAGA ACCACAGGG (SEQ ID NO: 374)
2109	3'UTR	AAATATGAGAG CATGCTAA (SEQ ID NO: 339)	TTAGCATGCTC TCATATTT (SEQ ID NO: 351)	TTAAATATGAG AGCATGCTAA (SEQ ID NO: 363)	TTAGCATGCTC TCATATTTAA (SEQ ID NO: 375)

[00490] In one embodiment, the inhibitor of an inhibitory signal can be, e.g., an antibody or antibody fragment that binds to an inhibitory molecule. For example, the agent can be an antibody or antibody fragment that binds to PD1, PD-Ll, PD-L2 or CTLA4 (e.g., ipilimumab (also referred to as MDX-010 and MDX-101, and marketed as Yervoy®; Bristol-Myers Squibb; Tremelimumab (IgG2 monoclonal antibody available from Pfizer, formerly known as ticilimumab, CP-675,206).). In an embodiment, the agent is an antibody or antibody fragment that binds to TΓM3. In an embodiment, the agent is an antibody or antibody fragment that binds to LAG3.

[00491] PD-1 is an inhibitory member of the CD28 family of receptors that also includes CD28, CTLA-4, ICOS, and BTLA. PD-1 is expressed on activated B cells, T cells and myeloid cells (Agata et al. 1996 Int. Immunol 8:765-75). Two ligands for PD-1, PD-Ll and PD-L2 have been shown to downregulate T cell activation upon binding to PD-1 (Freeman et a. 2000 J Exp Med 192: 1027-34; Latchman et al. 2001 Nat Immunol 2:261-8; Carter et al. 2002 Eur J Immunol 32:634-43). PD-Ll is abundant in human cancers (Dong et al. 2003 J Mol Med 81:281-7; Blank et al. 2005 Cancer Immunol. Immunother 54:307-314; Konishi et al. 2004 Clin Cancer Res 10:5094). Immune suppression can be reversed by inhibiting the local interaction of PD-1 with PD-Ll. Antibodies, antibody fragments, and other inhibitors of PD-1, PD-Ll and PD-L2 are available in the art and may be used combination with a CAR of the present invention described herein. For example, nivolumab (also referred to as BMS-936558 or MDXl 106; Bristol-Myers Squibb) is a fully human IgG4 monoclonal antibody which specifically blocks PD-1. Nivolumab

(clone 5C4) and other human monoclonal antibodies that specifically bind to PD-1 are disclosed in US 8,008,449 and WO2006/121 168. Pidilizumab (CT-01 1; Cure Tech) is a humanized IgGlk monoclonal antibody that binds to PD-1. Pidilizumab and other humanized anti-PD-1 monoclonal antibodies are disclosed in WO2009/10161 1. Pembrolizumab (formerly known as lambrolizumab, and also referred to as MK03475; Merck) is a humanized IgG4 monoclonal antibody that binds to PD-1. Pembrolizumab and other humanized anti-PD-1 antibodies are disclosed in US 8,354,509 and WO2009/1 14335. MEDI4736 (Medimmune) is a human monoclonal antibody that binds to PDL1, and inhibits interaction of the ligand with PD1. MDPL3280A (Genentech / Roche) is a human Fc optimized IgGl monoclonal antibody that binds to PD-Ll. MDPL3280A and other human monoclonal antibodies to PD-Ll are disclosed in U.S. Patent No.: 7,943,743 and U.S Publication No.: 20120039906. Other anti-PD-Ll binding agents include YW243.55.S70 (heavy and light chain variable regions are shown in SEQ ID NOs 20 and 21 in WO20 10/077634) and MDX-1 105 (also referred to as BMS-936559, and, e.g., anti-PD-Ll binding agents disclosed in WO2007/005874). AMP-224 (B7-DCIg; Amplimmune; e.g., disclosed in WO2010/027827 and WO201 1/066342), is a PD-L2 Fc fusion soluble receptor that blocks the interaction between PD-1 and B7-H1. Other anti-PD-1 antibodies include AMP 514 (Amplimmune), among others, e.g., anti-PD-1 antibodies disclosed in US 8,609,089, US 2010028330, and/or US 20120114649.

[00492] TFM3 (T cell immunoglobulin-3) also negatively regulates T cell function, particularly in IFN-g-secreting CD4+ T helper 1 and CD8+ T cytotoxic 1 cells, and plays a critical role in T cell exhaustion. Inhibition of the interaction between TIM3 and its ligands, e.g., galectin-9 (Gal9), phosphotidylserine (PS), and HMGB1, can increase immune response. Antibodies, antibody fragments, and other inhibitors of TIM3 and its ligands are available in the art and may be used combination with a CD 19 CAR described herein. For example, antibodies, antibody fragments, small molecules, or peptide inhibitors that target TFM3 binds to the IgV domain of TFM3 to inhibit interaction with its ligands. Antibodies and peptides that inhibit TFM3 are disclosed in WO2013/006490 and US20100247521. Other anti-TIM3 antibodies include humanized versions ofRMT3-23 (disclosed in Ngiow et al, 2011, Cancer Res, 71:3540-3551), and clone 8B.2C12 (disclosed in Monney et al, 2002, Nature, 415:536-541). Bi-specific antibodies that inhibit TFM3 and PD-1 are disclosed in US20130156774.

[00493] In other embodiments, the agent which enhances the activity of a CAR-expressing cell is a CEACAM inhibitor *(e.g.,* CEACAM-1, CEACAM-3, and/or CEACAM-5 inhibitor). In one embodiment, the inhibitor of CEACAM is an anti-CEACAM antibody molecule. Exemplary anti-CEACAM- 1 antibodies are described in WO 2010/125571, WO 2013/082366 WO 2014/059251 and WO 2014/022332, *e.g., a* monoclonal antibody 34B1, 26H7, and 5F4; or a recombinant form thereof, as described in, *e.g.,* US 2004/0047858, US 7,132,255 and WO 99/052552. In other embodiments, the anti-CEACAM antibody binds to CEACAM-5 as described in, *e.g.,* Zheng et al. *PLoS One.* 2010 Sep 2;5(9). pii: el2529 (DOI:10:1371/journal.pone.0021 146), or crossreacts with CEACAM-1 and CEACAM-5 as described in, *e.g.,* WO 2013/054331 and US 2014/0271618.

[00494] Without wishing to be bound by theory, carcinoembryonic antigen cell adhesion molecules (CEACAM), such as CEACAM-1 and CEACAM-5, are believed to mediate, at least in part, inhibition of an anti-tumor immune response *{see e.g.,* Markel *et al. J Immunol.* 2002 Mar 15;168(6):2803-10; Markel et al. *J Immunol.* 2006 Nov 1;177(9):6062-71; Markel et al. *Immunology.* 2009 Feb; 126(2): 186-200; Markel et al. *Cancer Immunol Immunother.* 2010 Feb;59(2):215-30; Ortenberg et al. *Mol Cancer Ther.* 2012 Jun;l 1(6): 1300-10; Stern et al. *J Immunol.* 2005 Jun 1;174(11):6692-701; Zheng et al. *PLoS One.* 2010 Sep 2;5(9). pii: el2529). For example, CEACAM-1 has been described as a heterophilic ligand for TFM-3 and as playing a role in TIM-3-mediated T cell tolerance and exhaustion *(see e.g.,* WO 2014/022332; Huang, *et al.* (2014) *Nature* doi:10.1038/naturel3848). In embodiments, co-blockade of CEACAM-1 and TFM-3 has been shown to enhance an anti-tumor immune response in xenograft colorectal cancer models *(see e.g.,* WO 2014/022332; Huang, *et al.* (2014), *supra).* In other embodiments, co-blockade of CEACAM-1 and PD-1 reduce T cell tolerance as described, *e.g.,* in WO 2014/059251 . Thus, CEACAM inhibitors can be used with the other immunomodulators described herein *(e.g.,* anti-PD-1 and/or anti-TIM-3 inhibitors) to enhance an immune response against a cancer, *e.g.,* a melanoma, a lung cancer (e.g., NSCLC), a bladder cancer, a colon cancer an ovarian cancer, and other cancers as described herein

[00495] LAG3 (lymphocyte activation gene-3 or CD223) is a cell surface molecule expressed on activated T cells and B cells that has been shown to play a role in CD8+ T cell exhaustion. Antibodies, antibody fragments, and other inhibitors of LAG3 and its ligands are available in the art and may be used combination with a CD19 CAR described herein. For example, BMS-

986016 (Bristol-Myers Squib) is a monoclonal antibody that targets LAG3. ГМР701 (Immutep) is an antagonist LAG3 antibody and IMP73 1 (Immutep and GlaxoSmithKline) is a depleting LAG3 antibody. Other LAG3 inhibitors include IMP321 (Immutep), which is a recombinant fusion protein of a soluble portion of LAG3 and Ig that binds to MHC class II molecules and activates antigen presenting cells (APC). Other antibodies are disclosed, e.g., in WO201 0/01 9570.

[00496] In some embodiments, the agent which enhances the activity of a CAR-expressing cell can be, e.g., a fusion protein comprising a first domain and a second domain, wherein the first domain is an inhibitory molecule, or fragment thereof, and the second domain is a polypeptide that is associated with a positive signal, e.g., a polypeptide comrpsing an antracellular signaling domain as described herein. In some embodiments, the polypeptide that is associated with a positive signal can include a costimulatory domain of CD28, CD27, ICOS, e.g., an intracellular signaling domain of CD28, CD27 and/or ICOS, and/or a primary signaling domain, e.g., of CD3 zeta, e.g., described herein. In one embodiment, the fusion protein is expressed by the same cell that expressed the CAR. In another embodiment, the fusion protein is expressed by a cell, e.g., a T cell that does not express a mesothelin CAR.

[00497] In one embodiment, the agent which enhances activity of a CAR-expressing cell described herein is miR- 17-92.

Combination with a low dose of an mTOR inhibitor

[00498] In one embodiment, the cells expressing a CAR molecule, e.g., a CAR molecule described herein, are administered in combination with a low, immune enhancing dose of an mTOR inhibitor.

[00499] In an embodiment, a dose of an mTOR inhibitor is associated with, or provides, mTOR inhibition of at least 5 but no more than 90%, at least 10 but no more than 90%, at least 15, but no more than 90%, at least 20 but no more than 90%, at least 30 but no more than 90%, at least 40 but no more than 90%, at least 50 but no more than 90%, at least 60 but no more than 90%, or at least 70 but no more than 90%.

[00500] In an embodiment, a dose of an mTOR inhibitor is associated with, or provides, mTOR inhibition of at least 5 but no more than 80%, at least 10 but no more than 80%, at least

15, but no more than 80%, at least 20 but no more than 80%, at least 30 but no more than 80%, at least 40 but no more than 80%, at least 50 but no more than 80%, or at least 60 but no more than 80%.

[00501] In an embodiment, a dose of an mTOR inhibitor is associated with, or provides, mTOR inhibition of at least 5 but no more than 70%, at least 10 but no more than 70%, at least 15, but no more than 70%, at least 20 but no more than 70%, at least 30 but no more than 70%, at least 40 but no more than 70%, or at least 50 but no more than 70%.

[00502] In an embodiment, a dose of an mTOR inhibitor is associated with, or provides, mTOR inhibition of at least 5 but no more than 60%, at least 10 but no more than 60%, at least 15, but no more than 60%, at least 20 but no more than 60%, at least 30 but no more than 60%, or at least 40 but no more than 60%.

[00503] In an embodiment, a dose of an mTOR inhibitor is associated with, or provides, mTOR inhibition of at least 5 but no more than 50%, at least 10 but no more than 50%, at least 15, but no more than 50%, at least 20 but no more than 50%, at least 30 but no more than 50%, or at least 40 but no more than 50%.

[00504] In an embodiment, a dose of an mTOR inhibitor is associated with, or provides, mTOR inhibition of at least 5 but no more than 40%, at least 10 but no more than 40%, at least 15, but no more than 40%, at least 20 but no more than 40%, at least 30 but no more than 40%, or at least 35 but no more than 40%.

[00505] In an embodiment, a dose of an mTOR inhibitor is associated with, or provides, mTOR inhibition of at least 5 but no more than 30%, at least 10 but no more than 30%, at least 15, but no more than 30%, at least 20 but no more than 30%, or at least 25 but no more than 30%.

[00506] In an embodiment, a dose of an mTOR inhibitor is associated with, or provides, mTOR inhibition of at least 1, 2, 3, 4 or 5 but no more than 20%, at least 1, 2, 3, 4 or 5 but no more than 30%, at least 1, 2, 3, 4 or 5, but no more than 35, at least 1, 2, 3, 4 or 5 but no more than 40%, or at least 1, 2, 3, 4 or 5 but no more than 45%.

[00507] In an embodiment, a dose of an mTOR inhibitor is associated with, or provides, mTOR inhibition of at least 1, 2, 3, 4 or 5 but no more than 90%.

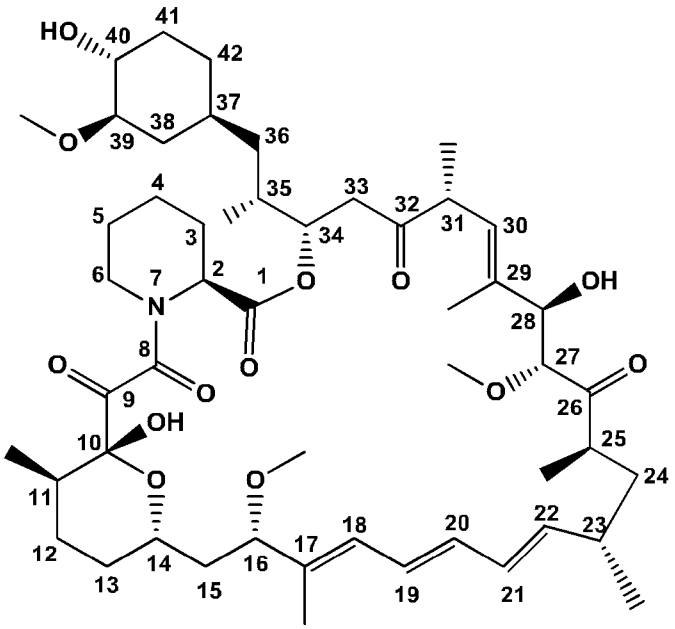
[00508] As is discussed herein, the extent of mTOR inhibition can be expressed as the extent of P70 S6 inhibition, e.g., the extent of mTOR inhibition can be determined by the level of decrease in P70 S6 activity, e.g., by the decrease in phosphorylation of a P70 S6 substrate. The level of mTOR inhibition can be evaluated by a method described herein, e.g. by the Boulay assay.

EXEMPLARY AtTOR INHIBITORS

[00509] As used herein, the term "mTOR inhibitor" refers to a compound or ligand, or a pharmaceutically acceptable salt thereof, which inhibits the mTOR kinase in a cell. In an embodiment an mTOR inhibitor is an allosteric inhibitor. In an embodiment an mTOR inhibitor is a catalytic inhibitor.

[005 10] Allosteric mTOR inhibitors include the neutral tricyclic compound rapamycin (sirolimus), rapamycin-related compounds, that is compounds having structural and functional similarity to rapamycin including, e.g., rapamycin derivatives, rapamycin analogs (also referred to as rapalogs) and other macrolide compounds that inhibit mTOR activity.

[0051 1] Rapamycin is a known macrolide antibiotic produced by Streptomyces hygroscopicus having the structure shown in Formula A.



[00512] (A)

[00513] See, e.g., McAlpine, J.B., et al, J. Antibiotics (1991) 44: 688; Schreiber, S.L., et al, J. Am. Chem. Soc. (1991) 113: 7433; U.S. Patent No. 3,929,992. There are various numbering schemes proposed for rapamycin. To avoid confusion, when specific rapamycin analogs are named herein, the names are given with reference to rapamycin using the numbering scheme of formula A.

[00514] Rapamycin analogs useful in the invention are, for example, O-substituted analogs in which the hydroxyl group on the cyclohexyl ring of rapamycin is replaced by ORi in which Ri is hydroxyalkyl, hydroxyalkoxyalkyl, acylaminoalkyl, or aminoalkyl; e.g. RAD001, also known as, everolimus as described in US 5,665,772 and WO94/09010 the contents of which are incorporated by reference. Other suitable rapamycin analogs include those substituted at the 26- or 28-position. The rapamycin analog may be an epimer of an analog mentioned above, particularly an epimer of an analog substituted in position 40, 28 or 26, and may optionally be further hydrogenated, e.g. as described in US 6,015,815, WO95/14023 and WO99/15530 the contents of which are incorporated by reference, e.g. ABT578 also known as zotarolimus or a rapamycin analog described in US 7,091,213, WO98/02441 and WO01/14387 the contents of which are incorporated by reference, e.g. AP23573 also known as ridaforolimus.

[00515] Examples of rapamycin analogs suitable for use in the present invention from US 5,665,772 include, but are not limited to, 40-O-benzyl-rapamycin, 40-O-(4'-hydroxymethyl)benzyl-rapamycin, 40-O-[4'-(1,2-dihydroxyethyl)]benzyl-rapamycin, 40-O-allyl-rapamycin, 40-O-[3'-(2,2-dimethyl-1,3-dioxolan-4(S)-yl)-prop-2'-en-1'-yl]-rapamycin, (2'E,4'S)-40-O-(4',5'-dihydroxypent-2'-en-l'-yl)-rapamycin, 40-O-(2-hydroxy)ethoxycarbonylmethyl-rapamycin, 40-O-(2-hydroxy)ethyl-rapamycin, 40-O-(3-hydroxy)propyl-rapamycin, 40-O-(6-hydroxy)hexyl-rapamycin, 40-O-[2-(2-hydroxy)ethoxy]ethyl-rapamycin, 40-O-[(3S)-2,2-dimethyldioxolan-3-yl]methyl-rapamycin, 40-O-[(2S)-2,3-dihydroxyprop-l-yl]-rapamycin, 40-O-(2-acetoxy)ethyl-rapamycin, 40-O-(2-nicotinoyloxy)ethyl-rapamycin, 40-O-[2-(N-morpholino)acetoxy]ethyl-rapamycin, 40-O-(2-N-imidazolylacetoxy)ethyl-rapamycin, 40-O-[2-(N-methyl-N'-piperazinyl)acetoxy]ethyl-rapamycin, 39-O-desmethyl-39,40-O,O-ethylene-rapamycin, (26R)-26-dihydro-40-O-(2-hydroxy)ethyl-rapamycin, 40-O-(2-aminoethyl)-rapamycin, 40-O-(2-acetaminoethyl)-rapamycin, 40-O-(2-nicotinamidoethyl)-rapamycin, 40-O-(2-(N-methyl-imidazo-2'-

ylcarbethoxamido)ethyl)-rapamycin, 40-O-(2-ethoxycarbonylaminoethyl)-rapamycin, 40-O-(2-tolylsulfonamidoethyl)-rapamycin and 40-O-[2-(4',5'-dicarboethoxy-l ',2',3'-triazol-l '-yl)-ethyl]-rapamycin.

[00516] Other rapamycin analogs useful in the present invention are analogs where the hydroxyl group on the cyclohexyl ring of rapamycin and/or the hydroxy group at the 28 position is replaced with an hydroxyester group are known, for example, rapamycin analogs found in US RE44,768, e.g. temsirolimus.

[00517] Other rapamycin analogs useful in the preset invention include those wherein the methoxy group at the 16 position is replaced with another substituent, preferably (optionally hydroxy-substituted) alkynyloxy, benzyl, orthomethoxybenzyl or chlorobenzyl and/or wherein the mexthoxy group at the 39 position is deleted together with the 39 carbon so that the cyclohexyl ring of rapamycin becomes a cyclopentyl ring lacking the 39 position methyoxy group; e.g. as described in W095/16691 and WO96/41807 the contents of which are incorporated by reference. The analogs can be further modified such that the hydroxy at the 40-position of rapamycin is alkylated and/or the 32-carbonyl is reduced.

[0051 8] Rapamycin analogs from W095/16691 include, but are not limited to, 16-demthoxy- 16-(pent-2-ynyl)oxy-rapamycin, 16-demthoxy- 16-(but-2-ynyl)oxy-rapamycin, 16-demthoxy- 16-(propargyl)oxy-rapamycin, 16-demethoxy- 16-(4-hydroxy-but-2-ynyl)oxy-rapamycin, 16-demthoxy- 16-benzyloxy-40-O-(2-hydroxyethyl)-rapamycin, 16-demthoxy- 16-benzyloxy-rapamycin, 16-demethoxy- 16-ortho-methoxybenzyl-rapamycin, 16-demethoxy-40-0-(2-methoxyethyl)-16-pent-2-ynyl)oxy-rapamycin, 39-demethoxy-40-desoxy-39-formyl-42-nor-rapamycin, 39-demethoxy-40-desoxy-39-hydroxymethyl-42-nor-rapamycin, 39-demethoxy-40-desoxy-39-carboxy-42-nor-rapamycin, 39-demethoxy-40-desoxy-39-(4-methyl-piperazin-l-yl)carbonyl-42-nor-rapamycin, 39-demethoxy-40-desoxy-39-(morpholin-4-yl)carbonyl-42-nor-rapamycin, 39-demethoxy-40-desoxy-39-[N-methyl, N-(2-pyridin-2-yl-ethyl)]carbamoyl-42-nor-rapamycin and 39-demethoxy-40-desoxy-39-(p-toluenesulfonylhydrazonomethyl)-42-nor-rapamycin.

[00519] Rapamycin analogs from WO96/41807 include, but are not limited to, 32-deoxo-rapamycin, 16-0-pent-2-ynyl-32-deoxo-rapamycin, 16-O-pent-2-ynyl-32-deoxo-40-O-(2-hydroxy-ethyl)-rapamycin, 16-O-pent-2-ynyl-32-(S)-dihydro-40-O-(2-hydroxyethyl)-

rapamycin, 32(S)-dihydro-40-O-(2-methoxy)ethyl-rapamycin and 32(S)-dihydro-40-O-(2-hydroxyethyl)-rapamycin.

[00520] Another suitable rapamycin analog is umirolimus as described in US2005/0101624 the contents of which are incorporated by reference.

[00521] RAD001, otherwise known as everolimus (Afinitor®), has the chemical name (lR,9S,12S,15R,16E,18R,19R,21R,23S,24E,26E,28E,30S,32S,35R)-l,18-dihydroxy-12-{(lR)-2-[(lS,3R,4R)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]-l-methylethyl}-19,30-dimethoxy-15, 17,21,23,29,35-hexamethyl-ll,36-dioxa-4-aza-tricyclo[30. 3.1. 04,9]hexatriaconta-16,24,26,28-tetraene-2,3 ,10, 14,20-pentaone

[00522] Further examples of allosteric mTOR inhibitors include sirolimus (rapamycin, AY-22989), 40-[3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate]-rapamycin (also called temsirolimus or CCI-779) and ridaforolimus (AP-23573/MK-8669). Other examples of allosteric mTor inhibtors include zotarolimus (ABT578) and umirolimus.

[00523] Alternatively or additionally, catalytic, ATP-competitive mTOR inhibitors have been found to target the mTOR kinase domain directly and target both mTORCl and mTORC2. These are also more effective inhibitors of mTORCl than such allosteric mTOR inhibitors as rapamycin, because they modulate rapamycin-resistant mTORCl outputs such as 4EBP1-T37/46 phosphorylation and cap-dependent translation.

[00524] Catalytic inhibitors include: BEZ235 or 2-methyl-2-[4-(3-methyl-2-oxo-8-quinolin-3-yl-2,3-dihydro-imidazo[4,5-c]quinolin-l-yl)-phenyl]-propionitrile, or the monotosylate salt form. the synthesis of BEZ235 is described in WO2006/1 22806; CCG168 (otherwise known as AZD-8055, Chresta, CM., et al., Cancer Res, 2010, 70(1), 288-298) which has the chemical name {5-[2,4-bis-((S)-3-methyl-morpholin-4-yl)-pyrido[2,3d]pyrimidin-7-yl]-2-methoxy-phenyl }-methanol; 3-[2,4-bis[(3 S)-3 -methylmorpholin-4-yl]pyrido[2,3 -d] pyrimidin-7-yl] -N-methylbenzamide (WO09 1040 19); 3-(2-aminobenzo[d] oxazol-5-yl)- 1-isopropyl- 1H-pyrazolo[3,4-d]pynmidin-4-amine (WO10051043 and WO2013023184); A N-(3-(N-(3-((3,5-dimethoxyphenyl)amino)quinoxaline-2-yl)sulfamoyl)phenyl)-3-methoxy-4-methylbenzamide (WO07044729 and WO12006552); PKI-587 (Venkatesan, AM., J. Med.Chem., 2010, 53, 2636-2645) which has the chemical name l-[4-[4-(dimethylamino)piperidine-l-carbonyl]phenyl]-3-[4-(4,6-dimorpholino-l,3,5-tnazin-2-yl)phenyl]urea; GSK-2126458 (ACS Med. Chem. Lett, 2010, 1, 39-43) which has the chemical name 2,4-difluoro-N-{2-methoxy-5-[4-(4-pyridazinyl)-6-

quinolinyl]-3-pyridinyl}benzenesulfonamide; ; 5-(9-isopropyl-8-methyl-2-morpholino-9H-purin-6-yl)pyrimidin-2-amine (WO101 14484); (E)-N-(8-(6-amino-5-(trifluoromethyl)pyridin-3-yl)-l-(6-(2-cyanopropan-2-yl)pyridin-3-yl)-3-methyl-lH-imidazo[4,5-c]quinolin-2(3H)-ylidene)cyanamide (WO 12007926).

[00525] Further examples of catalytic mTOR inhibitors include 8-(6-methoxy-pyridin-3-yl)-3-methyl-l-(4-piperazin-l-yl-3-trifluoromethyl-phenyl)-l,3-dihydro-imidazo[4,5-c]quinolin-2-one (WO2006/1 22806) and Ku-0063794 (Garcia-Martinez JM, et al.,Biochem I , 2009, 421(1), 29-42.. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR).) WYE-354 is another example of a catalytic mTor inhibitor (Yu K, et al. (2009). Biochemical, Cellular, and In vivo Activity of Novel ATP-Competitive and Selective Inhibitors of the Mammalian Target of Rapamycin. Cancer Res. 69(15): 6232-6240).

[00526] mTOR inhibitors useful according to the present invention also include prodrugs, derivatives, pharmaceutically acceptable salts, or analogs thereof of any of the foregoing.

[00527] mTOR inhibitors, such as RADOOl, may be formulated for delivery based on well-established methods in the art based on the particular dosages described herein. In particular, US Patent 6,004,973 (incorporated herein by reference) provides examples of formulations useable with the mTOR inhibitors described herein.

EVALUATIONOFMTOR INHIBITION

[00528] mTOR phosphorylates the kinase P70 S6, thereby activating P70 S6 kinase and allowing it to phosphorylate its substrate. The extent of mTOR inhibition can be expressed as the extent of P70 S6 kinase inhibition, e.g., the extent of mTOR inhibition can be determined by the level of decrease in P70 S6 kinase activity, e.g., by the decrease in phosphorylation of a P70 S6 kinase substrate. One can determine the level of mTOR inhibition, by measuring P70 S6 kinase activity (the ability of P70 S6 kinase to phsophorylate a substrate), in the absence of inhibitor, e.g., prior to administration of inhibitor, and in the presences of inhibitor, or after the administration of inhibitor. The level of inhibition of P70 S6 kinase gives the level of mTOR inhibition. Thus, if P70 S6 kinase is inhibited by 40%, mTOR activity, as measured by P70 S6 kinase activity, is inhibited by 40%. The extent or level of inhibition referred to herein is the average level of inhibition over the dosage interval. By way of example, if the inhibitor is given

once per week, the level of inhibition is given by the average level of inhibition over that interval, namely a week.

[00529] Boulay et al., Cancer Res, 2004, 64:252-61, hereby incorporated by reference, teaches an assay that can be used to assess the level of mTOR inhibition (referred to herein as the Boulay assay). In an embodiment, the assay relies on the measurement of P70 S6 kinase activity from biological samples before and after administration of an mTOR inhibitor, e.g., RADOOl. Samples can be taken at preselected times after treatment with an mTOR ihibitor, e.g., 24, 48, and 72 hours after treatment. Biological samples, e.g., from skin or peripheral blood mononuclear cells (PBMCs) can be used. Total protein extracts are prepared from the samples. P70 S6 kinase is isolated from the protein extracts by immunoprecipitation using an antibody that specifically recognizes the P70 S6 kinase. Activity of the isolated P70 S6 kinase can be measured in an in vitro kinase assay. The isolated kinase can be incubated with 40S ribosomal subunit substrates (which is an endogenous substrate of P70 S6 kinase) and gamma-^{32}P under conditions that allow phosphorylation of the substrate. Then the reaction mixture can be resolved on an SDS-PAGE gel, and ^{32}P signal analyzed using a Phosphorlmager. A ^{32}P signal corresponding to the size of the 40S ribosomal subunit indicates phosphorylated substrate and the activity of P70 S6 kinase. Increases and decreases in kinase activity can be calculated by quantifying the area and intensity of the ^{32}P signal of the phosphorylated substrate (e.g., using ImageQuant, Molecular Dynamics), assigning arbitrary unit values to the quantified signal, and comparing the values from after administration with values from before administration or with a reference value. For example, percent inhibition of kinase activity can be calculated with the following formula: 1-(value obtained after administration/value obtained before administration) X 100. As described above, the extent or level of inhibition referred to herein is the average level of inhibition over the dosage interval.

[00530] Methods for the evaluation of kinase activity, e.g., P70 S6 kinase activity, are also provided in US 7,727,950, hereby incorporated by reference.

[0053 1] The level of mTOR inhibition can also be evaluated by a change in the ration of PDl negative to PDl positive T cells. T cells from peripheral blood can be identified as PDl negative or positive by art-known methods.

Low-Dose mTOR Inhibitors

[00532] Methods described herein use low, immune enhancing, dose mTOR inhibitors, doses of mTOR inhibitors, e.g., allosteric mTOR inhibitors, including rapalogs such as RAD001. In contrast, levels of inhibitor that fully or near fully inhibit the mTOR pathway are immunosuppressive and are used, e.g., to prevent organ transplant rejection. In addition, high doses of rapalogs that fully inhibit mTOR also inhibit tumor cell growth and are used to treat a variety of cancers (See, e.g., Antineoplastic effects of mammalian target of rapamycine inhibitors. Salvadori M. World J Transplant. 2012 Oct 24;2(5): 74-83; Current and Future Treatment Strategies for Patients with Advanced Hepatocellular Carcinoma: Role of mTOR Inhibition. Finn RS. Liver Cancer. 2012 Nov; 1(3-4): 247-256; Emerging Signaling Pathways in Hepatocellular Carcinoma. Moeini A, Cornelia H, Villanueva A. Liver Cancer. 2012 Sep; 1(2): 83-93; Targeted cancer therapy - Are the days of systemic chemotherapy numbered? Joo WD, Visintin I, Mor G. Maturitas. 2013 Sep 20.; Role of natural and adaptive immunity in renal cell carcinoma response to VEGFR-TKIs and mTOR inhibitor. Santoni M, Berardi R, Amantini C, Burattini L, Santini D, Santoni G, Cascinu S. Int J Cancer. 2013 Oct 2).

[00533] The present invention is based, at least in part, on the surprising finding that doses of mTOR inhibitors well below those used in current clinical settings had a superior effect in increasing an immune response in a subject and increasing the ratio of PD-1 negative T cells/PD-1 positive T cells. It was surprising that low doses of mTOR inhibitors, producing only partial inhibition of mTOR activity, were able to effectively improve immune responses in human subjects and increase the ratio of PD-1 negative T cells/PD-1 positive T cells.

[00534] Alternatively, or in addition, without wishing to be bound by any theory, it is believed that low, a low, immune enhancing, dose of an mTOR inhibitor can increase naive T cell numbers, e.g., at least transiently, e.g., as compared to a non-treated subject. Alternatively or additionally, again while not wishing to be bound by theory, it is believed that treatment with an mTOR inhibitor after a sufficient amount of time or sufficient dosing results in one or more of the following:

an increase in the expression of one or more of the following markers: $CD62L^{hlgh}$, $CD127^{hlgh}$, $CD27^{+}$, and BCL2, e.g., on memory T cells, e.g., memory T cell precursors;

a decrease in the expression of KLRG1, e.g., on memory T cells, e.g., memory T cell precursors; and

an increase in the number of memory T cell precursors, e.g., cells with any one or combination of the following characteristics: increased $CD62L^{hlgh}$, increased $CD127^{hlgh}$, increased $CD27^{+}$, decreased KLRG1, and increased BCL2;

and wherein any of the changes described above occurs, e.g., at least transiently, e.g., as compared to a non-treated subject (Araki, K et al. (2009) *Nature* 460: 108-1 12). Memory T cell precursors are memory T cells that are early in the differentiation program. For example, memory T cells have one or more of the following characteristics: increased $CD62L^{hlgh}$, increased $CD127^{hlgh}$, increased $CD27^{+}$, decreased KLRG1, and/or increased BCL2.

[00535] In an embodiment, the invention relates to a composition, or dosage form, of an mTOR inhibitor, e.g., an allosteric mTOR inhibitor, e.g., a rapalog, rapamycin, or RAD001, or a catalytic mTOR inhibitor, which, when administered on a selected dosing regimen, e.g., once daily or once weekly, is associated with: a level of mTOR inhibition that is not associated with complete, or significant immune suppression, but is associated with enhancement of the immune response.

[00536] An mTOR inhibitor, e.g., an allosteric mTOR inhibitor, e.g., a rapalog, rapamycin, or RAD001, or a catalytic mTOR inhibitor, can be provided in a sustained relase formulation. Any of the compositions or unit dosage forms described herein can be provided in a sustained release formulation. In some embodiments, a sustained release formulation will have lower bioavailability than an immediate release formulation. E.g., in embodiments, to attain a similar therapeutic effect of an immediate release forlation a sustained release formulation will have from about 2 to about 5, about 2.5 to about 3.5, or about 3 times the amount of inhibitor provided in the immediate release formulation.

[00537] In an embodiment, immediate release forms, e.g., of RAD001, typically used for one administration per week, having 0.1 to 20, 0.5 to 10, 2.5 to 7.5, 3 to 6, or about 5, mgs per unit dosage form, are provided. For once per week administrations, these immediate release formulations correspond to sustained release forms, having, respectively, 0.3 to 60, 1.5 to 30, 7.5 to 22.5, 9 to 18, or about 15 mgs of an mTOR inhibitor, e.g., an allosteric mTOR inhibitor, e.g., rapamycin or RAD001. In embodiments both forms are administered on a once/week basis.

[00538] In an embodiment, immediate release forms, e.g., of RAD001, typically used for one administration per day, having having 0.005 to 1.5, 0.01 to 1.5, 0.1 to 1.5, 0.2 to 1.5, 0.3 to 1.5, 0.4 to 1.5, 0.5 to 1.5, 0.6 to 1.5, 0.7 to 1.5, 0.8 to 1.5, 1.0 to 1.5, 0.3 to 0.6, or about 0.5 mgs per

unit dosage form, are provided. For once per day administrations, these immediate release forms correspond to sustained release forms, having, respectively, 0.015 to 4.5, 0.03 to 4.5, 0.3 to 4.5, 0.6 to 4.5, 0.9 to 4.5, 1.2 to 4.5, 1.5 to 4.5, 1.8 to 4.5, 2.1 to 4.5, 2.4 to 4.5, 3.0 to 4.5, 0.9 to 1.8, or about 1.5 mgs of an mTOR inhibitor, e.g., an allosteric mTOR inhibitor, e.g., rapamycin or RAD001. For once per week administrations, these immediate release forms correspond to sustained release forms, having, respectively, 0.1 to 30, 0.2 to 30, 2 to 30, 4 to 30, 6 to 30, 8 to 30, 10 to 30, 1.2 to 30, 14 to 30, 16 to 30, 20 to 30, 6 to 12, or about 10 mgs of an mTOR inhibitor, e.g., an allosteric mTOR inhibitor, e.g., rapamycin or RAD001.

[00539] In an embodiment, immediate release forms, e.g., of RAD001, typically used for one administration per day, having having 0.01 to 1.0 mgs per unit dosage form, are provided. For once per day administrations, these immediate release forms correspond to sustained release forms, having, respectively, 0.03 to 3 mgs of an mTOR inhibitor, e.g., an allosteric mTOR inhibitor, e.g., rapamycin or RADOOl.For once per week administrations, these immediate release forms correspond to sustained release forms, having, respectively, 0.2 to 20 mgs of an mTOR inhibitor, e.g., an allosteric mTOR inhibitor, e.g., rapamycin or RAD001.

[00540] In an embodiment, immediate release forms, e.g., of RAD001, typically used for one administration per week, having having 0.5 to 5.0 mgs per unit dosage form, are provided. For once per week administrations, these immediate release forms correspond to sustained release forms, having, respectively, 1.5 to 15 mgs of an mTOR inhibitor, e.g., an allosteric mTOR inhibitor, e.g., rapamycin or RAD001.

[00541] As described above, one target of the mTOR pathway is the P70 S6 kinase. Thus, doses of mTOR inhibitors which are useful in the methods and compositions described herein are those which are sufficient to achieve no greater than 80% inhibition of P70 S6 kinase activity relative to the activity of the P70 S6 kinase in the absence of an mTOR inhibitor, e.g., as measured by an assay described herein, e.g., the Boulay assay. In a further aspect, the invention provides an amount of an mTOR inhibitor sufficient to achieve no greater than 38% inhibition of P70 S6 kinase activity relative to P70 S6 kinase activity in the absence of an mTOR inhibitor.

[00542] In one aspect the dose of mTOR inhibitor useful in the methods and compositions of the invention is sufficient to achieve, e.g., when administered to a human subject, 90 +/-5 % (i.e., 85-95%), 89+/-5 %, 88+/-5 %, 87+/-5 %, 86+/-5 %, 85+/-5 %, 84+/-5 %, 83+/-5 %, 82+/-5 %, 81+/-5 %, 80+/-5 %, 79+/-5 %, 78+/-5 %, 77+/-5 %, 76+/-5 %, 75+/-5 %, 74+/-5 %, 73+/-5 %,

72 +1-5%, 71 +/-5%, 70 +1-5%, 69 +1-5%, 68 +1-5%, 67 +/-5%, 66 +/-5%, 65 +1-5%, 64 +1-5%, 63 +1-5%, 62 +/-5%, 61 +/-5%, 60 +1-5%, 59 +1-5%, 58 +1-5%, 57 +1-5%, 56 +1-5%, 55 +1-5%, 54 +1-5%, 54 +/-5%, 53 +/-5%, 52 +/-5%, 51 +1-5%, 50 +/-5%, 49 +/-5%, 48 +1-5%, 47 +1-5%, 46 +1-5%, 45 +/-5%, 44 +1-5%, 43 +/-5%, 42 +/-5%, 41 +1-5%, 40 +/-5%, 39 +/-5%, 38 +/-5%, 37 +1-5%, 36 +1-5%, 35 +/-5%, 34 +1-5%, 33 +/-5%, 32 +/-5%, 31 +1-5%, 30 +1-5%, 29 +/-5%, 28 +/-5%, 27 +/-5%, 26 +1-5%, 25 +/-5%, 24 +1-5%, 23 +/-5%, 22 +/-5%, 21 +/-5%, 20 +1-5%, 19 +/-5%, 18 +/-5%, 11 +1-5%, 16 +/-5%, 15 +/-5%, 14 +/-5%, 13 +1-5%, 12 +/-5%, 11 +/-5%, or 10 +1-5%, inhibition of P70 S6 kinase activity , e.g., as measured by an assay described herein, e.g., the Boulay assay.

[00543] P70 S6 kinase activity in a subject may be measured using methods known in the art, such as, for example, according to the methods described in U.S. Pat. 7,727,950, by immunoblot analysis of phosphoP70 S6K levels and/or phosphoP70 S6 levels or by in vitro kinase activity assays.

[00544] As used herein, the term "about" in reference to a dose of mTOR inhibitor refers to up to a +/- 10% variability in the amount of mTOR inhibitor, but can include no variability around the stated dose.

[00545] In some embodiments, the invention provides methods comprising administering to a subject an mTOR inhibitor, e.g., an allosteric inhibitor, e.g., RAD001, at a dosage within a target trough level. In some embodiments, the trough level is significantly lower than trough levels associated with dosing regimens used in organ transplant and cancer patients. In an embodiment mTOR inhibitor, e.g., RAD001, or rapamycin, is administerd to result in a trough level that is less than ½, 1/4, 1/10, or 1/20 of the trough level that results in immunosuppression or an anticancer effect. In an embodiment mTOR inhibitor, e.g., RADOOl, or rapamycin, is administerd to result in a trough level that is less than ½, 1/4, 1/10, or 1/20 of the trough level provided on the FDA approved packaging insert for use in immunosuppression or an anticancer indications.

[00546] In an embodiment a method disclosed herein comprises administering to a subject an mTOR inhibitor, e.g., an allosteric inhibitor, e.g., RADOOl, at a dosage that provides a target trough level of 0.1 to 10 ng/ml, 0.1 tO 5 ng/ml, 0.1 to 3ng/ml, 0.1 to 2 ng/ml, or 0.1 to 1 ng/ml.

[00547] In an embodiment a method disclosed herein comprises administering to a subject an mTOR inhibitor, e.g., an allosteric inhibitor, e.g., RADOOl, at a dosage that provides a target trough level of 0.2 to 10 ng/ml, 0.2 to 5 ng/ml, 0.2 to 3ng/ml, 0.2 to 2 ng/ml, or 0.2 to 1 ng/ml.

[00548] In an embodiment a method disclosed herein comprises administering to a subject an mTOR inhibitor, e.g. an, allosteric inhibitor, e.g., RADOOl, at a dosage that provides a target trough level of 0.3 to 10 ng/ml, 0.3 to 5 ng/ml, 0.3 to 3ng/ml, 0.3 to 2 ng/ml, or 0.3 to 1 ng/ml.

[00549] In an embodiment a method disclosed herein comprises administering to a subject an mTOR inhibitor, e.g., an allosteric inhibitor, e.g., RADOOl, at a dosage that provides a target trough level of 0.4 to 10 ng/ml, 0.4 to 5 ng/ml, 0.4 to 3ng/ml, 0.4 to 2 ng/ml, or 0.4 to 1 ng/ml.

[00550] In an embodiment a method disclosed herein comprises administering to a subject an mTOR inhibitor, e.g., an allosteric inhibitor, e.g., RADOOl, at a dosage that provides a target trough level of 0.5 to 10 ng/ml, 0.5 to 5 ng/ml, 0.5 to 3ng/ml, 0.5 to 2 ng/ml, or 0.5 to 1 ng/ml.

[00551] In an embodiment a method disclosed herein comprises administering to a subject an mTOR inhibitor, e.g., an allosteric inhibitor, e.g., RADOOl, at a dosage that provides a target trough level of 1 to 10 ng/ml, 1 to 5 ng/ml, 1 to 3ng/ml, or 1 to 2 ng/ml.

[00552] As used herein, the term "trough level" refers to the concentration of a drug in plasma just before the next dose, or the minimum drug conce ntration between two doses.

[00553] In some embodiments, a target trough level of RADOOl is in a range of between about 0.1 and 4.9 ng/ml. In an embodiment, the target trough level is below 3ng/ml, e.g., is between 0.3 or less and 3 ng/ml. In an embodiment, the target trough level is below 3ng/ml, e.g., is between 0.3 or less and 1 ng/ml.

[00554] In a further aspect, the invention can utilize an mTOR inhibitor other than RADOOl in an amount that is associated with a target trough level that is bioequivalent to the specified target trough level for RADOOl. In an embodiment, the target trough level for an mTOR inhibitor other than RADOOl, is a level that gives the same level of mTOR inhibition (e.g., as measured by a method described herein, e.g., the inhibition of P70 S6) as does a trough level of RADOOl described herein.

Pharmaceutical compositions: mTOR Inhibitors

[00555] In one aspect, the present invention relates to pharmaceutical compositions comprising an mTOR inhibitor, e.g., an mTOR inhibitor as described herein, formulated for use in combination with CAR cells described herein.

[00556] In some embodiments, the mTOR inhibitor is formulated for administration in combination with an additional, e.g., as described herein.

[00557] In general, compounds of the invention will be administered in therapeutically effective amounts as described above via any of the usual and acceptable modes known in the art, either singly or in combination with one or more therapeutic agents.

[00558] The pharmaceutical formulations may be prepared using conventional dissolution and mixing procedures. For example, the bulk drug substance (e.g., an mTOR inhibitor or stabilized form of the compound (e.g., complex with a cyclodextrin derivative or other known complexation agent) is dissolved in a suitable solvent in the presence of one or more of the excipients described herein. The mTOR inhibitor is typically formulated into pharmaceutical dosage forms to provide an easily controllable dosage of the drug and to give the patient an elegant and easily handleable product.

[00559] Compounds of the invention can be administered as pharmaceutical compositions by any conventional route, in particular enterally, e.g., orally, e.g., in the form of tablets or capsules, or parenterally, e.g., in the form of injectable solutions or suspensions, topically, e.g., in the form of lotions, gels, ointments or creams, or in a nasal or suppository form. Where an mTOR inhibitor is administered in combination with (either simultaneously with or separately from) another agent as described herein, in one aspect, both components can be administered by the same route (e.g., parenterally). Alternatively, another agent may be administered by a different route relative to the mTOR inhibitor. For example, an mTOR inhibitor may be administered orally and the other agent may be administered parenterally.

SUSTAINED RELEASE

[00560] mTOR inhibitors, e.g., allosteric mTOR inhibitors or catalytic mTOR inhibitors, disclosed herein can be provided as pharmaceutical formulations in form of oral solid dosage forms comprising an mTOR inhibitor disclosed herein, e.g., rapamycin or RADOOl, which satisfy product stability requirements and/or have favorable pharmacokinetic properties over the immediate release (IR) tablets, such as reduced average plasma peak concentrations, reduced inter- and intra-patient variability in the extent of drug absorption and in the plasma peak

concentration, reduced C_{max} / C_{min} ratio and/or reduced food effects. Provided pharmaceutical formulations may allow for more precise dose adjustment and/or reduce frequency of adverse events thus providing safer treatments for patients with an mTOR inhibitor disclosed herein, e.g., rapamycin or RADOOl.

[00561] In some embodiments, the present disclosure provides stable extended release formulations of an mTOR inhibitor disclosed herein, e.g., rapamycin or RADOOl, which are multi-particulate systems and may have functional layers and coatings.

[00562] The term "extended release, multi-particulate formulation as used herein refers to a formulation which enables release of an mTOR inhibitor disclosed herein, e.g., rapamycin or RADOOl, over an extended period of time e.g. over at least 1, 2, 3, 4, 5 or 6 hours. The extended release formulation may contain matrices and coatings made of special excipients, e.g., as described herein, which are formulated in a manner as to make the active ingredient available over an extended period of time following ingestion.

[00563] The term "extended release" can be interchangeably used with the terms "sustained release" (SR) or "prolonged release". The term "extended release" relates to a pharmaceutical formulation that does not release active drug substance immediately after oral dosing but over an extended in accordance with the definition in the pharmacopoeias Ph. Eur. (7^{th} edition) mongraph for tablets and capsules and USP general chapter <1151> for pharmaceutical dosage forms. The term "Immediate Release" (IR) as used herein refers to a pharmaceutical formulation which releases 85% of the active drug substance within less than 60 minutes in accordance with the definition of "Guidance for Industry: "Dissolution Testing of Immediate Release Solid Oral Dosage Forms" (FDA CDER, 1997). In some embodiments, the term "immediate release" means release of everolismus from tablets within the time of 30 minutes, e.g., as measured in the dissolution assay described herein.

[00564] Stable extended release formulations of an mTOR inhibitor disclosed herein, e.g., rapamycin or RADOOl, can be characterized by an in-vitro release profile using assays known in the art, such as a dissolution assay as described herein: a dissolution vessel filled with 900 mL phosphate buffer pH 6.8 containing sodium dodecyl sulfate 0.2% at 37°C and the dissolution is performed using a paddle method at 75 rpm according to USP by according to USP testing monograph 711, and Ph.Eur. testing monograph 2.9.3. respectively.

[00565] In some embodiments, stable extended release formulations of an mTOR inhibitor disclosed herein, e.g., rapamycin or RAD001, release the mTOR inhibitor in the in-vitro release assay according to following release specifications:

0.5h: <45%, or <40, e.g., <30%

lh: 20-80%, e.g., 30-60%

2h: >50%, or >70%, e.g., >75%

3h: >60%, or >65%, e.g., >85%, e.g., >90%.

[00566] In some embodiments, stable extended release formulations of an mTOR inhibitor disclosed herein, e.g., rapamycin or RADOOl, release 50% of the mTOR inhibitor not earlier than 45, 60, 75, 90, 105 min or 120 min in the in-vitro dissolution assay.

Pharmaceutical compositions and treatments

[00567] Pharmaceutical compositions of the present invention may comprise a CAR-expressing cell, e.g., a plurality of CAR-expressing cells, as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions of the present invention are in one aspect formulated for intravenous administration.

[00568] Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

[00569] In one embodiment, the pharmaceutical composition is substantially free of, e.g., there are no detectable levels of a contaminant, e.g., selected from the group consisting of endotoxin, mycoplasma, replication competent lentivirus (RCL), p24, VSV-G nucleic acid, HIV gag, residual anti-CD3/anti-CD28 coated beads, mouse antibodies, pooled human serum, bovine

serum albumin, bovine serum, culture media components, vector packaging cell or plasmid components, a bacterium and a fungus. In one embodiment, the bacterium is at least one selected from the group consisting of Alcaligenes faecalis, Candida albicans, Escherichia coli, Haemophilus influenza, Neisseria meningitides, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumonia, and Streptococcus pyogenes group A.

[00570] When "an immunologically effective amount," "an anti-cancer effective amount," "a cancer-inhibiting effective amount," or "therapeutic amount" is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the immune effector cells described herein may be administered at a dosage of 10^4 to 10^9 cells/kg body weight, in some instances 10^5 to 10^6 cells/kg body weight, including all integer values within those ranges. The immune effector cell compositions may also be administered multiple times at these dosages. The cells can be administered by using infusion techniques that are commonly known in immunotherapy (see, e.g., Rosenberg *et ah,* New Eng. J. of Med. 319:1676, 1988).

[00571] In certain aspects, it may be desired to administer activated immune effector cells to a subject and then subsequently redraw blood (or have an apheresis performed), activate the cells therefrom according to the present invention, and reinfuse the patient with these activated and expanded cells. This process can be carried out multiple times every few weeks. In certain aspects, the cells can be activated from blood draws of from lOcc to 400cc. In certain aspects, the cells are activated from blood draws of 20cc, 30cc, 40cc, 50cc, 60cc, 70cc, 80cc, 90cc, or lOOcc.

[00572] The administration of the subject compositions may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient trans arterially, subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally. In one aspect, the T cell compositions of the present invention are administered to a patient by intradermal or subcutaneous injection. In one aspect, the immune effector cell compositions of the present

invention are administered by i.v. injection. The compositions of immune effector cells may be injected directly into a tumor, lymph node, or site of infection.

[00573] In a particular exemplary aspect, subjects may undergo leukapheresis, wherein leukocytes are collected, enriched, or depleted ex vivo to select and/or isolate the cells of interest, *e.g.*, T cells. These T cell isolates may be expanded by methods known in the art and treated such that one or more CAR constructs of the invention may be introduced, thereby creating a CAR T cell of the invention. Subjects in need thereof may subsequently undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain aspects, following or concurrent with the transplant, subjects receive an infusion of the expanded CAR T cells of the present invention. In an additional aspect, expanded cells are administered before or following surgery.

[00574] The dosage of the above treatments to be administered to a patient will vary with the precise nature of the condition being treated and the recipient of the treatment. The scaling of dosages for human administration can be performed according to art-accepted practices. The dose for CAMPATH, for example, will generally be in the range 1 to about 100 mg for an adult patient, usually administered daily for a period between 1 and 30 days. The preferred daily dose is 1 to 10 mg per day although in some instances larger doses of up to 40 mg per day may be used (described in U.S. Patent No. 6,120,766).

[00575] In one embodiment, the CAR is introduced into immune effector cells, e.g., using in vitro transcription, and the subject (e.g., human) receives an initial administration of CAR-expressingcells of the invention, and one or more subsequent administrations of the CAR-expressing cells of the invention, wherein the one or more subsequent administrations are administered less than 15 days, e.g., 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 days after the previous administration. In one embodiment, more than one administration of the CAR-expressing cells of the invention are administered to the subject (e.g., human) per week, e.g., 2, 3, or 4 administrations of the CAR-expressing cells of the invention are administered per week. In one embodiment, the subject (e.g., human subject) receives more than one administration of the CAR-expressing cells per week (e.g., 2, 3 or 4 administrations per week) (also referred to herein as a cycle), followed by a week of no CAR-expressing cells administration, and then one or more additional administration of the CAR-expressing cells (e.g., more than one administration of the

CAR-expressing cells per week) is administered to the subject. In another embodiment, the subject (e.g., human subject) receives more than one cycle of CAR-expressing cells, and the time between each cycle is less than 10, 9, 8, 7, 6, 5, 4, or 3 days. In one embodiment, the CAR-expressing cells are administered every other day for 3 administrations per week. In one embodiment, the CAR-expressing cells of the invention are administered for at least two, three, four, five, six, seven, eight or more weeks.

[00576] In one aspect, mesothelin CAR-expressing cells are generated using lentiviral viral vectors, such as lentivirus. CAR-expressing cells generated that way will have stable CAR expression.

[00577] In one aspect, the CAR-expressing cellss transiently express CAR vectors for 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 days after transduction. Transient expression of CARs can be effected by RNA CAR vector delivery. In one aspect, the CAR RNA is transduced into the T cell by electroporation.

[00578] In one embodiment, the dose and/or dosing schedule is one provided in Figure 6.

[00579] A potential issue that can arise in patients being treated using transiently expressing CAR-expressing cells (particularly with murine scFv bearing CAR-expressing cells) is anaphylaxis after multiple treatments.

[00580] Without being bound by this theory, it is believed that such an anaphylactic response might be caused by a patient developing humoral anti-CAR response, i.e., anti-CAR antibodies having an anti-IgE isotype. It is thought that a patient's antibody producing cells undergo a class switch from IgG isotype (that does not cause anaphylaxis) to IgE isotype when there is a ten to fourteen day break in exposure to antigen.

[00581] If a patient is at high risk of generating an anti-CAR antibody response during the course of transient CAR therapy (such as those generated by RNA transductions), CAR-expressing cell infusion breaks should not last more than ten to fourteen days.

[00582] Using CARs with human (instead of murine) scFvs can reduce the likelihood and intensity of a patient having an anti-CAR response.

[00583]

[00584] Table 2: Amino Acid Sequences of Human scFvs and CARs (bold underline is the leader sequence and grey box is a linker sequence). In the case of the scFvs, the remaining amino acids are the heavy chain variable region and light chain variable regions, with each of the HC CDRs (HC CDR1, HC CDR2, HC CDR3) and LC CDRs (LC CDR1, LC CDR2, LCCDR3) underlined). In the case of the CARs, the further remaining amino acids are the remaining amino acids of the CARs.)

SEQ ID NO:	Description	Amino Acid Sequence
39	M1 (ScFv domain)	QVQLQQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQ APGQGLEWMGRINPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSEDTAVYYCARG RYYGMDVWGQGTMVTVSSGGGGSGGGGSGGGGSGGGGSEIVLTQSPATLSLSPGERATIS CRASQSVSSNFAWYQQRPGQAPRLLIYDASNRATGIPPRFSGSGSGTDFTLTISSLEPED FAAYYCHQRSNWLYTFGQGTKVDIK
63	M1 (full) >ZA53-27BC (M1 ZA53-27BC R001-A11 126161)	**MALPVTALLLPLALLLHAARP**QVQLQQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQ APGQGLEWMGRINPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSEDTAVYYCARG RYYGMDVWGQGTMVTVSSGGGGSGGGGSGGGGSGGGGSEIVLTQSPATLSLSPGERATIS CRASQSVSSNFAWYQQRPGQAPRLLIYDASNRATGIPPRFSGSGSGTDFTLTISSLEPED FAAYYCHQRSNWLYTFGQGTKVDIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAV HTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEED GCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPE MGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL HMQALPPR
40	M2 (ScFv domain)	QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQ APGQGLEWMGWINPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARD LRRTVVTPRAYYGMDVWGQGTTVTVSSGGGGSGGGGSGGGGSGGGGSDIQLTQSPSTLSA SVGDRVTITCQASQDISNSLNWYQQKAGKAPKLLIYDASTLETGVPSRFSGSGSGTDFSF TISSLQPEDIATYYCQQHDNLPLTFGQGTKVEIK
64	M2 (full) >FA56-26RC (M2 FA56-26RC R001-A10 126162)	**MALPVTALLLPLALLLHAARP**QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQ APGQGLEWMGWINPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARD LRRTVVTPRAYYGMDVWGQGTTVTVSSGGGGSGGGGSGGGGSGGGGSDIQLTQSPSTLSA SVGDRVTITCQASQDISNSLNWYQQKAGKAPKLLIYDASTLETGVPSRFSGSGSGTDFSF TISSLQPEDIATYYCQQHDNLPLTFGQGTKVEIKTTTPAPRPPTPAPTIASQPLSLRPEA CRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMR PVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVL DKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPR
41	M3 (ScFv domain)	QVQLVQSGAEVKKPGAPVKVSCKASGYTFTGYYMHWVRQ APGQGLEWMGWINPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARG EWDGSYYYDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIVLTQTPSSLSASVGDRV TITCRASQSINTYLNWYQHKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQ PEDFATYYCQQSFSPLTFGGGTKLEIK
65	M3 >VA58-21LC	**MALPVTALLLPLALLLHAARP**QVQLVQSGAEVKKPGAPVKVSCKASGYTFTGYYMHWVRQ APGQGLEWMGWINPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARG EWDGSYYYDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIVLTQTPSSLSASVGDRV TITCRASQSINTYLNWYQHKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQ

	(M3 VA58-21LC R001-A1 126163)	PEDFATYYCQQSFS PLTFGGGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQE EDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRD PEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD ALHMQALPPR
42	M4 (ScFv **domain)**	QVQLVESGGGLVQPGGSLRLSCAASGFTFSSYWMHWVRQ VPGKGLVWVSRINTDGSTTTYADSVEGRFTISRDNAKNTLYLQMNSLRDDDTAVYYCVGG HWAVWGQGT TVTVS S**GGGGS** GGGGSGGGGSGGGGSDI QMTQS PSTLSASVGDRVT ITCRA SQSISDRLAWYQQKPGKAPKLLIYKASSLESGVPSRFSGSGSGTEFTLTISSLQPDDFAV YYCQQYGHLPMYTFGQGTKVEIK
66	M4 >DP37-07IC (M4 DP37-07IC R001-C6 126164)	**MALPVTALLLPLALLLHAARPOVQLVESGGGLVOPGGSLRLSCAASGFTFSSYWMHWVRQ** VPGKGLVWVSRINTDGSTTTYADSVEGRFTISRDNAKNTLYLQMNSLRDDDTAVYYCVGG HWAVWGQGT TVTVS SGGGGS GGGGSGGGGSGGGGSDI QMTQS PSTLSASVGDRVT ITCRA SQSISDRLAWYQQKPGKAPKLLIYKASSLESGVPSRFSGSGSGTEFTLTISSLQPDDFAV YYCQQYGHLPMYTFGQGTKVEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHT RGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGC SCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMG GKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR
43	M5 (ScFv **domain)**	QVQLVQS GAEVE KPGAS VKVS CKAS GYTFTDYYMHWVRQ APGQGLEWMGWINPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCASG WDFDYWGQGTLVTVSSGGGGS GGGGSGGGGSGGGGSDI VMTQS PSSLSASVGDRVT ITCR ASQSIRYYLSWYQQKPGKAPKLLIYTASILQNGVPSRFSGSGSGTDFTLTISSLQPEDFA TYYCLQTYTTPDFGPGTKVEIK
67	M5 >XP31-20LC (M5 XP31-20LC R001-B4 126165)	**MALPVTALLLPLALLLHAARPQVQLVQS** GAEVE KPGAS VKVS CKAS GYTFTDY**YMHWVRQ** APGQGLEWMGWINPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCASG WDFDYWGQGTLVTVSSGGGGS GGGGSGGGGSGGGGSDI VMTQS PSSLSASVGDRVT ITCR ASQSIRYYLSWYQQKPGKAPKLLIYTASILQNGVPSRFSGSGSGTDFTLTISSLQPEDFA TYYCLQTYTTPDFGPGTKVEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTR GLDFACDI YIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCS CRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDALHMQ ALP PR
44	M6 (ScFv **domain)**	QVQLVQS GAEVKKPGAS VKVS CKAS GYTFTSYYMHWVRQ APGQGLEWMGIINPSGGSTSYAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARY RLIAVAGDYYYYGMDVWGQGTMVTVSS GGGGS GGGGSGGGGSGGGGSDI QMTQS PSSVSA SVGDRVTITCRASQGVGRWLAWYQQKPGTAPKLLIYAASTLQSGVPSRFSGSGSGTDFTL TINNLQPEDFATYYCQQANS FPLTFGGGTRLEIK
68	M6 >FE10-06ID (M6 46FE10-06ID R001-A4	**MALPVTALLLPLALLLHAARPQVQLVQS** GAEVKKPGAS VKVS CKAS GYTFTSY**YMHWVRQ** APGQGLEWMGIINPSGGSTSYAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARY RLIAVAGDYYYYGMDVWGQGTMVTVSS GGGGS GGGGSGGGGSGGGGSDI QMTQS PSSVSA SVGDRVTITCRASQGVGRWLAWYQQKPGTAPKLLIYAASTLQSGVPSRFSGSGSGTDFTL TINNLQPEDFATYYCQQANS FPLTFGGGTRLEIKTTTPAPRPPTPAPTIASQPLSLRPEA CRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYI FKQPFMR PVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVL DKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST

	126166)	ATKDTYDALHMQALPPR
45	M7 (ScFv domain)	QVQLVQSGGGWQPGRSLRLSCAASGFTFSSYAMHWVRQ APGKGLEWVAVISYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARW KVSSSSPAFDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSEIVLTQSPATLSLSPGER AILSCRASQSVYTKYLGWYQQKPGQAPRLLIYDASTRATGI PDRFSGSGSGTDFTLTINR LEPEDFAVYYCQHYGGSPLITFGQGTRLEIK
69	M7 >VE12-01CD (M7 VE12-01CD R001-A5 126167)	**MALPVTALLLPLALLLHAARPQVQLVQSGGGWQPGRSLRLSCAASGFTFSSYAMHWVRQ** APGKGLEWVAVISYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARW KVSSSSPAFDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSEIVLTQSPATLSLSPGER AILSCRASQSVYTKYLGWYQQKPGQAPRLLIYDASTRATGI PDRFSGSGSGTDFTLTINR LEPEDFAVYYCQHYGGSPLITFGQGTRLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRP AAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYI FKQPFMRPVQ TTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATK DTYDALHMQALPPR
46	M8 (ScFv domain)	QVQLQQSGAEVKKPGASVKVSCKTSGYPFTGYSLHWVRQ APGQGLEWMGWINPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARD HYGGNSLFYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIQLTQSPSSISASVGDTVS ITCRASQDSGTWLAWYQQKPGKAPNLLMYDASTLEDGVPSRFSGSASGTEFTLTVNRLQP EDSATYYCQQYNSYPLTFGGGTKVDIK
70	M8 >LE13-05XD (M8 LE13-05XD R001-E5 126168)	**MALPVTALLLPLALLLHAARPQVQLQQSGAEVKKPGASVKVSCKTSGYPFTGYSLHWVRQ** APGQGLEWMGWINPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARD HYGGNSLFYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIQLTQSPSSISASVGDTVS ITCRASQDSGTWLAWYQQKPGKAPNLLMYDASTLEDGVPSRFSGSASGTEFTLTVNRLQP EDSATYYCQQYNSYPLTFGGGTKVDIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQE EDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRD PEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD ALHMQALPPR
47	M9 (ScFv domain)	QVQLVQSGAEVKKPGASVEVSCKASGYTFTSYYMHWVRQ APGQGLEWMGIINPSGGSTGYAQKFQGRVTMTRDTSTSTVHMELSSLRSEDTAVYYCARG GYSSSSDAFDIWGQGTMVTVSSGGGGSGGGGSGGGGSGGGGSDIQMTQSPPSLSASVGDR VTITCRASQDISSALAWYQQKPGTPPKLLIYDASSLESGVPSRFSGSGSGTDFTLTISSL QPEDFATYYCQQFSSYPLTFGGGTRLEIK
71	M9 >BE15-00SD (M9 BE15-00SD R001-A3 126169)	**MALPVTALLLPLALLLHAARPQVQLVQS**GAEVKKPGASVEVSCKASGYTFTSY**YMHWVRQ** APGQGLEWMGIINPSGGSTGYAQKFQGRVTMTRDTSTSTVHMELSSLRSEDTAVYYCARG GYSSSSDAFDIWGQGTMVTVSSGGGGSGGGGSGGGGSGGGGSDIQMTQSPPSLSASVGDR VTITCRASQDISSALAWYQQKPGTPPKLLIYDASSLESGVPSRFSGSGSGTDFTLTISSL QPEDFATYYCQQFSSYPLTFGGGTRLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAA GGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDT YDALHMQALPPR
48	M10 (ScFv domain)	QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQ APGQGLEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCARV AGGIYYYYGMDVWGQGTTITVSSGGGGSGGGGSGGGGSGGGGSDIVMTQTPDSLAVSLGE

		RATISCKSSHSVLYNRNNKNYLAWYQQKPGQPPKLLFYWASTRKSGVPDRFSGSGSGTDF TLTISSLQPEDFATYFCQQTQTFPLTFGQGTRLEIN
72	**M10** >RE16-05MD (M10 RE16-05MD R001-D10 126170)	**MALPVTALLLPLALLLHAARPOVOLVOS** GAEVKKPGAS VKVS CKAS GYTFTSYGI **SWVRQ** APGQGLEWMGWISAYNGNTNYAQKLQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCARV AGGIYYYYGMDVWGQGTTITVSSGGGGSGGGGSGGGGSGGGGSDIVMTQTPDSLAVSLGE RATISCKSSHSVLYNRNNKNYLAWYQQKPGQPPKLLFYWASTRKSGVPDRFSGSGSGTDF TLTISSLQPEDFATYFCQQTQTFPLTFGQGTRLEINTTTPAPRPPTPAPTIASQPLSLRP EACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPF MRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYD VLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGL STATKDTYDALHMQAL PPR
49	**Mil (ScFv domain)**	QVQLQQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQ APGQGLEWMGWINPNSGGTNYAQNFQGRVTMTRDTSISTAYMELRRLRSDDTAVYYCASG WDFDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIRMTQSPSSLSASVGDRVTITCR ASQSIRYYLSWYQQKPGKAPKLLIYTASILQNGVPSRFSGSGSGTDFTLTISSLQPEDFA TYYCLQTYTTPDFGPGTKVEIK
73	**Mil** >NE10-19WD (Mil NE10-19WD R001-G2 126171)	MALPVTALLLPLALLLHAARPOVOLOOSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQ APGQGLEWMGWINPNSGGTNYAQNFQGRVTMTRDTSISTAYMELRRLRSDDTAVYYCASG WDFDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIRMTQSPSSLSASVGDRVTITCR ASQSIRYYLSWYQQKPGKAPKLLIYTASILQNGVPSRFSGSGSGTDFTLTISSLQPEDFA TYYCLQTYTTPDFGPGTKVEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTR GLDFACDI YIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYI FKQPFMRPVQTTQEEDGCS CRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDALHMQ ALP PR
50	**M12 (ScFv domain)**	QVQLVQS GAEVKKPGAS VKVS CKAS GYTFTGYYMHWVRQ APGQGLEWMGRINPNSGGTNYAQKFQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCART TTSYAFDIWGQGTMVTVSSGGGGSGGGGSGGGGSGGGGSDIQLTQSPSTLSASVGDRVTI TCRASQS ISTWLAWYQQKPGKAPNLLIYKASTLESGVPSRFSGSGSGTEFTLTISSLQPD DFATYYCQQYNTYSPYTFGQGTKLEIK
74	**M12** >DE12-14RD (M12 DE12-14RD R001-G9 126172)	**MALPVTALLLPLALLLHAARPOVOLVOS** GAEVKKPGAS VKVS CKAS GYTFTGY**YMHWVRQ** APGQGLEWMGRINPNSGGTNYAQKFQGRVTMTTDTSTSTAYMELRSLRSDDTAVYYCART TTSYAFDIWGQGTMVTVSSGGGGSGGGGSGGGGSGGGGSDIQLTQSPSTLSASVGDRVTI TCRASQS ISTWLAWYQQKPGKAPNL LIYKASTLESGVPSR FSGSGSGTEFTLT ISSLQPD DFATYYCQQYNTYS PYTFGQGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQE EDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRD PEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD ALHMQALPPR
51	**M13 (ScFv domain)**	QVQLVQS GGGLVKPGGSLRLSCEASGF IFSDYYMGWIRQ APGKGLEWVSYIGRSGSSMYYADSVKGRFTFSRDNAKNSLYLQMNSLRAEDTAVYYCAAS PVVAATEDFQHWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIVMTQTPATLSLSPGER ATLSCRASQSVTSNYLAWYQQKPGQAPRLLLFGASTRATGI PDRFSGSGSGTDFTLTINR LEPEDFAMYYCQQYGSAPVTFGQGTKLEIK
75	**M13** >TE13-19LD	**MALPVTALLLPLALLLHAARPOVOLVOS** GGGLVKPGGSLRLSCEASGF I**FSDYYMGWIRQ** APGKGLEWVSYIGRSGSSMYYADSVKGRFTFSRDNAKNSLYLQMNSLRAEDTAVYYCAAS PVVAATEDFQHWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIVMTQTPATLSLSPGER

	(M13 TE13-19LD R002-C3 126173)	ATLSCRASQSVTSNYLAWYQQKPGQAPRLLLFGASTRATGI PDRFSGSGSGTDFTLTINR LEPEDFAMYYCQQYGSAPVTFGQGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPA AGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQT TQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRR GRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPR
52	M14 (ScFv domain)	QVQLVQSGAEVRAPGASVKISCKASGFTFRGYYIHWVRQ APGQGLEWMGIINPSGGSRAYAQKFQGRVTMTRDTSTSTVYMELSSLRSDDTAMYYCART ASCGGDCYYLDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIQMTQSPPTLSASVGD RVTITCRASENVNIWLAWYQQKPGKAPKLLIYKSSSLASGVPSRFSGSGSGAEFTLTISS LQPDDFATYYCQQYQS YPLTFGGGTKVDIK
76	M14 >BS83-95ID (M14 BS83-95ID R001-E8 126174)	MALPVTALLLPLALLLHAARPQVQLVQSGAEVRAPGASVKISCKASGFTFRGYYIHWVRQ APGQGLEWMGIINPSGGSRAYAQKFQGRVTMTRDTSTSTVYMELSSLRSDDTAMYYCART ASCGGDCYYLDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIQMTQSPPTLSASVGD RVTITCRASENVNIWLAWYQQKPGKAPKLLIYKSSSLASGVPSRFSGSGSGAEFTLTISS LQPDDFATYYCQQYQS YPLTFGGGTKVDIKTTTPAPRPPTPAPTIASQPLSLRPEACRPA AGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQT TQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRR GRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPR
53	M15 (ScFv domain)	QVQLVQSGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQ APGKGLEWVSGISWNSGS IGYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAKD GSSSWSWGYFDYWGQGTLVTVSSGGGGSGGGGSGGGGSSSELTQDPAVSVALGQTVRTTC QGDALRSYYASWYQQKPGQAPMLVIYGKNNRPSGI PDRFSGSDSGDTASLTITGAQAEDE ADYYCNSRDSSGYPVFGTGTKVTVL
77	M15 >HS86-94XD (M15 HS86-94XD NT 127553)	MALPVTALLLPLALLLHAARPQVQLVQSGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQ APGKGLEWVSGISWNSGS IGYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAKD GSSSWSWGYFDYWGQGTLVTVSSGGGGSGGGGSGGGGSSSELTQDPAVSVALGQTVRTTC QGDALRSYYASWYQQKPGQAPMLVIYGKNNRPSGI PDRFSGSDSGDTASLTITGAQAEDE ADYYCNSRDSSGYPVFGTGTKVTVLTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAV HTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEED GCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPE MGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDAL HMQALPPR
54	M16 (ScFv domain)	EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQ APGKGLEWVSGISWNSGS TGYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTALYYCAKD SSSWYGGGSAFD IWGQGTMVTVS SGGGGSGGGGSGGGGS SSELTQE PAVSVALGQTVRI T CQGDSLRSYYASWYQQKPGQAPVLVIFGRSRRPSGI PDRFSGSSSGNTASLI ITGAQAED EADYYCNSRDNTANHYVFGTGTKLTVL
78	M16 >XS87-99RD (M16 XS87-99RD NT 127554)	MALPVTALLLPLALLLHAARPEVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQ APGKGLEWVSGISWNSGS TGYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTALYYCAKD SSSWYGGGSAFD IWGQGTMVTVS SGGGGSGGGGSGGGGS SSELTQE PAVSVALGQTVRI T CQGDSLRSYYASWYQQKPGQAPVLVIFGRSRRPSGI PDRFSGSSSGNTASLI ITGAQAED EADYYCNSRDNTANHYVFGTGTKLTVLTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQE EDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRD PEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYD ALHMQALPPR
55	M17 (ScFv domain)	EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQ APGKGLEWVSGISWNSGS TGYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTALYYCAKD SSSWYGGGSAFD IWGQGTMVTVS SGGGGSGGGGSGGGGS SSELTQD PAVSVALGQTVRI T

		CQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGI PDRFSGSSSGNTASLTITGAQAED EADYYCNSRGSSGNHYVFGTGTKVTVL
79	M17 >NS89-94MD (M17 NS89-94MD NT 127555)	MALPVTALLLPLALLLHAARPEVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQ APGKGLEWVSGISWNSGSTGYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTALYYCAKD SSSWYGGGSAFD IWGQGTMVTVS SGGGGSGGGGSGGGGS SSELTQDPAVSVALGQTVRI T CQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGI PDRFSGSSSGNTASLTITGAQAED EADYYCNSRGSSGNHYVFGTGTKVTVLTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQE EDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRD PEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD ALHMQALPPR
56	M18 (ScFv domain)	QVQLVQSGGGLVQPGGSLRLSCAASGFTFSSYWMHWVRQ APGKGLVWVSRINSDGSSTSYADSVKGRFTISRDNAKNTLYLQMNSLRAEDTAVYYCVRT GWVGS YYYYMDVWGKGTTVTVSSGGGGSGGGGSGGGGSGGGGSEIVLTQSPGTLSLSPGE RATLSCRASQSVSSNYLAWYQQKPGQPPRLLIYDVSTRATGI PARFSGGGSGTDFTLTIS SLEPEDFAVYYCQQRSNWPPWTFGQGTKVEIK
80	M18 >DS90-09HD (M18 DS90-09HD R003-A05 127556)	MALPVTALLLPLALLLHAARPQVQLVQSGGGLVQPGGSLRLSCAASGFTFSSYWMHWVRQ APGKGLVWVSRINSDGSSTSYADSVKGRFTISRDNAKNTLYLQMNSLRAEDTAVYYCVRT GWVGSYYYYMDVWGKGTTVTVSSGGGGSGGGGSGGGGSGGGGSEIVLTQSPGTLSLSPGE RATLSCRASQSVSSNYLAWYQQKPGQPPRLLIYDVSTRATGI PARFSGGGSGTDFTLTIS SLEPEDFAVYYCQQRSNWPPWTFGQGTKVEIKTTTPAPRPPTPAPTIASQPLSLRPEACR PAAGGAVHTRGLDFACDI YIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPV QTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDK RRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDALHMQALPPR
57	M19 (ScFv domain)	QVQLVQSGGGWQPGRSLRLSCAASGFTFSSYGMHWVRQ APGKGLEWVAVISYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKG YSRYYYYGMDVWGQGTTVTVSSGGGGSGGGGSGGGGSGGGGSEIVMTQSPATLSLSPGER AILSCRASQSVYTKYLGWYQQKPGQAPRLLIYDASTRATGI PDRFSGSGSGTDFTLTINR LEPEDFAVYYCQHYGGSPLITFGQGTKVDIK
81	M19 >TS92-04BD (M19 TS92-04BD R003-C06 127557)	MALPVTALLLPLALLLHAARPQVQLVQSGGGWQPGRSLRLSCAASGFTFSSYGMHWVRQ APGKGLEWVAVISYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKG YSRYYYYGMDVWGQGTTVTVSSGGGGSGGGGSGGGGSGGGGSEIVMTQSPATLSLSPGER AILSCRASQSVYTKYLGWYQQKPGQAPRLLIYDASTRATGI PDRFSGSGSGTDFTLTINR LEPEDFAVYYCQHYGGSPLITFGQGTKVDIKTTTPAPRPPTPAPTIASQPLSLRPEACRP AAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYI FKQPFMRPVQ TTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATK DTYDALHMQALPPR
58	M20 (ScFv domain)	QVQLVQSGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQ APGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKR EAAAGHDWYFDLWGRGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIRVTQSPSSLSASVGD RVTITCRASQS ISS YLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQSYS IPLTFGQGTKVEIK
82	M20	MALPVTALLLPLALLLHAARPQVQLVQSGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQ APGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKR

	>JS93-08WD (M20 JS93-08WD R003-E07 127558)	EAAAGHDWYFDLWGRGTLVTVSSGGGGSGGGGSGGGGSGGGGSDIRVTQSPSSLSASVGD RVTITCRASQS ISS YLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQSYS IPLTFGQGTKVEIKTTTPAPRPPTPAPTIASQPLSLRPEACRPA AGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQT TQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRR GRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPR
275	Ssl (scFv domain)	QVQLQQSGPELEKPGASVKISCKASGYSFTGYTMNWVKQSHGKSLEWIGLITPYNGASS YNQKFRGKATLTVDKS SSTAYMDLLS LTSEDSAVY FCARGGYDGRGFDYWGQGT TVTVS SGGGGSGGGGSGGGGSDIELTQSPAIMSASPGEKVTMTCSASSSVSYMHWYQQKSGTSP KRWIYDTSKLASGVPGRFSGSGSGNSYSLTISSVEAEDDATYYCQQWSGYPLTFGAGTK LEI
278	Ssl (full)	MALPVTALLLPLALLLHAARPQVQLQQSGPELEKPGASVKISCKASGYSFTGYTMNWVK QSHGKSLEWIGLITPYNGASS YNQKFRGKATLTVDKS SSTAYMDLLS LTSEDSAVYFCA RGGYDGRGFDYWGQGT TVTVS SGGGGS GGGGSGGGGSDI ELTQS PAIMSASPGEKVTMT CSASSSVSYMHWYQQKSGTSPKRWIYDTSKLASGVPGRFSGSGSGNSYSLTISSVEAED DATYYCQQWSGYPLTFGAGTKLEITTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAV HTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEE DGCSCRFPEEEEGGCELRVKFSRSADAPA

Table 3: Nucleic Acid Sequences encoding CAR molecules (underlined is the leader sequence)

SEQ ID NO:	Desc.	Nucleic Acid Sequence
87	M1 (ScFv domain) >ZA53-27BC (M1)	CAAGTCCAACTGCAGCAGTCAGGAGCGGAAGTGAAGAAACCAGGAGCGTCAGTCAAAGTGTCGTGCAAGGCTAGCGGCTA CACCTTCACCGGCTACTA CATGCACTGGGTTCGACAGGCTCCAGGGCAGGGTCTGGAGTGGATGGGCCGCATCAACCCGAATTCCGGTGGGACTAACT ACGCCCAGAAGTTCCAGGGAAGAGTGACCATGACTAGGGACACGTCGATCAGCACTGCGTACATGGAACTGAGCCGCCTG CGGTCCGAGGATACTGCCGTCTACTACTGCGCACGCGGAAGGTACTATGGAATGGACGTGTGGGGCCAAGGGACTATGGT GACTGTGAGCTCGGGAGGGGGAGGCTCCGGTGGCGGGGGATCAGGAGGAGGAGGATCAGGGGGAGGAGGTTCCGAAATTG TCCTCACCCAGAGCCCGGCAACCCTCTCACTTTCCCCGGGAGAGCGCGCAACCATCTCTTGCCGGGCTAGCCAATCCGTG TCGTCCAATTTCGCCTGGTACCAGCAACGGCCGGGACAAGCCCCTAGACTCCTGATCTACGACGCCAGCAACAGAGCGAC TGGAATTCCTCCACGCTTTTCGGGATCAGGCTCCGGTACCGACTTCACCCTGACTATCTCGTCGCTCGAACCCGAGGATT TCGCCGCCTACTACTGTCATCAGCGGTCGAACTGGTTGTATACGTTTGGCCAGGGCACCAAGGTGGATATCAAG
111	M1 (Full) >ZA53-27BC (M1)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAACTGCAGCA G TCAGGAGCGGAAGTGAAGAAACCAGGAGCGTCAGTCAAAGTGTCGTGCAAGGCTAGCGGCTACACCTTCACCGGCTACTA CATGCACTGGGTTCGACAGGCTCCAGGGCAGGGTCTGGAGTGGATGGGCCGCATCAACCCGAATTCCGGTGGGACTAACT ACGCCCAGAAGTTCCAGGGAAGAGTGACCATGACTAGGGACACGTCGATCAGCACTGCGTACATGGAACTGAGCCGCCTG CGGTCCGAGGATACTGCCGTCTACTACTGCGCACGCGGAAGGTACTATGGAATGGACGTGTGGGGCCAAGGGACTATGGT GACTGTGAGCTCGGGAGGGGGAGGCTCCGGTGGCGGGGGATCAGGAGGAGGAGGATCAGGGGGAGGAGGTTCCGAAATTG TCCTCACCCAGAGCCCGGCAACCCTCTCACTTTCCCCGGGAGAGCGCGCAACCATCTCTTGCCGGGCTAGCCAATCCGTG TCGTCCAATTTCGCCTGGTACCAGCAACGGCCGGGACAAGCCCCTAGACTCCTGATCTACGACGCCAGCAACAGAGCGAC TGGAATTCCTCCACGCTTTTCGGGATCAGGCTCCGGTACCGACTTCACCCTGACTATCTCGTCGCTCGAACCCGAGGATT TCGCCGCCTACTACTGTCATCAGCGGTCGAACTGGTTGTATACGTTTGGCCAGGGCACCAAGGTGGATATCAAGACCACT ACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATGTAGACC CGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTGGTACTT GCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTTAAGCAA CCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGGCGGCTG CGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACGAACTCA ATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCGCGCAGA AAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTATGAAAGG

		GGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACGCTCTTC ACATGCAGGCCCTGCCGCCTCGG
88	M2 (ScFv domain) >FA56- 26RC (M2)	CAAGTCCAACTCGTCCAGTCAGGAGCAGAAGTCAAGAAACCAGGTGCTAGCGTGAAAGTGTCGTGCAAGGCGTCGGGATA CACTTTCACCGGATACTAC ATGCACTGGGTCCGCCAGGCCCCCGGACAAGGACTGGAATGGATGGGCTGGATCAACCCGAATAGCGGGGGAACTAATTA CGCCCAGAAGTTTCAGGGACGAGTGACCATGACCCGCGATACCTCTATCTCGACCGCCTACATGGAGCTCTCCAGACTGC GCTCCGACGATACTGCAGTGTACTACTGCGCCCGGGACCTGAGGCGGACTGTGGTTACTCCTCGCGCCTATTATGGCATG GACGTGTGGGGCCAAGGAACTACTGTGACTGTGAGCTCGGGAGGCGGTGGGTCAGGCGGAGGAGGGTCGGGCGGTGGTGG CTCGGGAGGGGGAGGAAGCGACATTCAACTTACGCAGAGCCCGTCAACCCTGTCAGCGTCAGTGGGAGATCGGGTGACCA TCACGTGTCAGGCCAGCCAGGATATCTCCAACTCGCTCAACTGGTACCAGCAAAAGGCGGGTAAAGCTCCGAAGCTGCTG ATCTACGACGCTTCCACCCTCGAGACTGGAGTCCCATCCAGATTTTCCGGGTCAGGAAGCGGCACCGATTTCTCCTTCAC CATTTCGTCCTTGCAACCGGAGGACATCGCAACCTACTACTGCCAGCAGCATGACAACTTGCCTCTGACGTTCGGGCAGG GCACCAAGGTGGAAATCAAG
112	M2 (Full) >FA56- 26RC (M2)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAACTCGTCCA GTCAGGAGCAGAAGTCAAGAAACCAGGTGCTAGCGTGAAAGTGTCGTGCAAGGCGTCGGGATACACTTTCACCGGATACT AC ATGCACTGGGTCCGCCAGGCCCCCGGACAAGGACTGGAATGGATGGGCTGGATCAACCCGAATAGCGGGGGAACTAATTA CGCCCAGAAGTTTCAGGGACGAGTGACCATGACCCGCGATACCTCTATCTCGACCGCCTACATGGAGCTCTCCAGACTGC GCTCCGACGATACTGCAGTGTACTACTGCGCCCGGGACCTGAGGCGGACTGTGGTTACTCCTCGCGCCTATTATGGCATG GACGTGTGGGGCCAAGGAACTACTGTGACTGTGAGCTCGGGAGGCGGTGGGTCAGGCGGAGGAGGGTCGGGCGGTGGTGG CTCGGGAGGGGGAGGAAGCGACATTCAACTTACGCAGAGCCCGTCAACCCTGTCAGCGTCAGTGGGAGATCGGGTGACCA TCACGTGTCAGGCCAGCCAGGATATCTCCAACTCGCTCAACTGGTACCAGCAAAAGGCGGGTAAAGCTCCGAAGCTGCTG ATCTACGACGCTTCCACCCTCGAGACTGGAGTCCCATCCAGATTTTCCGGGTCAGGAAGCGGCACCGATTTCTCCTTCAC CATTTCGTCCTTGCAACCGGAGGACATCGCAACCTACTACTGCCAGCAGCATGACAACTTGCCTCTGACGTTCGGGCAGG GCACCAAGGTGGAAATCAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTG TCCCTGCGTCCGGAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTA CATTTGGGCCCCTCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGA AGAAGCTGCTGTACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGG TTCCCAGAGGAGGAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGG GCAGAACCAGCTCTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACC CAGAAATGGGCGGGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAA GCCTATAGCGAGATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGC CACCAAGGACACCTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
89	M3 (ScFv domain) >VA58- 21LC (M3)	CAAGTCCAACTCGTCCAA TCAGGAGCGGAAGTCAAAAAGCCCGGAGCTCCAGTGAAAGTGTCATGCAAGGCCTCCGGCTACACCTTCACCGGTTACTA TATGCACTGGGTGCGGCAGGCCCCGGGCCAGGGGTTGGAATGGATGGGATGGATCAATCCAAACTCGGGTGGGACTAACT ACGCCCAGAAGTTCCAAGGACGGGTGACCATGACTAGGGACACCTCGATCTCCACCGCATACATGGAGCTTAGCAGACTC CGCTCCGACGATACCGCAGTCTACTATTGCGCGCGGGGAGAGTGGGACGGATCGTACTACTACGATTACTGGGGCCAGGG AACTCTGGTGACTGTTTCCTCGGGTGGAGGAGGTTCAGGCGGAGGCGGCTCGGGCGGGGGAGGATCTGGAGGAGGAGGGT CCGACATTGTGCTGACCCAAACTCCTTCGTCCCTGTCGGCCAGCGTGGGCGACCGCGTGACGATTACGTGCAGAGCTAGC CAATCCATCAATACTTACCTCAACTGGTACCAGCATAAGCCGGGGAAAGCACCAAAGCTGCTGATCTACGCCGCCTCATC CTTGCAGAGCGGTGTGCCTTCACGCTTTAGCGGATCGGGATCGGGAACGGATTTCACCCTGACTATCAGCTCCCTCCAGC CGGAGGATTTTGCGACCTACTACTGTCAGCAGAGCTTCTCACCGCTGACTTTCGGCGGCGGGACCAAGCTGGAAATCAAG
113	M3 (Full) >VA58- 21LC (M3)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAACTCGTCCA A TCAGGAGCGGAAGTCAAAAAGCCCGGAGCTCCAGTGAAAGTGTCATGCAAGGCCTCCGGCTACACCTTCACCGGTTACTA TATGCACTGGGTGCGGCAGGCCCCGGGCCAGGGGTTGGAATGGATGGGATGGATCAATCCAAACTCGGGTGGGACTAACT ACGCCCAGAAGTTCCAAGGACGGGTGACCATGACTAGGGACACCTCGATCTCCACCGCATACATGGAGCTTAGCAGACTC CGCTCCGACGATACCGCAGTCTACTATTGCGCGCGGGGAGAGTGGGACGGATCGTACTACTACGATTACTGGGGCCAGGG AACTCTGGTGACTGTTTCCTCGGGTGGAGGAGGTTCAGGCGGAGGCGGCTCGGGCGGGGGAGGATCTGGAGGAGGAGGGT CCGACATTGTGCTGACCCAAACTCCTTCGTCCCTGTCGGCCAGCGTGGGCGACCGCGTGACGATTACGTGCAGAGCTAGC CAATCCATCAATACTTACCTCAACTGGTACCAGCATAAGCCGGGGAAAGCACCAAAGCTGCTGATCTACGCCGCCTCATC CTTGCAGAGCGGTGTGCCTTCACGCTTTAGCGGATCGGGATCGGGAACGGATTTCACCCTGACTATCAGCTCCCTCCAGC CGGAGGATTTTGCGACCTACTACTGTCAGCAGAGCTTCTCACCGCTGACTTTCGGCGGCGGGACCAAGCTGGAAATCAAG ACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATG TAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTG GTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTT AAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGG CGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACG AACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCG CGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTAT GAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACG CTCTTCACATGCAGGCCCTGCCGCCTCGG
90	M4	CAAGTGCAACTCGTTGAA TCAGGTGGAGGTTTGGTGCAACCCGGAGGATCTCTCAGACTGTCGTGTGCGGCGTCCGGGTTCACCTTTTCGTCCTACTG

	(ScFv domain) >DP37-07IC (M4)	GATGCACTGGGTGCGCCAGGTGCCGGGAAAAGGACTGGTGTGGGTGTCCAGAATCAACACCGACGGGTCAACGACTACCT ACGCAGATAGCGTGGAAGGTCGGTTCACCATTTCGCGGGACAACGCTAAAAACACTCTGTACCTTCAGATGAATTCACTG CGCGATGACGACACCGCAGTCTACTACTGCGTCGGTGGACACTGGGCGGTCTGGGGACAGGGAACTACGGTGACTGTGTC CAGCGGCGGGGGAGGAAGCGGCGGAGGGGGGAGCGGAGGCGGAGGATCAGGAGGAGGCGGCTCCGATATCCAGATGACCC AGTCGCCATCGACCCTCTCCGCTAGCGTGGGGGATAGGGTCACTATCACTTGCCGAGCCAGCCAATCCATTAGCGACCGG CTTGCCTGGTACCAACAGAAACCTGGAAAGGCCCCGAAGCTGCTCATCTACAAGGCCTCGTCACTGGAGTCGGGAGTCCC GTCCCGCTTTTCCGGCTCGGGCTCAGGCACCGAGTTCACTCTGACCATCTCGAGCCTGCAGCCGGACGATTTCGCCGTGT ATTACTGCCAGCAATACGGACATCTCCCAATGTACACGTTCGGTCAGGGCACCAAGGTCGAAATCAAG
114	M4 >DP37-07IC (M4)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTGCAACTCGTTGAA TCAGGTGGAGGTTTGGTGCAACCCGGAGGATCTCTCAGACTGTCGTGTGCGGCGTCCGGGTTCACCTTTTCGTCCTACTG GATGCACTGGGTGCGCCAGGTGCCGGGAAAAGGACTGGTGTGGGTGTCCAGAATCAACACCGACGGGTCAACGACTACCT ACGCAGATAGCGTGGAAGGTCGGTTCACCATTTCGCGGGACAACGCTAAAAACACTCTGTACCTTCAGATGAATTCACTG CGCGATGACGACACCGCAGTCTACTACTGCGTCGGTGGACACTGGGCGGTCTGGGGACAGGGAACTACGGTGACTGTGTC CAGCGGCGGGGGAGGAAGCGGCGGAGGGGGGAGCGGAGGCGGAGGATCAGGAGGAGGCGGCTCCGATATCCAGATGACCC AGTCGCCATCGACCCTCTCCGCTAGCGTGGGGGATAGGGTCACTATCACTTGCCGAGCCAGCCAATCCATTAGCGACCGG CTTGCCTGGTACCAACAGAAACCTGGAAAGGCCCCGAAGCTGCTCATCTACAAGGCCTCGTCACTGGAGTCGGGAGTCCC GTCCCGCTTTTCCGGCTCGGGCTCAGGCACCGAGTTCACTCTGACCATCTCGAGCCTGCAGCCGGACGATTTCGCCGTGT ATTACTGCCAGCAATACGGACATCTCCCAATGTACACGTTCGGTCAGGGCACCAAGGTCGAAATCAAGACCACTACCCCA GCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATGTAGACCCGCAGC TGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTGGTACTTGCGGGG TCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTTAAGCAACCCTTC ATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGGCGGCTGCGAACT GCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACGAACTCAATCTTG GTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCGCGCAGAAAGAAT CCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTATGAAAGGGGAACG CAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACGCTCTTCACATGC AGGCCCTGCCGCCTCGG
91	M5 (ScFv domain) >XP31-20LC (M5)	CAAGTCCAACTCGTTCAATCAGGCGCAGAAGTCGAAAAGCCCGGAGCATCAGTCAAAGTCTCTTGCAAGGCTTCCGGCTA CACCTTCACGGACTACTAC ATGCACTGGGTGCGCCAGGCTCCAGGCCAGGGACTGGAGTGGATGGGATGGATCAACCCGAATTCCGGGGGAACTAACTA CGCCCAGAAGTTTCAGGGCCGGGTGACTATGACTCGCGATACCTCGATCTCGACTGCGTACATGGAGCTCAGCCGCCTCC GGTCGGACGATACCGCCGTGTACTATTGTGCGTCGGGATGGGACTTCGACTACTGGGGGCAGGGCACTCTGGTCACTGTG TCAAGCGGAGGAGGTGGATCAGGTGGAGGTGGAAGCGGGGGAGGAGGTTCCGGCGGCGGAGGATCAGATATCGTGATGAC GCAATCGCCTTCCTCGTTGTCCGCATCCGTGGGAGACAGGGTGACCATTACTTGCAGAGCGTCCCAGTCCATTCGGTACT ACCTGTCGTGGTACCAGCAGAAGCCGGGGAAAGCCCCAAAACTGCTTATCTATACTGCCTCGATCCTCCAAAACGGCGTG CCATCAAGATTCAGCGGTTCGGGCAGCGGGACCGACTTTACCCTGACTATCAGCAGCCTGCAGCCGGAAGATTTCGCCAC GTACTACTGCCTGCAAACCTACACCACCCCGGACTTCGGACCTGGAACCAAGGTGGAGATCAAG
115	M5 (Full) >XP31-20LC (M5)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAACTCGTTCA ATCAGGCGCAGAAGTCGAAAAGCCCGGAGCATCAGTCAAAGTCTCTTGCAAGGCTTCCGGCTACACCTTCACGGACTACT AC ATGCACTGGGTGCGCCAGGCTCCAGGCCAGGGACTGGAGTGGATGGGATGGATCAACCCGAATTCCGGGGGAACTAACTA CGCCCAGAAGTTTCAGGGCCGGGTGACTATGACTCGCGATACCTCGATCTCGACTGCGTACATGGAGCTCAGCCGCCTCC GGTCGGACGATACCGCCGTGTACTATTGTGCGTCGGGATGGGACTTCGACTACTGGGGGCAGGGCACTCTGGTCACTGTG TCAAGCGGAGGAGGTGGATCAGGTGGAGGTGGAAGCGGGGGAGGAGGTTCCGGCGGCGGAGGATCAGATATCGTGATGAC GCAATCGCCTTCCTCGTTGTCCGCATCCGTGGGAGACAGGGTGACCATTACTTGCAGAGCGTCCCAGTCCATTCGGTACT ACCTGTCGTGGTACCAGCAGAAGCCGGGGAAAGCCCCAAAACTGCTTATCTATACTGCCTCGATCCTCCAAAACGGCGTG CCATCAAGATTCAGCGGTTCGGGCAGCGGGACCGACTTTACCCTGACTATCAGCAGCCTGCAGCCGGAAGATTTCGCCAC GTACTACTGCCTGCAAACCTACACCACCCCGGACTTCGGACCTGGAACCAAGGTGGAGATCAAGACCACTACCCCAGCAC CGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATGTAGACCCGCAGCTGGT GGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTGGTACTTGCGGGGTCCT GCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTTAAGCAACCCTTCATGA GGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGGCGGCTGCGAACTGCGC GTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACGAACTCAATCTTGGTCG GAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCGCGCAGAAAGAATCCCC AAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTATGAAAGGGGAACGCAGA AGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACGCTCTTCACATGCAGGC CCTGCCGCCTCGG
92	M6 (ScFv domain) >FE10-06ID (M6)	CAAGTGCAACTCGTCCAGTCAGGTGCAGAAGTGAAGAAACCCGGAGCGTCAGTCAAAGTGTCATGCAAGGCGTCAGGCTA CACCTTCACCAGCTACTAC ATGCACTGGGTGCGGCAGGCCCCAGGCCAAGGCTTGGAGTGGATGGGAATCATTAACCCGTCAGGAGGCTCCACCTCCTA CGCCCAGAAGTTTCAGGGAAGAGTGACGATGACTCGGGATACGTCGACCTCGACCGTGTACATGGAACTGAGCTCGCTGC GCTCCGAGGACACTGCTGTGTACTACTGCGCACGGTACAGACTCATTGCCGTGGCAGGAGACTACTACTACTATGGCATG GACGTCTGGGGGCAGGGCACTATGGTCACTGTGTCGTCCGGCGGAGGAGGCTCGGGTGGAGGAGGTAGCGGAGGAGGGGG AAGCGGAGGGGGGGGCTCCGATATCCAGATGACTCAGTCGCCTTCCTCCGTGTCGGCCTCGGTTGGAGATCGCGTCACCA TCACTTGTCGAGCTTCCCAAGGAGTCGGTAGGTGGCTGGCGTGGTACCAGCAAAAGCCGGGAACTGCCCCGAAGCTCCTG ATCTACGCGGCTAGCACCCTGCAGTCGGGAGTGCCATCCCGCTTCAGCGGATCTGGGTCAGGTACCGACTTCACCCTTAC GATCAACAATCTCCAGCCGGAGGACTTTGCCACCTATTACTGCCAACAGGCCAACAGCTTCCCTCTGACTTTCGGAGGGG

		GCACTCGCCTGGAAATCAAG
116	M6 (Full) >FE10-06ID (M6)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTGCAACTCGTCCA GTCAGGTGCAGAAGTGAAGAAACCCGGAGCGTCAGTCAAAGTGTCATGCAAGGCGTCAGGCTACACCTTCACCAGCTACT AC ATGCACTGGGTGCGGCAGGCCCCAGGCCAAGGCTTGGAGTGGATGGGAATCATTAACCCGTCAGGAGGCTCCACCTCCTA CGCCCAGAAGTTTCAGGGAAGAGTGACGATGACTCGGGATACGTCGACCTCGACCGTGTACATGGAACTGAGCTCGCTGC GCTCCGAGGACACTGCTGTGTACTACTGCGCACGGTACAGACTCATTGCCGTGGCAGGAGACTACTACTACTATGGCATG GACGTCTGGGGGCAGGGCACTATGGTCACTGTGTCGTCCGGCGGAGGAGGCTCGGGTGGAGGAGGTAGCGGAGGAGGGGG AAGCGGAGGGGGGGGCTCCGATATCCAGATGACTCAGTCGCCTTCCTCCGTGTCGGCCTCGGTTGGAGATCGCGTCACCA TCACTTGTCGAGCTTCCCAAGGAGTCGGTAGGTGGCTGGCGTGGTACCAGCAAAAGCCGGGAACTGCCCCGAAGCTCCTG ATCTACGCGGCTAGCACCCTGCAGTCGGGAGTGCCATCCCGCTTCAGCGGATCTGGGTCAGGTACCGACTTCACCCTTAC GATCAACAATCTCCAGCCGGAGGACTTTGCCACCTATTACTGCCAACAGGCCAACAGCTTCCCTCTGACTTTCGGAGGGG GCACTCGCCTGGAAATCAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTG TCCCTGCGTCCGGAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTA CATTTGGGCCCCTCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGA AGAAGCTGCTGTACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGG TTCCCAGAGGAGGAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGG GCAGAACCAGCTCTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACC CAGAAATGGGCGGGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAA GCCTATAGCGAGATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGC CACCAAGGACACCTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
93	M7 (ScFv domain) >VE12-01CD (M7)	CAAGTGCAATTGGTTCAA TCAGGAGGAGGAGTGGTGCAACCTGGAAGATCTCTCAGACTGTCGTGTGCGGCATCGGGATTCACTTTCTCATCATACGC AATGCACTGGGTCCGCCAGGCCCCGGGCAAAGGCTTGGAATGGGTGGCGGTCATTTCATACGACGGCTCGAACAAGTACT ACGCTGACAGCGTGAAGGGACGCTTTACTATTTCCCGGGACAATTCGAAGAACACTCTGTACCTCCAGATGAACTCCCTT AGGGCTGAGGACACCGCCGTCTACTACTGCGCACGCTGGAAAGTGTCGTCCAGCTCCCCAGCTTTTGACTACTGGGGACA GGGAACCCTTGTGACCGTGTCGTCCGGTGGAGGGGGAAGCGGCGGAGGGGGATCAGGTGGCGGCGGATCGGGAGGCGGGG GATCAGAAATCGTGCTGACTCAGTCCCCGGCCACGCTGTCTCTCAGCCCGGGAGAGAGAGCGATCCTGTCCTGCCGCGCC TCGCAGAGCGTGTACACTAAGTACCTGGGGTGGTACCAGCAGAAACCGGGTCAAGCGCCTCGGCTGCTGATCTACGATGC CTCCACCCGGGCCACCGGAATCCCCGATCGGTTCTCCGGCAGCGGCTCGGGAACTGATTTCACGCTGACCATCAATCGCC TGGAGCCGGAAGATTTCGCCGTCTATTACTGCCAGCATTACGGCGGGAGCCCACTCATCACCTTCGGTCAAGGAACCCGA CTCGAAATCAAG
117	M7 (Full) >VE12-01CD (M7)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTGCAATTGGTTCA A TCAGGAGGAGGAGTGGTGCAACCTGGAAGATCTCTCAGACTGTCGTGTGCGGCATCGGGATTCACTTTCTCATCATACGC AATGCACTGGGTCCGCCAGGCCCCGGGCAAAGGCTTGGAATGGGTGGCGGTCATTTCATACGACGGCTCGAACAAGTACT ACGCTGACAGCGTGAAGGGACGCTTTACTATTTCCCGGGACAATTCGAAGAACACTCTGTACCTCCAGATGAACTCCCTT AGGGCTGAGGACACCGCCGTCTACTACTGCGCACGCTGGAAAGTGTCGTCCAGCTCCCCAGCTTTTGACTACTGGGGACA GGGAACCCTTGTGACCGTGTCGTCCGGTGGAGGGGGAAGCGGCGGAGGGGGATCAGGTGGCGGCGGATCGGGAGGCGGGG GATCAGAAATCGTGCTGACTCAGTCCCCGGCCACGCTGTCTCTCAGCCCGGGAGAGAGAGCGATCCTGTCCTGCCGCGCC TCGCAGAGCGTGTACACTAAGTACCTGGGGTGGTACCAGCAGAAACCGGGTCAAGCGCCTCGGCTGCTGATCTACGATGC CTCCACCCGGGCCACCGGAATCCCCGATCGGTTCTCCGGCAGCGGCTCGGGAACTGATTTCACGCTGACCATCAATCGCC TGGAGCCGGAAGATTTCGCCGTCTATTACTGCCAGCATTACGGCGGGAGCCCACTCATCACCTTCGGTCAAGGAACCCGA CTCGAAATCAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCG TCCGGAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGG CCCCTCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTG CTGTACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGA GGAGGAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACC AGCTCTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATG GGCGGGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAG CGAGATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGG ACACCTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
94	M8 (ScFv domain) >LE13-05XD (M8)	CAAGTCCAACTCCAGCAG TCAGGTGCAGAAGTCAAAAAGCCAGGAGCATCCGTGAAGGTTTCGTGCAAGACTTCCGGCTACCCTTTTACCGGGTACTC CCTCCATTGGGTGAGACAAGCACCGGGCCAGGGACTGGAGTGGATGGGATGGATCAACCCAAATTCGGGCGGCACCAACT ATGCGCAGAAGTTCCAGGGACGGGTGACCATGACTCGCGACACTTCGATCTCCACTGCCTACATGGAGCTGTCCCGCTTG AGATCTGACGACACGGCCGTCTACTACTGCGCCCGGGATCACTACGGAGGTAATTCGCTGTTCTACTGGGGGCAGGGAAC CCTTGTGACTGTGTCCTCGGGTGGTGGAGGGTCAGGAGGCGGAGGCTCAGGGGGAGGAGGTAGCGGAGGAGGCGGATCAG ACATCCAACTGACCCAGTCACCATCCTCCATCTCGGCTAGCGTCGGAGACACCGTGTCGATTACTTGTAGGGCCTCCCAA GACTCAGGGACGTGGCTGGCGTGGTATCAGCAAAAACCGGGCAAAGCTCCGAACCTGTTGATGTACGACGCCAGCACCCT CGAAGATGGAGTGCCTAGCCGCTTCAGCGGAAGCGCCTCGGGCACTGAATTCACGCTGACTGTGAATCGGCTCCAGCCGG AGGATTCGGCGACCTACTACTGCCAGCAGTACAACAGCTACCCCCTGACCTTTGGAGGCGGGACCAAGGTGGATATCAAG
118	M8 (Full)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAACTCCAGCA G TCAGGTGCAGAAGTCAAAAAGCCAGGAGCATCCGTGAAGGTTTCGTGCAAGACTTCCGGCTACCCTTTTACCGGGTACTC

	>LE13-05XD (M8)	CCTCCATTGGGTGAGACAAGCACCGGGCCAGGGACTGGAGTGGATGGGATGGATCAACCCAAATTCGGGCGGCACCAACT ATGCGCAGAAGTTCCAGGGACGGGTGACCATGACTCGCGACACTTCGATCTCCACTGCCTACATGGAGCTGTCCCGCTTG AGATCTGACGACACGGCCGTCTACTACTGCGCCCGGGATCACTACGGAGGTAATTCGCTGTTCTACTGGGGGCAGGGAAC CCTTGTGACTGTGTCCTCGGGTGGTGGAGGGTCAGGAGGCGGAGGCTCAGGGGGAGGAGGTAGCGGAGGAGGCGGATCAG ACATCCAACTGACCCAGTCACCATCCTCCATCTCGGCTAGCGTCGGAGACACCGTGTCGATTACTTGTAGGGCCTCCCAA GACTCAGGGACGTGGCTGGCGTGGTATCAGCAAAAACCGGGCAAAGCTCCGAACCTGTTGATGTACGACGCCAGCACCCT CGAAGATGGAGTGCCTAGCCGCTTCAGCGGAAGCGCCTCGGGCACTGAATTCACGCTGACTGTGAATCGGCTCCAGCCGG AGGATTCGGCGACCTACTACTGCCAGCAGTACAACAGCTACCCCCTGACCTTTGGAGGCGGGACCAAGGTGGATATCAAG ACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATG TAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTG GTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTT AAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGG CGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACG AACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCG CGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTAT GAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACG CTCTTCACATGCAGGCCCTGCCGCCTCGG
95	M9 (ScFv domain) >BE15-00SD (M9)	CAAGTGCAACTCGTCCAG TCAGGTGCAGAAGTGAAGAAACCAGGAGCGTCCGTCGAAGTGTCGTGTAAGGCGTCCGGCTACACTTTCACCTCGTACTA CATGCACTGGGTGCGGCAGGCCCCGGGACAAGGCCTCGAATGGATGGGAATCATCAACCCGAGCGGAGGCTCGACTGGTT ACGCCCAGAAGTTCCAGGGAAGGGTGACGATGACCCGCGATACCTCGACTTCGACCGTTCATATGGAGCTCTCGTCCCTG CGGAGCGAGGACACTGCTGTCTACTATTGCGCGCGGGGAGGATACTCTAGCTCCTCCGATGCATTTGACATTTGGGGCCA GGGAACTATGGTGACCGTGTCATCAGGCGGAGGTGGATCAGGAGGAGGAGGGTCGGGAGGGGGAGGCAGCGGCGGGGGTG GGTCGGACATTCAGATGACGCAGTCCCCTCCTAGCCTGAGCGCCTCGGTGGGTGACAGAGTGACCATCACTTGCAGAGCC TCGCAAGACATCTCCTCCGCATTGGCTTGGTACCAGCAAAAGCCGGGCACTCCGCCGAAACTGCTCATCTACGATGCCTC CTCACTGGAGTCAGGAGTCCCATCTCGCTTCTCGGGGTCAGGAAGCGGCACCGATTTTACCCTTACCATCTCCAGCCTGC AGCCCGAGGACTTCGCCACGTACTACTGCCAACAGTTCAGCTCCTACCCACTGACCTTCGGGGGCGGAACTCGCCTGGAA AⓉCAAG
119	M9 (Full) >BE15-OOSD (M9)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTGCAACTCGTCCA G TCAGGTGCAGAAGTGAAGAAACCAGGAGCGTCCGTCGAAGTGTCGTGTAAGGCGTCCGGCTACACTTTCACCTCGTACTA CATGCACTGGGTGCGGCAGGCCCCGGGACAAGGCCTCGAATGGATGGGAATCATCAACCCGAGCGGAGGCTCGACTGGTT ACGCCCAGAAGTTCCAGGGAAGGGTGACGATGACCCGCGATACCTCGACTTCGACCGTTCATATGGAGCTCTCGTCCCTG CGGAGCGAGGACACTGCTGTCTACTATTGCGCGCGGGGAGGATACTCTAGCTCCTCCGATGCATTTGACATTTGGGGCCA GGGAACTATGGTGACCGTGTCATCAGGCGGAGGTGGATCAGGAGGAGGAGGGTCGGGAGGGGGAGGCAGCGGCGGGGGTG GGTCGGACATTCAGATGACGCAGTCCCCTCCTAGCCTGAGCGCCTCGGTGGGTGACAGAGTGACCATCACTTGCAGAGCC TCGCAAGACATCTCCTCCGCATTGGCTTGGTACCAGCAAAAGCCGGGCACTCCGCCGAAACTGCTCATCTACGATGCCTC CTCACTGGAGTCAGGAGTCCCATCTCGCTTCTCGGGGTCAGGAAGCGGCACCGATTTTACCCTTACCATCTCCAGCCTGC AGCCCGAGGACTTCGCCACGTACTACTGCCAACAGTTCAGCTCCTACCCACTGACCTTCGGGGGCGGAACTCGCCTGGAA ATCAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGA GGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTC TGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTAC ATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGA GGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCT ACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGG AAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGAT TGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCT ATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
96	MIO (ScFv domain) >RE16-05MD (MIO)	CAAGTGCAACTCGTCCAGAGCGGAGCAGAAGTCAAGAAGCCAGGAGCGTCAGTGAAAGTGTCATGCAAGGCCAGCGGCTA TACCTTTACTTCGTATGGG ATCTCCTGGGTGCGGCAGGCACCGGGCCAAGGACTGGAGTGGATGGGATGGATCTCAGCCTACAACGGTAACACCAACTA CGCCCAGAAGCTGCAAGGACGCGTGACCATGACTACTGATACGAGCACCTCCACTGCCTACATGGAATTGCGGTCCCTTC GGTCGGACGATACTGCTGTGTACTACTGCGCAAGAGTCGCCGGAGGGATCTACTACTACTACGGCATGGACGTCTGGGGA CAGGGAACCACCATTACGGTGTCGAGCGGAGGGGGAGGCTCGGGGGGAGGAGGAAGCGGAGGTGGCGGCTCCGGGGGCGG CGGATCGGACATTGTGATGACCCAGACTCCTGACTCCCTGGCTGTTTCGTTGGGAGAGCGCGCGACTATCTCGTGTAAGT CCAGCCACTCAGTCCTGTACAATCGCAATAACAAGAACTACCTCGCGTGGTACCAGCAAAAACCGGGTCAGCCGCCTAAA CTCCTGTTCTACTGGGCCTCCACCAGAAAGAGCGGGGTGCCAGATCGATTCTCTGGATCAGGATCAGGTACCGACTTTAC GCTGACCATCTCGTCCCTGCAGCCGGAGGATTTCGCGACTTACTTCTGCCAGCAGACTCAGACTTTCCCCCTCACCTTCG GTCAAGGCACCAGGCTGGAAATCAAT
120	MIO (Full) >RE16-05MD (MIO)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTGCAACTCGTCCA GAGCGGAGCAGAAGTCAAGAAGCCAGGAGCGTCAGTGAAAGTGTCATGCAAGGCCAGCGGCTATACCTTTACTTCGTATG GG ATCTCCTGGGTGCGGCAGGCACCGGGCCAAGGACTGGAGTGGATGGGATGGATCTCAGCCTACAACGGTAACACCAACTA CGCCCAGAAGCTGCAAGGACGCGTGACCATGACTACTGATACGAGCACCTCCACTGCCTACATGGAATTGCGGTCCCTTC GGTCGGACGATACTGCTGTGTACTACTGCGCAAGAGTCGCCGGAGGGATCTACTACTACTACGGCATGGACGTCTGGGGA CAGGGAACCACCATTACGGTGTCGAGCGGAGGGGGAGGCTCGGGGGGAGGAGGAAGCGGAGGTGGCGGCTCCGGGGGCGG CGGATCGGACATTGTGATGACCCAGACTCCTGACTCCCTGGCTGTTTCGTTGGGAGAGCGCGCGACTATCTCGTGTAAGT CCAGCCACTCAGTCCTGTACAATCGCAATAACAAGAACTACCTCGCGTGGTACCAGCAAAAACCGGGTCAGCCGCCTAAA CTCCTGTTCTACTGGGCCTCCACCAGAAAGAGCGGGGTGCCAGATCGATTCTCTGGATCAGGATCAGGTACCGACTTTAC GCTGACCATCTCGTCCCTGCAGCCGGAGGATTTCGCGACTTACTTCTGCCAGCAGACTCAGACTTTCCCCCTCACCTTCG GTCAAGGCACCAGGCTGGAAATCAATACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAG

		CCTCTGTCCCTGCGTCCGGAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGA TATCTACATTTGGGCCCCTCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCG GTCGGAAGAAGCTGCTGTACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCA TGCCGGTTCCCAGAGGAGGAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAA GCAGGGGCAGAACCAGCTCTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGAC GGGACCCAGAAATGGGCGGGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATG GCAGAAGCCTATAGCGAGATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAG CACCGCCACCAAGGACACCTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
97	**Mil (ScFv domain)** >NE10-19WD (Mil)	CAAGTCCAATTGCAGCAGAGCGGAGCAGAAGTGAAGAAGCCAGGAGCGTCAGTCAAAGTGTCGTGTAAGGCGTCAGGATA CACCTTCACGGGATACTAC ATGCACTGGGTGCGCCAGGCCCCGGGCCAAGGACTCGAGTGGATGGGCTGGATCAACCCTAACTCTGGAGGCACCAACTA CGCCCAGAATTTCCAAGGCAGAGTGACCATGACCCGGGACACCTCCATCTCGACTGCCTATATGGAACTGCGGCGGCTGC GCTCGGACGATACTGCTGTGTATTACTGCGCCAGCGGCTGGGACTTTGACTACTGGGGACAGGGTACTCTGGTGACTGTT TCCTCGGGAGGAGGCGGATCGGGTGGAGGAGGTAGCGGGGGAGGGGGGTCGGGAGGCGGAGGCAGCGATATTCGCATGAC TCAATCGCCGTCCTCCCTGAGCGCTAGCGTGGGAGATCGAGTCACCATCACTTGCAGAGCGTCACAGTCGATTCGCTACT ACCTGTCCTGGTACCAGCAGAAACCGGGAAAGGCACCAAAGCTTCTGATCTACACGGCCTCCATCCTGCAAAATGGTGTC CCATCAAGGTTCTCCGGGTCAGGGAGCGGCACTGACTTCACTCTCACCATCTCCTCACTCCAGCCCGAGGACTTTGCAAC CTACTACTGCCTCCAGACGTACACCACCCCGGATTTCGGTCCTGGAACCAAGGTGGAAATCAAA
121	**Mil (Full)** >NE10-19WD (Mil)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAATTGCAGCA GAGCGGAGCAGAAGTGAAGAAGCCAGGAGCGTCAGTCAAAGTGTCGTGTAAGGCGTCAGGATACACCTTCACGGGATACT AC ATGCACTGGGTGCGCCAGGCCCCGGGCCAAGGACTCGAGTGGATGGGCTGGATCAACCCTAACTCTGGAGGCACCAACTA CGCCCAGAATTTCCAAGGCAGAGTGACCATGACCCGGGACACCTCCATCTCGACTGCCTATATGGAACTGCGGCGGCTGC GCTCGGACGATACTGCTGTGTATTACTGCGCCAGCGGCTGGGACTTTGACTACTGGGGACAGGGTACTCTGGTGACTGTT TCCTCGGGAGGAGGCGGATCGGGTGGAGGAGGTAGCGGGGGAGGGGGGTCGGGAGGCGGAGGCAGCGATATTCGCATGAC TCAATCGCCGTCCTCCCTGAGCGCTAGCGTGGGAGATCGAGTCACCATCACTTGCAGAGCGTCACAGTCGATTCGCTACT ACCTGTCCTGGTACCAGCAGAAACCGGGAAAGGCACCAAAGCTTCTGATCTACACGGCCTCCATCCTGCAAAATGGTGTC CCATCAAGGTTCTCCGGGTCAGGGAGCGGCACTGACTTCACTCTCACCATCTCCTCACTCCAGCCCGAGGACTTTGCAAC CTACTACTGCCTCCAGACGTACACCACCCCGGATTTCGGTCCTGGAACCAAGGTGGAAATCAAAACCACTACCCCAGCAC CGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATGTAGACCCGCAGCTGGT GGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTGGTACTTGCGGGGTCCT GCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTTAAGCAACCCTTCATGA GGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGGCGGCTGCGAACTGCGC GTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACGAACTCAATCTTGGTCG GAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCGCGCAGAAAGAATCCCC AAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTATGAAAGGGGAACGCAGA AGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACGCTCTTCACATGCAGGC CCTGCCGCCTCGG
98	**M12 (ScFv domain)** >DE12-14RD (M12)	CAAGTCCAACTCGTCCAA AGCGGAGCAGAAGTCAAAAAGCCAGGAGCGTCGGTGAAAGTGTCTTGCAAAGCCAGCGGCTACACCTTCACGGGTTACTA CATGCACTGGGTGCGCCAGGCGCCGGGCCAGGGGCTGGAGTGGATGGGCCGGATTAACCCTAACAGCGGGGGAACTAATT ACGCTCAGAAGTTCCAGGGTAGAGTCACCATGACTACGGACACTTCCACTTCCACCGCCTATATGGAACTGCGCTCCCTC CGCTCAGATGATACTGCCGTGTATTACTGCGCGCGGACTACCACGTCATACGCATTTGACATCTGGGGCCAGGGAACTAT GGTGACCGTGAGCTCGGGCGGAGGCGGTTCAGGGGGAGGAGGAAGCGGAGGAGGAGGATCGGGAGGAGGTGGCTCCGATA TCCAGCTGACTCAGTCCCCGAGCACCCTGTCGGCGTCGGTGGGGGACAGGGTTACCATCACCTGTAGAGCTTCCCAATCC ATTTCGACTTGGCTGGCCTGGTACCAGCAAAAGCCGGGAAAGGCCCCTAATTTGCTTATCTACAAGGCATCGACCCTCGA AAGCGGTGTGCCCTCCCGGTTTTCGGGATCAGGATCAGGGACCGAGTTCACCCTGACCATCTCATCCCTCCAGCCGGACG ACTTCGCCACTTACTACTGCCAGCAGTACAACACCTACTCGCCATACACTTTCGGCCAAGGCACCAAGCTGGAGATCAAG
122	**M12 (Full)** >DE12-14RD (M12)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAACTCGTCCA A AGCGGAGCAGAAGTCAAAAAGCCAGGAGCGTCGGTGAAAGTGTCTTGCAAAGCCAGCGGCTACACCTTCACGGGTTACTA CATGCACTGGGTGCGCCAGGCGCCGGGCCAGGGGCTGGAGTGGATGGGCCGGATTAACCCTAACAGCGGGGGAACTAATT ACGCTCAGAAGTTCCAGGGTAGAGTCACCATGACTACGGACACTTCCACTTCCACCGCCTATATGGAACTGCGCTCCCTC CGCTCAGATGATACTGCCGTGTATTACTGCGCGCGGACTACCACGTCATACGCATTTGACATCTGGGGCCAGGGAACTAT GGTGACCGTGAGCTCGGGCGGAGGCGGTTCAGGGGGAGGAGGAAGCGGAGGAGGAGGATCGGGAGGAGGTGGCTCCGATA TCCAGCTGACTCAGTCCCCGAGCACCCTGTCGGCGTCGGTGGGGGACAGGGTTACCATCACCTGTAGAGCTTCCCAATCC ATTTCGACTTGGCTGGCCTGGTACCAGCAAAAGCCGGGAAAGGCCCCTAATTTGCTTATCTACAAGGCATCGACCCTCGA AAGCGGTGTGCCCTCCCGGTTTTCGGGATCAGGATCAGGGACCGAGTTCACCCTGACCATCTCATCCCTCCAGCCGGACG ACTTCGCCACTTACTACTGCCAGCAGTACAACACCTACTCGCCATACACTTTCGGCCAAGGCACCAAGCTGGAGATCAAG ACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATG TAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTG GTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTT AAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGG CGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACG AACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCG CGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTAT GAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACG CTCTTCACATGCAGGCCCTGCCGCCTCGG
99	**M13**	CAAGTTCAACTCGTGCAATCAGGTGGAGGACTCGTCAAACCCGGAGGATCATTGAGACTGTCATGCGAAGCGAGCGGTTT

	(ScFv domain) >TE13-19LD (M13)	TATCTTCTCCGATTACTAT ATGGGATGGATTCGGCAGGCCCCGGGAAAGGGACTCGAATGGGTGTCATACATCGGAAGGTCAGGCTCGTCCATGTACTA CGCAGACTCGGTGAAAGGCAGATTCACCTTTAGCCGGGACAACGCCAAGAATTCCCTCTACTTGCAGATGAACAGCCTGC GAGCCGAGGATACTGCTGTCTACTACTGTGCCGCGTCGCCGGTGGTGGCAGCTACTGAAGATTTCCAGCACTGGGGACAG GGAACTCTGGTCACGGTGTCGAGCGGTGGGGGCGGAAGCGGAGGCGGAGGATCGGGCGGCGGAGGTTCGGGGGGGGGAGG GTCTGACATCGTGATGACCCAAACCCCAGCCACCCTGAGCCTCTCCCCTGGAGAGCGCGCGACTCTTTCGTGCCGCGCTT CCCAGTCAGTGACCAGCAATTACTTGGCTTGGTACCAACAGAAGCCGGGACAGGCGCCACGGCTGCTGCTTTTTGGTGCC AGCACTCGCGCCACCGGAATCCCGGATCGCTTCTCGGGCTCAGGGTCCGGGACGGACTTCACCCTGACTATCAACCGGCT GGAACCTGAGGACTTCGCGATGTACTACTGCCAGCAGTACGGCTCCGCACCAGTCACTTTCGGACAAGGCACCAAGCTGG AGATCAAG
123	M13 (Full) >TE13-19LD (M13)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTTCAACTCGTGCA ATCAGGTGGAGGACTCGTCAAACCCGGAGGATCATTGAGACTGTCATGCGAAGCGAGCGGTTTTATCTTCTCCGATTACT AT ATGGGATGGATTCGGCAGGCCCCGGGAAAGGGACTCGAATGGGTGTCATACATCGGAAGGTCAGGCTCGTCCATGTACTA CGCAGACTCGGTGAAAGGCAGATTCACCTTTAGCCGGGACAACGCCAAGAATTCCCTCTACTTGCAGATGAACAGCCTGC GAGCCGAGGATACTGCTGTCTACTACTGTGCCGCGTCGCCGGTGGTGGCAGCTACTGAAGATTTCCAGCACTGGGGACAG GGAACTCTGGTCACGGTGTCGAGCGGTGGGGGCGGAAGCGGAGGCGGAGGATCGGGCGGCGGAGGTTCGGGGGGGGGAGG GTCTGACATCGTGATGACCCAAACCCCAGCCACCCTGAGCCTCTCCCCTGGAGAGCGCGCGACTCTTTCGTGCCGCGCTT CCCAGTCAGTGACCAGCAATTACTTGGCTTGGTACCAACAGAAGCCGGGACAGGCGCCACGGCTGCTGCTTTTTGGTGCC AGCACTCGCGCCACCGGAATCCCGGATCGCTTCTCGGGCTCAGGGTCCGGGACGGACTTCACCCTGACTATCAACCGGCT GGAACCTGAGGACTTCGCGATGTACTACTGCCAGCAGTACGGCTCCGCACCAGTCACTTTCGGACAAGGCACCAAGCTGG AGATCAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCG GAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCC TCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGT ACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAG GAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCT CTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCG GGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAG ATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACAC CTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
100	M14 (ScFv domain) >BS83-95ID (M14)	CAAGTCCAACTCGTCCAGTCGGGAGCAGAAGTTAGAGCACCAGGAGCGTCAGTGAAAATCTCATGCAAGGCCTCGGGCTT CACGTTCCGCGGATACTAC ATCCACTGGGTGCGCCAAGCCCCGGGTCAGGGATTGGAGTGGATGGGAATCATTAACCCATCAGGAGGGAGCCGGGCTTA CGCGCAGAAGTTCCAGGGACGCGTCACTATGACCCGAGATACTTCCACCTCGACTGTGTACATGGAACTCTCGTCCCTGA GGTCCGACGACACTGCGATGTATTACTGTGCTCGGACTGCCAGCTGCGGTGGGGACTGTTACTACCTCGATTACTGGGGC CAGGGAACTCTGGTGACCGTGTCCAGCGGAGGTGGCGGGTCAGGGGGTGGCGGAAGCGGAGGCGGCGGTTCAGGCGGAGG AGGCTCGGACATCCAAATGACGCAATCGCCGCCTACCCTGAGCGCTTCCGTGGGAGATCGGGTGACCATTACTTGCAGAG CATCCGAGAACGTCAATATCTGGCTGGCCTGGTACCAACAGAAGCCGGGGAAGGCCCCTAAACTGCTGATCTACAAGTCG AGCAGCCTTGCCTCTGGAGTGCCCTCCCGCTTCTCGGGCTCGGGATCAGGAGCGGAATTCACCCTCACCATCTCCTCCCT GCAGCCAGATGACTTTGCCACCTACTACTGCCAGCAGTACCAGAGCTATCCGTTGACCTTTGGGGGAGGCACTAAAGTGG ACATCAAG
124	M14 (Full) >BS83-95ID (M14)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAACTCGTCCA GTCGGGAGCAGAAGTTAGAGCACCAGGAGCGTCAGTGAAAATCTCATGCAAGGCCTCGGGCTTCACGTTCCGCGGATACT AC ATCCACTGGGTGCGCCAAGCCCCGGGTCAGGGATTGGAGTGGATGGGAATCATTAACCCATCAGGAGGGAGCCGGGCTTA CGCGCAGAAGTTCCAGGGACGCGTCACTATGACCCGAGATACTTCCACCTCGACTGTGTACATGGAACTCTCGTCCCTGA GGTCCGACGACACTGCGATGTATTACTGTGCTCGGACTGCCAGCTGCGGTGGGGACTGTTACTACCTCGATTACTGGGGC CAGGGAACTCTGGTGACCGTGTCCAGCGGAGGTGGCGGGTCAGGGGGTGGCGGAAGCGGAGGCGGCGGTTCAGGCGGAGG AGGCTCGGACATCCAAATGACGCAATCGCCGCCTACCCTGAGCGCTTCCGTGGGAGATCGGGTGACCATTACTTGCAGAG CATCCGAGAACGTCAATATCTGGCTGGCCTGGTACCAACAGAAGCCGGGGAAGGCCCCTAAACTGCTGATCTACAAGTCG AGCAGCCTTGCCTCTGGAGTGCCCTCCCGCTTCTCGGGCTCGGGATCAGGAGCGGAATTCACCCTCACCATCTCCTCCCT GCAGCCAGATGACTTTGCCACCTACTACTGCCAGCAGTACCAGAGCTATCCGTTGACCTTTGGGGGAGGCACTAAAGTGG ACATCAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCG GAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCC TCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGT ACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAG GAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCT CTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCG GGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAG ATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACAC CTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
101	M15 (ScFv domain) >HS86-94XD	CAAGTTCAACTCGTTCAA TCAGGTGGAGGACTCGTGCAACCAGGAAGATCACTCAGACTCAGCTGCGCCGCGTCGGGATTCACTTTCGATGACTACGC AATGCACTGGGTGCGGCAGGCCCCGGGCAAAGGACTGGAATGGGTGAGCGGAATTAGCTGGAACTCGGGGTCCATCGGGT ACGCCGACTCGGTGAAGGGACGCTTTACGATCTCCCGGGACAATGCCAAGAACTCCCTGTATTTGCAGATGAACTCCTTG AGGGCTGAGGACACCGCCGTGTACTACTGCGCTAAAGATGGATCATCGTCCTGGTCCTGGGGATACTTCGATTACTGGGG CCAGGGCACTCTGGTGACCGTGTCGTCAGGCGGTGGAGGGTCGGGCGGAGGAGGTAGCGGAGGCGGAGGGAGCAGCTCTG AACTGACCCAAGACCCGGCGGTGTCGGTCGCCCTTGGTCAGACTGTGCGGACTACCTGTCAGGGGGACGCGCTGCGCTCG

	(M15)	TACTACGCTTCATGGTACCAGCAGAAGCCCGGACAGGCACCTATGCTGGTCATCTACGGAAAGAATAACCGCCCATCCGG CATCCCGGATCGCTTCTCGGGTTCGGACAGCGGCGACACCGCATCCCTGACGATCACTGGAGCGCAGGCCGAGGATGAAG CCGACTACTACTGCAATTCCCGAGATTCAAGCGGCTACCCTGTGTTTGGGACCGGAACTAAGGTCACCGTCCTG
125	M15 (Full) >HS86- 94XD (M15)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTTCAACTCGTTCA A TCAGGTGGAGGACTCGTGCAACCAGGAAGATCACTCAGACTCAGCTGCGCCGCGTCGGGATTCACTTTCGATGACTACGC AATGCACTGGGTGCGGCAGGCCCCGGGCAAAGGACTGGAATGGGTGAGCGGAATTAGCTGGAACTCGGGGTCCATCGGGT ACGCCGACTCGGTGAAGGGACGCTTTACGATCTCCCGGGACAATGCCAAGAACTCCCTGTATTTGCAGATGAACTCCTTG AGGGCTGAGGACACCGCCGTGTACTACTGCGCTAAAGATGGATCATCGTCCTGGTCCTGGGGATACTTCGATTACTGGGG CCAGGGCACT CTGGT GACCGT GTCGT CAGGCGGTGGAGGGT CGGGCGGAGGAGGT AGCGGAGGCGGAGGGAGCAGCT CTG AACTGACCCAAGACCCGGCGGTGTCGGTCGCCCTTGGTCAGACTGTGCGGACTACCTGTCAGGGGGACGCGCTGCGCTCG TACTACGCTTCATGGTACCAGCAGAAGCCCGGACAGGCACCTATGCTGGTCATCTACGGAAAGAATAACCGCCCATCCGG CATCCCGGATCGCTTCTCGGGTTCGGACAGCGGCGACACCGCATCCCTGACGATCACTGGAGCGCAGGCCGAGGATGAAG CCGACTACTACTGCAATTCCCGAGATTCAAGCGGCTACCCTGTGTTTGGGACCGGAACTAAGGTCACCGTCCTGACCACT ACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATGTAGACC CGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTGGTACTT GCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTTAAGCAA CCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGGCGGCTG CGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACGAACTCA ATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCGCGCAGA AAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTATGAAAGG GGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACGCTCTTC ACATGCAGGCCCTGCCGCCTCGG
102	M16 (ScFv domain) >XS87- 99RD (M16)	GAAGTGCAACTCGTGGAA TCTGGTGGAGGACTTGTGCAACCTGGAAGATCGTTGAGACTCTCATGTGCTGCCTCCGGGTTCACCTTTGACGACTACGC CATGCACTGGGTGCGCCAGGCACCAGGAAAGGGTCTGGAGTGGGTTTCGGGTATCTCGTGGAACTCCGGGAGCACTGGCT ACGCTGATTCGGTGAAAGGCCGGTTTACCATCTCCCGAGACAATGCGAAGAATTCCCTCTATCTGCAGATGAACAGCCTC CGGGCCGAGGATACTGCCCTGTACTACTGCGCCAAGGATAGCTCATCATGGTACGGAGGTGGATCGGCTTTCGATATCTG GGGCCAGGGCACGATGGTCACCGTGTCCTCGGGGGGCGGAGGCTCCGGGGGAGGAGGTAGCGGAGGAGGAGGATCGAGCT CAGAGTTGACTCAAGAACCCGCAGTGTCCGTGGCACTGGGCCAAACCGTCAGGATCACTTGCCAGGGAGACAGCCTGAGG TCGTACTACGCGTCCTGGTACCAGCAGAAGCCGGGACAGGCCCCGGTCCTGGTCATTTTCGGACGCTCAAGACGCCCATC GGGCATCCCGGACCGGTTCAGCGGAAGCTCCTCGGGAAACACCGCGTCACTTATCATTACCGGCGCACAGGCTGAGGACG AAGCGGATTACTACTGCAACTCCCGCGACAATACTGCCAACCATTACGTGTTCGGGACCGGAACGAAACTGACTGTCCTG
126	M16 (Full) >XS87- 99RD (M16)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCGAAGTGCAACTCGTGGA A TCTGGTGGAGGACTTGTGCAACCTGGAAGATCGTTGAGACTCTCATGTGCTGCCTCCGGGTTCACCTTTGACGACTACGC CATGCACTGGGTGCGCCAGGCACCAGGAAAGGGTCTGGAGTGGGTTTCGGGTATCTCGTGGAACTCCGGGAGCACTGGCT ACGCTGATTCGGTGAAAGGCCGGTTTACCATCTCCCGAGACAATGCGAAGAATTCCCTCTATCTGCAGATGAACAGCCTC CGGGCCGAGGATACTGCCCTGTACTACTGCGCCAAGGATAGCTCATCATGGTACGGAGGTGGATCGGCTTTCGATATCTG GGGCCAGGGCACGATGGTCACCGTGTCCTCGGGGGGCGGAGGCTCCGGGGGAGGAGGTAGCGGAGGAGGAGGATCGAGCT CAGAGTTGACTCAAGAACCCGCAGTGTCCGTGGCACTGGGCCAAACCGTCAGGATCACTTGCCAGGGAGACAGCCTGAGG TCGTACTACGCGTCCTGGTACCAGCAGAAGCCGGGACAGGCCCCGGTCCTGGTCATTTTCGGACGCTCAAGACGCCCATC GGGCATCCCGGACCGGTTCAGCGGAAGCTCCTCGGGAAACACCGCGTCACTTATCATTACCGGCGCACAGGCTGAGGACG AAGCGGATTACTACTGCAACTCCCGCGACAATACTGCCAACCATTACGTGTTCGGGACCGGAACGAAACTGACTGTCCTG ACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATG TAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTG GTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTT AAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGG CGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACG AACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCG CGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTAT GAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACG CTCTTCACATGCAGGCCCTGCCGCCTCGG
103	M17 (ScFv domain) >NS89- 94MD (M17)	GAAGTTCAATTGGTGGAA TCTGGAGGAGGACTTGTGCAACCCGGTAGATCTCTGAGACTGTCCTGTGCGGCATCGGGATTCACCTTCGACGACTACGC TATGCACTGGGTGAGACAAGCCCCTGGAAAAGGACTGGAGTGGGTGTCAGGCATCTCCTGGAATAGCGGGTCCACTGGAT ACGCCGATTCGGTCAAGGGTCGCTTCACCATTTCCCGGGACAATGCCAAGAACTCCCTGTACCTTCAAATGAACTCCCTC CGGGCCGAGGATACCGCCCTCTACTACTGCGCCAAAGACAGCTCGTCATGGTATGGCGGAGGGTCGGCATTTGACATCTG GGGACAGGGAACTATGGTGACTGTGTCATCAGGAGGCGGCGGAAGCGGCGGCGGCGGGTCCGGCGGAGGAGGGTCGTCCA GCGAACTCACCCAAGATCCAGCAGTGAGCGTCGCGCTGGGCCAGACCGTCAGGATCACGTGCCAGGGAGATTCACTGCGC TCATACTACGCGTCCTGGTACCAGCAGAAGCCGGGGCAGGCCCCGGTCCTCGTGATCTACGGAAAGAACAACCGCCCGTC GGGTATCCCAGACCGCTTTTCGGGTAGCTCCAGCGGAAATACGGCTAGCCTGACCATCACTGGAGCACAGGCTGAGGATG AAGCGGACTACTACTGCAATTCGCGGGGCTCATCGGGGAACCATTACGTGTTCGGAACTGGTACCAAGGTGACTGTCCTG
127	M17	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCGAAGTTCAATTGGTGGA A

	(Full) >NS8 9-94MD (M17)	TCTGGAGGAGGACTTGTGCAACCCGGTAGATCTCTGAGACTGTCCTGTGCGGCATCGGGATTCACCTTCGACGACTACGC TATGCACTGGGTGAGACAAGCCCCTGGAAAAGGACTGGAGTGGGTGTCAGGCATCTCCTGGAATAGCGGGTCCACTGGAT ACGCCGATTCGGTCAAGGGTCGCTTCACCATTTCCCGGGACAATGCCAAGAACTCCCTGTACCTTCAAATGAACTCCCTC CGGGCCGAGGATACCGCCCTCTACTACTGCGCCAAAGACAGCTCGTCATGGTATGGCGGAGGGTCGGCATTTGACATCTG GGGACAGGGAACTATGGTGACTGTGTCATCAGGAGGCGGCGGAAGCGGCGGCGGCGGGTCCGGCGGAGGAGGGTCGTCCA GCGAACTCACCCAAGATCCAGCAGTGAGCGTCGCGCTGGGCCAGACCGTCAGGATCACGTGCCAGGGAGATTCACTGCGC TCATACTACGCGTCCTGGTACCAGCAGAAGCCGGGGCAGGCCCCGGTCCTCGTGATCTACGGAAAGAACAACCGCCCGTC GGGTATCCCAGACCGCTTTTCGGGTAGCTCCAGCGGAAATACGGCTAGCCTGACCATCACTGGAGCACAGGCTGAGGATG AAGCGGACTACTACTGCAATTCGCGGGGCTCATCGGGGAACCATTACGTGTTCGGAACTGGTACCAAGGTGACTGTCCTG ACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATG TAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTG GTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTT AAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGG CGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACG AACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCG CGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTAT GAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACG CTCTTCACATGCAGGCCCTGCCGCCTCGG
104	Ml8 (ScFv domain) >DS90-09HD (M18)	CAAGTGCAGCTCGTTCAATCAGGCGGAGGACTCGTTCAACCAGGAGGATCATTGCGACTCTCATGTGCGGCCTCTGGATT CACGTTTAGCTCATATTGG ATGCACTGGGTGCGGCAGGCGCCGGGGAAAGGTCTGGTGTGGGTCAGCCGCATCAACTCAGACGGCTCCTCGACTTCGTA CGCCGACTCCGTGAAGGGACGCTTTACCATTTCCCGCGACAACGCCAAGAATACCCTTTACCTTCAGATGAACTCCCTCC GCGCTGAGGATACCGCCGTGTACTACTGCGTGAGGACTGGCTGGGTCGGCAGCTACTACTACTACATGGACGTGTGGGGC AAAGGAACTACTGTCACCGTGTCAAGCGGCGGTGGAGGTTCCGGCGGGGGAGGATCGGGGGGGGGCGGATCGGGTGGCGG AGGATCGGAGATCGTGTTGACCCAGTCGCCGGGAACCCTGTCGCTGTCGCCTGGGGAGAGAGCAACTCTGTCCTGCCGGG CTTCCCAGTCGGTGTCGAGCAATTACCTGGCATGGTACCAACAGAAGCCGGGACAGCCGCCACGCCTGCTGATCTATGAC GTGTCAACTCGGGCAACTGGAATCCCTGCGCGGTTCAGCGGCGGAGGGAGCGGTACCGATTTCACCCTGACTATTTCCTC CCTCGAACCAGAAGATTTCGCCGTCTACTACTGCCAGCAGAGAAGCAACTGGCCGCCCTGGACGTTCGGACAAGGAACCA AGGT CGAAAT CAAG
128	Ml8 (Full) >DS90-09HD (M18)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTGCAGCTCGTTCA ATCAGGCGGAGGACTCGTTCAACCAGGAGGATCATTGCGACTCTCATGTGCGGCCTCTGGATTCACGTTTAGCTCATATT GG ATGCACTGGGTGCGGCAGGCGCCGGGGAAAGGTCTGGTGTGGGTCAGCCGCATCAACTCAGACGGCTCCTCGACTTCGTA CGCCGACTCCGTGAAGGGACGCTTTACCATTTCCCGCGACAACGCCAAGAATACCCTTTACCTTCAGATGAACTCCCTCC GCGCTGAGGATACCGCCGTGTACTACTGCGTGAGGACTGGCTGGGTCGGCAGCTACTACTACTACATGGACGTGTGGGGC AAAGGAACTACTGTCACCGTGTCAAGCGGCGGTGGAGGTTCCGGCGGGGGAGGATCGGGGGGGGGCGGATCGGGTGGCGG AGGATCGGAGATCGTGTTGACCCAGTCGCCGGGAACCCTGTCGCTGTCGCCTGGGGAGAGAGCAACTCTGTCCTGCCGGG CTTCCCAGTCGGTGTCGAGCAATTACCTGGCATGGTACCAACAGAAGCCGGGACAGCCGCCACGCCTGCTGATCTATGAC GTGTCAACTCGGGCAACTGGAATCCCTGCGCGGTTCAGCGGCGGAGGGAGCGGTACCGATTTCACCCTGACTATTTCCTC CCTCGAACCAGAAGATTTCGCCGTCTACTACTGCCAGCAGAGAAGCAACTGGCCGCCCTGGACGTTCGGACAAGGAACCA AGGT CGAAAT CAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTG CGTCCGGAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTG GGCCCCTCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGC TGCTGTACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCA GAGGAGGAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAA CCAGCTCTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAA TGGGCGGGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTAT AGCGAGATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAA GGACACCTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
105	M19 (ScFv domain) >TS92-04BD (M19)	CAAGTGCAATTGGTTCAA TCAGGAGGAGGAGTCGTGCAGCCCGGAAGATCGTTGAGACTGTCATGTGCCGCGAGCGGCTTTACTTTCTCAAGCTACGG AATGCATTGGGTGCGACAGGCTCCGGGAAAAGGACTGGAATGGGTCGCAGTGATCTCATACGACGGCTCGAACAAGTACT ACGCCGACTCCGTCAAGGGTCGGTTCACGATTTCGCGCGATAATTCCAAGAACACTCTGTACCTCCAAATGAACAGCCTC CGGGCAGAGGACACCGCCGTCTACTACTGCGCTAAGGGATACTCGCGCTACTACTACTATGGAATGGATGTGTGGGGCCA GGGAACTACCGTGACGGTGTCGTCCGGCGGCGGTGGGTCGGGCGGAGGCGGATCAGGTGGAGGTGGAAGCGGAGGAGGAG GGAGCGAAATCGTCATGACTCAGTCCCCTGCTACCCTTTCTCTGTCGCCGGGAGAAAGAGCCATCCTGAGCTGCCGGGCC TCCCAGAGCGTGTACACCAAATACCTGGGATGGTACCAGCAGAAGCCGGGGCAGGCACCAAGGCTCCTGATCTACGATGC GTCCACCCGCGCGACTGGTATCCCAGACCGCTTTTCCGGCTCGGGGTCAGGGACTGACTTCACCCTTACTATCAATCGGC TCGAGCCTGAGGATTTCGCCGTGTATTACTGCCAGCACTACGGAGGGTCCCCGCTGATTACCTTCGGCCAAGGCACCAAA GTGGAC A TCAAG
129	M19 (Full) >TS92-04BD (M19)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTGCAATTGGTTCA A TCAGGAGGAGGAGTCGTGCAGCCCGGAAGATCGTTGAGACTGTCATGTGCCGCGAGCGGCTTTACTTTCTCAAGCTACGG AATGCATTGGGTGCGACAGGCTCCGGGAAAAGGACTGGAATGGGTCGCAGTGATCTCATACGACGGCTCGAACAAGTACT ACGCCGACTCCGTCAAGGGTCGGTTCACGATTTCGCGCGATAATTCCAAGAACACTCTGTACCTCCAAATGAACAGCCTC CGGGCAGAGGACACCGCCGTCTACTACTGCGCTAAGGGATACTCGCGCTACTACTACTATGGAATGGATGTGTGGGGCCA GGGAACTACCGTGACGGTGTCGTCCGGCGGCGGTGGGTCGGGCGGAGGCGGATCAGGTGGAGGTGGAAGCGGAGGAGGAG

		GGAGCGAAATCGTCATGACTCAGTCCCCTGCTACCCTTTCTCTGTCGCCGGGAGAAAGAGCCATCCTGAGCTGCCGGGCC TCCCAGAGCGTGTACACCAAATACCTGGGATGGTACCAGCAGAAGCCGGGGCAGGCACCAAGGCTCCTGATCTACGATGC GTCCACCCGCGCGACTGGTATCCCAGACCGCTTTTCCGGCTCGGGGTCAGGGACTGACTTCACCCTTACTATCAATCGGC TCGAGCCTGAGGATTTCGCCGTGTATTACTGCCAGCACTACGGAGGGTCCCCGCTGATTACCTTCGGCCAAGGCACCAAA GTGGACATCAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCG TCCGGAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGG CCCCTCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTG CTGTACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGA GGAGGAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACC AGCTCTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATG GGCGGGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAG CGAGATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGG ACACCTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
106	M20 (ScFv domain) >JS93- 08WD (M20)	CAAGTGCAACTTGTTCAATCAGGAGGAGGACTCGTTCAACCCGGAGGATCACTGCGACTCTCATGTGCAGCGTCGGGGTT CACCTTCTCCAGCTACGCA ATGTCCTGGGTGCGCCAAGCCCCTGGAAAAGGCCTGGAGTGGGTGTCGGCCATCTCTGGGAGCGGGGGATCAACTTACTA CGCTGACTCCGTCAAGGGCCGCTTTACCATCTCCCGGGACAACAGCAAGAACACTCTCTATCTCCAGATGAACTCGCTGA GAGCCGAAGATACCGCTGTCTACTACTGCGCGAAGAGAGAAGCTGCCGCAGGGCACGATTGGTACTTCGACTTGTGGGGC AGGGGCACCCTTGTGACCGTGTCCTCCGGTGGAGGCGGATCAGGAGGTGGGGGATCGGGTGGAGGAGGAAGCGGAGGCGG CGGTTCGGACATTCGCGTCACCCAGTCACCGAGCTCCCTCAGCGCATCGGTGGGCGACCGGGTCACTATCACTTGCCGGG CGTCCCAGTCGATCTCATCGTATCTGAATTGGTACCAGCAGAAACCGGGGAAAGGCGCCGAAGCTGTTGATCTACGCTGCC AGCTCCCTGCAGTCGGGTGTGCCATCACGCTTTTCCGGCTCGGGATCGGGAACCGATTTCACTCTGACGATCTCTAGCCT GCAGCCAGAAGATTTCGCCACTTACTACTGCCAGCAGTCCTACAGCATCCCTCTGACTTTCGGACAAGGGACGAAAGTGG AGATTAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCG GAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCC TCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGT ACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAG GAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCT CTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCG GGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAG ATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACAC CTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
130	M20 (Full) >JS93- 08WD (M20)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTGCAACTTGTTCA ATCAGGAGGAGGACTCGTTCAACCCGGAGGATCACTGCGACTCTCATGTGCAGCGTCGGGGTTCACCTTCTCCAGCTACG CA ATGTCCTGGGTGCGCCAAGCCCCTGGAAAAGGCCTGGAGTGGGTGTCGGCCATCTCTGGGAGCGGGGGATCAACTTACTA CGCTGACTCCGTCAAGGGCCGCTTTACCATCTCCCGGGACAACAGCAAGAACACTCTCTATCTCCAGATGAACTCGCTGA GAGCCGAAGATACCGCTGTCTACTACTGCGCGAAGAGAGAAGCTGCCGCAGGGCACGATTGGTACTTCGACTTGTGGGGC AGGGGCACCCTTGTGACCGTGTCCTCCGGTGGAGGCGGATCAGGAGGTGGGGGATCGGGTGGAGGAGGAAGCGGAGGCGG CGGTTCGGACATTCGCGTCACCCAGTCACCGAGCTCCCTCAGCGCATCGGTGGGCGACCGGGTCACTATCACTTGCCGGG CGTCCCAGTCGATCTCATCGTATCTGAATTGGTACCAGCAGAAACCGGGGAAAGGCGCCGAAGCTGTTGATCTACGCTGCC AGCTCCCTGCAGTCGGGTGTGCCATCACGCTTTTCCGGCTCGGGATCGGGAACCGATTTCACTCTGACGATCTCTAGCCT GCAGCCAGAAGATTTCGCCACTTACTACTGCCAGCAGTCCTACAGCATCCCTCTGACTTTCGGACAAGGGACGAAAGTGG AGATTAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCG GAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCC TCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGT ACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAG GAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCT CTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCG GGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAG ATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACAC CTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
107	M21 (ScFv domain) >ZS95- 03QD (M21)	CAAGTCCAACTCGTTCAGTCATGGGCAGAAGTCAAGAAACCCGGTGCAAGCGTCAAAGTGTCGTGTAAGGCCTCCGGCTA CACTTTCACTTCCTACTAC ATGCACTGGGTGCGCCAAGCCCCGGGACAGGGCCTTGAATGGATGGGCATCATCAACCCATCAGGAGGTTCCACGAGCTA CGCGCAGAAGTTCCAGGGGAGAGTGACGATGACTAGAGATACCTCCACGAGCACCGTCTACATGGAGCTGTCGAATCTGC GGTCAGAGGACACTGCTGTGTATTACTGCGCGCGCTCCCCGCGGGTGACCACTGGCTACTTTGACTACTGGGGACAAGGG ACCCTGGTGACCGTCAGCTCGGGAGGCGGAGGATCGGGAGGTGGAGGGTCCGGTGGAGGCGGCTCTGGAGGAGGCGGGTC GGACATTCAATTGACCCAGAGCCCATCCACCCTCTCAGCCTCGGTGGGGGATAGGGTGACTATCACTTGCCGGGCCTCCC AGTCAATTTCCAGCTGGCTGGCTTGGTACCAGCAAAAGCCTGGAAAGGCACCGAAGCTCCTGATCTACAAGGCCTCATCT CTGGAATCAGGAGTGCCTTCGCGCTTCAGCGGAAGCGGCTCGGGAACTGAGTTTACCCTGACCATCTCGAGCCTGCAGCC AGATGACTTCGCGACCTATTACTGCCAGCAGTACTCGTCCTACCCGTTGACTTTCGGAGGAGGTACCCGCCTCGAAATCA AA
131	M21 (Full) >ZS95- 03QD (M21)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAACTCGTTCA GTCATGGGCAGAAGTCAAGAAACCCGGTGCAAGCGTCAAAGTGTCGTGTAAGGCCTCCGGCTACACTTTCACTTCCTACT AC ATGCACTGGGTGCGCCAAGCCCCGGGACAGGGCCTTGAATGGATGGGCATCATCAACCCATCAGGAGGTTCCACGAGCTA CGCGCAGAAGTTCCAGGGGAGAGTGACGATGACTAGAGATACCTCCACGAGCACCGTCTACATGGAGCTGTCGAATCTGC GGTCAGAGGACACTGCTGTGTATTACTGCGCGCGCTCCCCGCGGGTGACCACTGGCTACTTTGACTACTGGGGACAAGGG ACCCTGGTGACCGTCAGCTCGGGAGGCGGAGGATCGGGAGGTGGAGGGTCCGGTGGAGGCGGCTCTGGAGGAGGCGGGTC GGACATTCAATTGACCCAGAGCCCATCCACCCTCTCAGCCTCGGTGGGGGATAGGGTGACTATCACTTGCCGGGCCTCCC AGTCAATTTCCAGCTGGCTGGCTTGGTACCAGCAAAAGCCTGGAAAGGCACCGAAGCTCCTGATCTACAAGGCCTCATCT CTGGAATCAGGAGTGCCTTCGCGCTTCAGCGGAAGCGGCTCGGGAACTGAGTTTACCCTGACCATCTCGAGCCTGCAGCC AGATGACTTCGCGACCTATTACTGCCAGCAGTACTCGTCCTACCCGTTGACTTTCGGAGGAGGTACCCGCCTCGAAATCA AAACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCA TGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGC TGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCT

		TTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAA GGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAA CGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGC CGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGT ATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGA CGCTCTTCACATGCAGGCCCTGCCGCCTCGG
108	M22 (ScFv domain) >PS96- 08LD (M22)	CAAGTCCAACTCGTCCAGTCCGGTGCAGAAGTCAGAAGGCCAGGAGCAAGCGTGAAGATCTCGTGTAGAGCGTCAGGAGA CACCAGCACTCGCCATTAC ATCCACTGGCTGCGCCAGGCTCCGGGCCAAGGGCCGGAGTGGATGGGTGTGATCAACCCGACTACGGGACCGGCTACCGG AAGCCCTGCGTACGCACAGATGCTGCAGGGACGGGTGACTATGACCCGCGATACTAGCACTAGGACCGTGTACATGGAAC TCCGCTCGTTGCGGTTCGAAGATACCGCCGTCTACTACTGCGCCCGGTCCGTGGTGGGCCGAAGCGCCCCTTACTACTTC GATTACTGGGGACAGGGCACTCTGGTGACCGTTAGCTCCGGTGGGGGAGGCTCGGGTGGAGGCGGATCGGGAGGAGGAGG CAGCGGTGGAGGGGGATCGGACATTCAGATGACCCAGTCACCCTCCTCCCTCTCAGCCTCGGTCGGGGACCGGGTGACCA TTACGTGCAGAGCCTCACAAGGGATCTCGGACTACTCCGCCTGGTACCAGCAGAAACCGGGAAAAGCGCCAAAGCTCCTG ATCTACGCCGCGAGCACCCTGCAATCAGGAGTGCCATCGCGCTTTTCTGGATCGGGCTCAGGGACTGACTTCACGCTGAC TATCTCCTACCTTCAGTCCGAGGATTTCGCTACCTACTACTGCCAACAGTATTACTCCTATCCCCTGACCTTTGGCGGAG GCACTAAGGTGGACATCAAG
132	M22 (Full) >PS96- 08LD (M22)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAACTCGTCCA GTCCGGTGCAGAAGTCAGAAGGCCAGGAGCAAGCGTGAAGATCTCGTGTAGAGCGTCAGGAGACACCAGCACTCGCCATT AC ATCCACTGGCTGCGCCAGGCTCCGGGCCAAGGGCCGGAGTGGATGGGTGTGATCAACCCGACTACGGGACCGGCTACCGG AAGCCCTGCGTACGCACAGATGCTGCAGGGACGGGTGACTATGACCCGCGATACTAGCACTAGGACCGTGTACATGGAAC TCCGCTCGTTGCGGTTCGAAGATACCGCCGTCTACTACTGCGCCCGGTCCGTGGTGGGCCGAAGCGCCCCTTACTACTTC GATTACTGGGGACAGGGCACTCTGGTGACCGTTAGCTCCGGTGGGGGAGGCTCGGGTGGAGGCGGATCGGGAGGAGGAGG CAGCGGTGGAGGGGGATCGGACATTCAGATGACCCAGTCACCCTCCTCCCTCTCAGCCTCGGTCGGGGACCGGGTGACCA TTACGTGCAGAGCCTCACAAGGGATCTCGGACTACTCCGCCTGGTACCAGCAGAAACCGGGAAAAGCGCCAAAGCTCCTG ATCTACGCCGCGAGCACCCTGCAATCAGGAGTGCCATCGCGCTTTTCTGGATCGGGCTCAGGGACTGACTTCACGCTGAC TATCTCCTACCTTCAGTCCGAGGATTTCGCTACCTACTACTGCCAACAGTATTACTCCTATCCCCTGACCTTTGGCGGAG GCACTAAGGTGGACATCAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTG TCCCTGCGTCCGGAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTA CATTTGGGCCCCTCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGA AGAAGCTGCTGTACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGG TTCCCAGAGGAGGAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGG GCAGAACCAGCTCTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACC CAGAAATGGGCGGGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAA GCCTATAGCGAGATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGC CACCAAGGACACCTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG
109	M23 (ScFv domain) >XH66- 84HE (M23)	CAAGTCCAACTCCAGCAATCGGGAGCAGAAGTCAAGAAACCAGGCGCATCGGTGAAAGTGTCGTGTAAGGCGTCAGGGTA CACCTTCACCAACTACTAT ATGCACTGGGTGCGCCAGGCTCCAGGCCAGGGGTTGGAGTGGATGGGGATCATCAATCCGTCAGGTGGCTACACCACTTA CGCTCAGAAGTTCCAGGGACGCCTCACTATGACTCGCGATACTAGCACCTCCACGGTGTACATGGAACTGTCATCGCTGA GGTCCGAAGATACCGCCGTCTACTACTGCGCACGGATCAGATCCTGCGGAGGAGATTGTTACTACTTTGACAACTGGGGA CAGGGCACCCTTGTTACTGTGTCATCGGGAGGAGGGGGAAGCGGAGGAGGTGGATCAGGCGGCGGTGGCAGCGGGGGCGG AGGATCGGACATTCAGCTGACTCAGTCCCCCTCCACTTTGTCGGCCAGCGTGGGAGACAGAGTGACCATCACTTGCCGGG CGTCCGAGAACGTCAATATCTGGCTGGCCTGGTACCAGCAAAAGCCTGGAAAAGCCCCGAAGCTGCTCATCTATAAGTCA TCCAGCCTGGCGTCTGGTGTGCCGTCGCGGTTCTCCGGCAGCGGGAGCGGAGCCGAGTTCACTCTCACCATTTCGAGCCT TCAACCGGACGATTTCGCCACCTACTACTGCCAGCAGTACCAATCCTACCCTCTGACGTTTGGAGGTGGAACCAAGGTGG ACATCAAG
133	M23 (Full) >XH66- 84HE (M23)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAGTCCAACTCCAGCA ATCGGGAGCAGAAGTCAAGAAACCAGGCGCATCGGTGAAAGTGTCGTGTAAGGCGTCAGGGTACACCTTCACCAACTACT AT ATGCACTGGGTGCGCCAGGCTCCAGGCCAGGGGTTGGAGTGGATGGGGATCATCAATCCGTCAGGTGGCTACACCACTTA CGCTCAGAAGTTCCAGGGACGCCTCACTATGACTCGCGATACTAGCACCTCCACGGTGTACATGGAACTGTCATCGCTGA GGTCCGAAGATACCGCCGTCTACTACTGCGCACGGATCAGATCCTGCGGAGGAGATTGTTACTACTTTGACAACTGGGGA CAGGGCACCCTTGTTACTGTGTCATCGGGAGGAGGGGGAAGCGGAGGAGGTGGATCAGGCGGCGGTGGCAGCGGGGGCGG AGGATCGGACATTCAGCTGACTCAGTCCCCCTCCACTTTGTCGGCCAGCGTGGGAGACAGAGTGACCATCACTTGCCGGG CGTCCGAGAACGTCAATATCTGGCTGGCCTGGTACCAGCAAAAGCCTGGAAAAGCCCCGAAGCTGCTCATCTATAAGTCA TCCAGCCTGGCGTCTGGTGTGCCGTCGCGGTTCTCCGGCAGCGGGAGCGGAGCCGAGTTCACTCTCACCATTTCGAGCCT TCAACCGGACGATTTCGCCACCTACTACTGCCAGCAGTACCAATCCTACCCTCTGACGTTTGGAGGTGGAACCAAGGTGG ACATCAAGACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCG GAGGCATGTAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCC TCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGT ACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAG GAGGAAGGCGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCT CTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCG GGAAGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAG ATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACAC CTATGACGCTCTTCACATGCAGGCCCTGCCGCCTCGG

110	M24 (ScFv domain) >NH67-89CE (M24)	CAAAT CACTCTGAAAGAA TCTGGACCGGCCCTGGTTAAGCCGACTCAAACGCTCACCCTTACTTGCACCTTCAGCGGATTCTCACTCAGCACTGCTGG TGTGCACGTCGGATGGATTAGACAGCCGCCTGGAAAGGCCCTGGAATGGCTCGCCCTCATCTCCTGGGCCGATGACAAGA GATACAGGCCCTCGCTTCGATCCCGGTTGGACATTACCCGGGTGACCTCGAAAGATCAGGTGGTGCTCTCAATGACCAAT ATGCAGCCGGAGGACACCGCTACGTACTACTGCGCACTGCAAGGATTTGACGGCTACGAGGCTAACTGGGGACCAGGTAC TCTGGTCACCGTGAGCTCCGGCGGGGGAGGATCAGGCGGGGGGGGGTCAGGAGGCGGAGGCTCCGGTGGAGGAGGATCGG ATATCGTCATGACCCAGTCCCCAAGCTCGCTGAGCGCGTCAGCGGGCGACCGCGTGACTATCACTTGCCGGGCCAGCCGC GGCATCTCCTCCGCACTGGCGTGGTACCAGCAGAAGCCTGGAAAACCGCCAAAGCTCCTGATCTATGATGCCTCCAGCCT GGAGTCAGGTGTCCCCAGCCGCTTCTCGGGTTCGGGCTCGGGAACCGACTTCACTTTGACCATCGACTCGCTGGAACCGG AAGATTTCGCAACCTACTACTGTCAGCAGTCCTACTCGACCCCTTGGACTTTTGGACAAGGGACGAAGGTGGACATCAAG
134	M24 (Full) >NH67-89CE (M24)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCGGCCCCAAATCACTCTGAAAGA A TCTGGACCGGCCCTGGTTAAGCCGACTCAAACGCTCACCCTTACTTGCACCTTCAGCGGATTCTCACTCAGCACTGCTGG TGTGCACGTCGGATGGATTAGACAGCCGCCTGGAAAGGCCCTGGAATGGCTCGCCCTCATCTCCTGGGCCGATGACAAGA GATACAGGCCCTCGCTTCGATCCCGGTTGGACATTACCCGGGTGACCTCGAAAGATCAGGTGGTGCTCTCAATGACCAAT ATGCAGCCGGAGGACACCGCTACGTACTACTGCGCACTGCAAGGATTTGACGGCTACGAGGCTAACTGGGGACCAGGTAC TCTGGTCACCGTGAGCTCCGGCGGGGGAGGATCAGGCGGGGGGGGGTCAGGAGGCGGAGGCTCCGGTGGAGGAGGATCGG ATATCGTCATGACCCAGTCCCCAAGCTCGCTGAGCGCGTCAGCGGGCGACCGCGTGACTATCACTTGCCGGGCCAGCCGC GGCATCTCCTCCGCACTGGCGTGGTACCAGCAGAAGCCTGGAAAACCGCCAAAGCTCCTGATCTATGATGCCTCCAGCCT GGAGTCAGGTGTCCCCAGCCGCTTCTCGGGTTCGGGCTCGGGAACCGACTTCACTTTGACCATCGACTCGCTGGAACCGG AAGATTTCGCAACCTACTACTGTCAGCAGTCCTACTCGACCCCTTGGACTTTTGGACAAGGGACGAAGGTGGACATCAAG ACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATG TAGACCCGCAGCTGGTGGGGCCGTGCATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTG GTACTTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGAAGCTGCTGTACATCTTT AAGCAACCCTTCATGAGGCCTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGG CGGCTGCGAACTGCGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAGAACCAGCTCTACAACG AACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGCTGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCG CGCAGAAAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGATTGGTAT GAAAGGGGAACGCAGAAGAGGCAAAGGCCACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACG CTCTTCACATGCAGGCCCTGCCGCCTCGG
279	SsI (scFv domain)	CAAGTCCAGCTCCAGCAGTCGGGCCCAGAGTTGGAGAAGCCTGGGGCGAGCGTGAAGAT CTCATGCAAAGCCTCAGGCTACTCCTTTACTGGATACACGATGAATTGGGTGAAACAGT CGCATGGAAAGTCACTGGAATGGATCGGTCTGATTACGCCCTACAACGGCGCCTCCAGC TACAACCAGAAGTTCAGGGGAAAGGCGACCCTTACTGTCGACAAGTCGTCAAGCACCGC CTACATGGACCTCCTGTCCCTGACCTCCGAAGATAGCGCGGTCTAC ĪĪĪĪGTGCACGCG GAGGTTACGATGGACGGGGATTCGACTACTGGGGCCAGGGAACCACTGTCACCGTGTCG AGCGGAGGCGGAGGGAGCGGAGGAGGAGGCAGCGGAGGTGGAGGGTCGGATATCGAACT CACTCAGTCCCCAGCAATCATGTCCGCTTCACCGGGAGAAAAGGTGACCATGACTTGCT CGGCCTCCTCGTCCGTGTCATACATGCACTGGTACCAACAAAAATCGGGGACCTCCCCT AAGAGATGGATCTACGATACCAGCAAACTGGCTTCAGGCGTGCCGGGACGCTTCTCGGG TTCGGGGAGCGGAAATTCGTATTCGTTGACCATTTCGTCCGTGGAAGCCGAGGACGACG CAACTTATTACTGCCAACAGTGGTCAGGCTACCCGCTCACTTTCGGAGCCGGCACTAAG CTGGAGATC
280	SsI (full)	ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGCCGCTCG GCCCCAAGTCCAGCTCCAGCAGTCGGGCCCAGAGTTGGAGAAGCCTGGGGCGAGCGTGA AGAT CTCATGCAAAGCC TCAGGC TACTCCTTTACT GGATACACGAT GAAT TGGGTGAAA CAGTCGCATGGAAAGTCACTGGAATGGATCGGTCTGAT TACGCCCTACAACGGCGCCTC CAGCTACAACCAGAAGTTCAGGGGAAAGGCGACCCTTACTGTCGACAAGTCGTCAAGCA CCGCCTACATGGACCTCCTGTCCCTGACCTCCGAAGATAGCGCGGTCTACTTTTGTGCA CGCGGAGGTTACGATGGACGGGGATTCGACTACTGGGGCCAGGGAACCACTGTCACCGT GTCGAGCGGAGGCGGAGGGAGCGGAGGAGGAGGCAGCGGAGGTGGAGGGTCGGATATCG AAC TCACTCAGTCCCCAGCAAT CATGTCCGCTTCACCGGGAGAAAAGG TGAC CATGAC T TGCTCGGCCTCCTCGTCCGTGTCATACATGCACTGGTACCAACAAAAATCGGGGACCTC CCCTAAGAGAT GGAT CTACGAT ACCAGCAAAC TGGCTT CAGGC GTGCCGGGAC GC TTCT CGGGTTCGGGGAGCGGAAATTCGTATTCGTTGACCATTTCGTCCGTGGAAGCCGAGGAC GACGCAACTTAT TACTGCCAACAGTGGTCAGGCTACCCGCTCACTTTCGGAGCCGGCAC TAAGC TGGAGAT CACCACTACCCCAGC ACCGAGGC CACCCACCCCGGCTCCTACCATCG CCTCCCAGCCTCTGTCCCTGCGTCCGGAGGCATGTAGACCCGCAGCTGGTGGGGCCGTG CATACCCGGGGTCTTGACTTCGCCTGCGATATCTACATTTGGGCCCCTCTGGCTGGTAC TTGCGGGGTCCTGCTGCTTTCACTCGTGATCACTCTTTACTGTAAGCGCGGTCGGAAGA

		AGCTGCTGTACATCTTTAAGCAACCCTTCATGAGGCCTGTGCAGAC TACTCAAGAGGAG GACGGCTGTTCATGCCGGTTCCCAGAGGAGGAGGAAGGCGGCTGCGAACTGCGCGTGAA ATTCAGCCGCAGCGCAGATGCTCCAGCC

Table 4. Amino acid sequences for the heavy chain (HC) CDRl , CDR2, and CDR3 regions of human anti-mesothelin scFvs

Descrip.	HC-CDR1	SEQ ID NO:	HC-CDR2	SEQ ID NO:	HC-CDR3	SEQ ID NO:
M1	GYTFTGYYMH	136	RINPNSGGTNYAQKFQG	155	GRYYGMDV	175
M2	GYTFTGYYMH	136	WINPNSGGTNYAQKFQG	156	DLRRTVVTPRAYYG MDV	176
M3	GYTFTGYYMH	136	WINPNSGGTNYAQKFQG	156	GEWDGSYYYDY	177
M4	GFTFSSYWMH	137	RINTDGSTTTYADSVEG	157	GHWAV	178
M5	GYTFTDYYMH	138	WINPNSGGTNYAQKFQG	156	GWDFDY	179
M6	GYTFTSYYMH	139	IINPSGGSTSYAQKFQ	158	YRLIAVAGDYYYYG MDV	180
M7	GFTFSSYAMH	140	VISYDGSNKYYADSVKG	274	WKVSSSSPAFDY	181
M8	GYPFTGYSLH	141	WINPNSGGTNYAQKFQG	159	DHYGGNSLFY	182
M9	GYTFTSYYMH	142	IINPSGGSTGYAQKFQG	160	GGYSSSSDAFDI	183
M10	GYTFTSYGIS	143	WISAYNGNTNYAQKLQ	161	VAGGIYYYYGMDV	184
M11	GYTFTGYYMH	144	WINPNSGGTNYAQNFQG	162	GWDFDY	185
M12	GYTFTGYYMH	144	RINPNSGGTNYAQKFQG	163	TTTSYAFDI	186
M13	GFIFSDYYMG	145	YIGRSGSSMYYADSVKG	164	SPVVAATEDFQH	187
M14	GFTFRGYYIH	146	IINPSGGSRAYAQKFQG	165	TASCGGDCYYLDY	188
M15	GFTFDDYAMH	147	GISWNSGSIGYADSVK	166	DGSSSWSWGYFDY	189
M16	GFTFDDYAMH	147	GISWNSGSTGYADSVKG	167	DSSSWYGGGSAFDI	190
M17	GFTFDDYAMH	147	GISWNSGSTGYADSVKG	167	DSSSWYGGGSAFDI	191
M18	GFTFSSYWMH	148	RINSDGSSTSYADSVKG	168	TGWVGSYYYYMDV	192
M19	GFTFSSYGMH	149	VISYDGSNKYYADSVKG	169	GYSRYYYYGMDV	193
M20	GFTFSSYAMS	150	AISGSGGSTYYADSVKG	170	REAAAGHDWYFDL	194
M21	GYTFTSYYMH	151	IINPSGGSTSYAQKFQG	171	SPRVTTGYFDY	195
M22	GDTSTRHYIH	152	VINPTTGPATGSPAYAQMLQ G	172	SVVGRSAPYYFDY	196
M23	GYTFTNYYMH	153	IINPSGGYTTYAQKFQG	173	IRSCGGDCYYFDN	197
M24	GFSLSTAGVHVG	154	LISWADDKRYRPSLRS	174	QGFDGYEAN	198
Ss1	GYSFTGYTMN	281	LITPYNGASSYNQKFRG	282	GGYDGRGFDY	283

Table 5. Amino acid sequences for the light chain (LC) CDRl, CDR2, and CDR3 regions of human anti-mesothelin scFvs

Description	LC-CDR1	SEQ ID NO:	LC-CDR2	SEQ ID NO:	LC-CDR3	SEQ ID NO:
Ml	RASQSVSSNFA	199	DASNRAT	223	HQRSNWLYT	247
M2	QASQDISNSLN	200	DASTLET	224	QQHDNLPLT	248
M3	RASQSINTYLN	201	AASSLQS	225	QQSFSPLT	249
M4	RASQSISDRLA	202	KASSLES	226	QQYGHLPMYT	250
M5	RASQSIRYYLS	203	TASILQN	227	LQTYTTPD	251
M6	RASQGVGRWLA	204	AASTLQS	228	QQANSFPLT	252
M7	RASQSVYTKYLG	205	DASTRAT	229	QHYGGSPLIT	253
M8	RASQDSGTWLA	206	DASTLED	230	QQYNSYPLT	254
M9	RASQDISSALA	207	DASSLES	231	QQFSSYPLT	255
M10	KSSHSVLYNRNNKNYLA	208	WASTRKS	232	QQTQTFPLT	256
Mil	RASQSIRYYLS	209	TASILQN	233	LQTYTTPD	257
M12	RASQSISTWLA	210	KASTLES	234	QQYNTYSPYT	258
M13	RASQSVTSNYLA	211	GASTRAT	235	QQYGSAPVT	259
M14	RASENVNIWLA	212	KSSSLAS	236	QQYQSYPLT	260
M15	QGDALRSYYAS	213	GKNNRPS	237	NSRDSSGYPV	261
M16	QGDSLRSYYAS	214	GRSRRPS	238	NSRDNTANHYV	262
M17	QGDSLRSYYAS	215	GKNNRPS	239	NSRGSSGNHYV	263
M18	RASQSVSSNYLA	216	DVSTRAT	240	QQRSNWPPWT	264
M19	RASQSVYTKYLG	217	DASTRAT	241	QHYGGSPLIT	265
M20	RASQSISSYLN	218	AASSLQS	242	QQSYSIPLT	266
M21	RASQSISSWLA	219	KASSLES	243	QQYSSYPLT	267
M22	RASQGISDYS	220	AASTLQS	244	QQYYSYPLT	268
M23	RASENVNIWLA	221	KSSSLAS	245	QQYQSYPLT	269
M24	RASRGISSALA	222	DASSLES	246	QQSYSTPWT	270
Ssl	SASSSVSYMH	284	DTSKLAS	285	QQWSGYPLT	286

EXAMPLES

[00585] The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1: Generating CAR constructs

[00586] ScFv to be used in the final CAR construct were derived from the pannings of human scFV libraries.

The amino acid sequences of the human scFv fragments are provided above in Table 2 and the nucleic acid sequences of the human scFv fragments are provided above in Table 3. Full CAR constructs were generated using the scFv fragments of Table 2 with additional sequences, SEQ ID NOs: 1-2, 6-7, 9-10, 12-13, 17-18, 20-21, and 36-37, shown below, to generate full CAR constructs.

- leader (amino acid sequence) (SEQ ID NO: 1)

  MALPVTALLLPLALLLHAARP

- leader (nucleic acid sequence) (SEQ ID NO: 12)

  ATGGCCCTCCCTGTCACCGCCCTGCTGCTTCCGCTGGCTCTTCTGCTCCACGC CGCTCGGCCC

- CD8 hinge (amino acid sequence) (SEQ ID NO: 2)

  TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD

- CD8 hinge (nucleic acid sequence) (SEQ ID NO: 13)

- ACCACTACCCCAGCACCGAGGCCACCCACCCCGGCTCCTACCATCGCCTCCC AGCCTCTGTCCCTGCGTCCGGAGGCATGTAGACCCGCAGCTGGTGGGGCCGT GCATACCCGGGGTCTTGACTTCGCCTGCGATCD8 transmembrane (amino acid sequence) (SEQ ID NO: 6)

  IYIWAPLAGTCGVLLLSLVITLYC

- CD8 transmembrane (nucleic acid sequence) (SEQ ID NO: 17)

- ATCTACATTTGGGCCCCTCTGGCTGGTACTTGCGGGGTCCTGCTGCTTTCACT CGTGATCACTCTTTACTGT4-1BB Intracellular domain (amino acid sequence) (SEQ ID NO: 7)

  KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

- 4-IBB Intracellular domain (nucleic acid sequence) (SEQ ID NO: 18)

- AAGCGCGGTCGGAAGAAGCTGCTGTACATCTTTAAGCAACCCTTCATGAGGC
  CTGTGCAGACTACTCAAGAGGAGGACGGCTGTTCATGCCGGTTCCCAGAGGA
  GGAGGAAGGCGGCTGCGAACTGCD3 zeta domain (amino acid sequence) (SEQ ID NO: 9)

  RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRK
  NPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL
  HMQALPPR
- CD3 zeta (nucleic acid sequence) (SEQ ID NO: 20)
- CGCGTGAAATTCAGCCGCAGCGCAGATGCTCCAGCCTACAAGCAGGGGCAG
  AACCAGCTCTACAACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGC
  TGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGAAGCCGCGCAGA
  AAGAATCCCCAAGAGGGCCTGTACAACGAGCTCCAAAAGGATAAGATGGCA
  GAAGCCTATAGCGAGATTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGC
  CACGACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACACCTATGACG
  CTCTTCACATGCAGGCCCTGCCGCCTCGGCD3 zeta domain (amino acid sequence; NCBI Reference Sequence NM_000734.3) (SEQ ID NO: 10)

  RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRK
  NPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL
  HMQALPPR
- CD3 zeta (nucleic acid sequence; NCBI Reference Sequence NM_000734.3); (SEQ ID NO:21)

  AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAGCAGGGCCAG
  AACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTT
  TGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGA
  AGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGG
  AGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGC
  ACGATGGCCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGC
  CCTTCACATGCAGGCCCTGCCCCCTCGC
- IgG4 Hinge (amino acid sequence) (SEQ ID NO: 36)

  ESKYGPPCPPCPAPEFLGGPSWLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQ
  FNWYVDGVEVHNAKTKPREEQFNSTYRWSVLTVLHQDWLNGKEYKCKVSNK
  GLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWE
  SNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHY
  TQKSLSLSLGKM

- IgG4 Hinge (nucleotide sequence) (SEQ ID NO:37)

  GAGAGCAAGTACGGCCCTCCCTGCCCCCCTTGCCCTGCCCCCGAGTTCCTGGG
  CGGACCCAGCGTGTTCCTGTTCCCCCCCAAGCCCAAGGACACCCTGATGATC
  AGCCGGACCCCCGAGGTGACCTGTGTGGTGGTGGACGTGTCCCAGGAGGACC
  CCGAGGTCCAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCACAACGCCAA
  GACCAAGCCCCGGGAGGAGCAGTTCAATAGCACCTACCGGGTGGTGTCCGTG
  CTGACCGTGCTGCACCAGGACTGGCTGAACGGCAAGGAATACAAGTGTAAGG
  TGTCCAACAAGGGCCTGCCCAGCAGCATCGAGAAAACCATCAGCAAGGCCA
  AGGGCCAGCCTCGGGAGCCCCAGGTGTACACCCTGCCCCCTAGCCAAGAGGA
  GATGACCAAGAACCAGGTGTCCCTGACCTGCCTGGTGAAGGGCTTCTACCCC
  AGCGACATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCCGAGAACAACTAC
  AAGACCACCCCCCCTGTGCTGGACAGCGACGGCAGCTTCTTCCTGTACAGCC
  GGCTGACCGTGGACAAGAGCCGGTGGCAGGAGGGCAACGTCTTTAGCTGCTC
  CGTGATGCACGAGGCCCTGCACAACCACTACACCCAGAAGAGCCTGAGCCTG
  TCCCTGGGCAAGATG

  These clones all contained a Q/K residue change in the signal domain of the co-stimulatory domain derived from CD3zeta chain.

[00587] The CAR scFv fragments were then cloned into lentiviral vectors to create a full length CAR construct in a single coding frame, and using the EFl alpha promoter for expression (SEQ ID NO: 11).

EFl alpha promoter

CGTGAGGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTCGGC
AATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCC
CGAGGGTGGGGGAGAACCGTATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAA
CACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTGCCTTGAAT TACT
TCCACCTGGCTGCAGTACGTGATTCTTGATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGAGGCCTTGCGC
TTAAGGAGCCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGC
ACCTTCGCGCCTGTCTCGCTGCTTTCGATAAGTCTCTAGCCATTTAAAATTTTTGATGACCTGCTGCGACGCTTTTT
TTCTGGCAAGATAGTCTTGTAAATGCGGGCCAAGATCTGCACACTGGTATTTCGGTTTTTGGGGCCGCGGGCGGCGA
CGGGGCCCGTGCGTCCCAGCGCACATGTTCGGCGAGGCGGGGCCTGCGAGCGCGGCCACCGAGAATCGGACGGGGGT
AGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGGCCTCGCGCCGCCGTGTATCGCCCCGCCCTGGGCGGCAAGGCTG
GCCCGGTCGGCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTGCAGGGAGCTCAAAATGGAGGAC
GCGGCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGGCCTTTCCGTCCTCAGCCGTCGCTTCAT
GTGACTCCACGGAGTACCGGGCGCCGTCCAGGCACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTCGTCTTTAGGT
TGGGGGGAGGGGTTTTATGCGATGGAGTTTCCCCACACTGAGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTT
GATGTAATTCTCCTTGGAATTTGCCCTTTTTGAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAA
GTTTTTTTCTTCCATTTCAGGTGTCGTGA (SEQ ID NO: 11).

Gly/Ser (SEQ ID NO:25)

GGGGS

Gly/Ser (SEQ ID NO:26): This sequence may encompass 1-6 "Gly Gly Gly Gly Ser" repeating units

GGGGSGGGGS GGGGSGGGGS GGGGSGGGGS

Gly/Ser (SEQ ID NO:27)

GGGGSGGGGS GGGGSGGGGS

Gly/Ser (SEQ ID NO:28)

GGGGSGGGGS GGGGS

Gly/Ser (SEQ ID NO:29)

GGGS

PolyA (SEQ ID NO:30)

This sequence may encompass 50-5000 adenines.

aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 60
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 120
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 180
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 240
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 300
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 360
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 420
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 480
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aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 720
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aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1080
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1140
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aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 2160
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 2220

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aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 4680
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aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 4860
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 4920
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 4980
aaaaaaaaaa aaaaaaaaaa 5000

PolyA (SEQ ID NO:31)

tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 60
tttttttttt tttttttttt tttttttttt tttttttttt 100

PolyA (SEQ ID NO:32)

This sequence may encompass 50-5000 thymines.

tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 60
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 120
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 180
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 240
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 300
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 360
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 420

tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 480
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 540
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 600
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 660
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 720
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 780
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 840
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 900
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 960
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1020
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1080
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1140
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1200
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1260
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1320
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1380
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1440
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1500
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1560
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1620
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1680
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1740
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1800
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1860
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1920
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1980
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tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2100
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2160
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2220
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2280
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2340
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2400
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2460
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2520
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2580
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2640
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2700
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2760
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2820
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2880
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2940
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3000
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3060
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3120
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3180
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3240
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3300
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3360
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3420
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3480
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3540
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3600
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3660
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3720
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3780
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3840
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3900
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3960
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4020
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4080
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4140
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4200
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4260
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4320
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4380
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4440

tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4500
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4560
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4620
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4680
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4740
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4800
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4860
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4920
tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 4980
tttttttttt tttttttttt 5000

PolyA (SEQ ID NO:33)

This sequence may encompass 100-5000 adenines.

aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 60
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 120
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 180
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 240
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 300
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 360
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 420
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aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 900
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 960
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1020
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aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 4980
aaaaaaaaaa aaaaaaaaaa 5000

PolyA (SEQ ID NO:34)

aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 60
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 120
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 180
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 240
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 300
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 360
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 400

PolyA (SEQ ID NO:35)

aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 60
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 120
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 180
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 240
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 300
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 360
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 420
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 480
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 540
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 600
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 660
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 720
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 780
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 840
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 900
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 960
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1020
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1080

aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1140
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1200
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1260
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 132 0
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1380
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aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1800
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1860
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1920
aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 1980
aaaaaaaaaa aaaaaaaaaa 2000

Gly/Ser (SEQ ID NO:38): This sequence may encompass 1-10 "Gly Gly Gly Ser" repeating units

GGGSGGGSGG GSGGGSGGGS GGGSGGGSGG GSGGGSGGGS

[00588]

Example 2: Expression and characterization of human anti-mesothelin scFv constructs for CAR therapy

[00589] Additional analysis was performed to characteraize the human anti-mesothelin scFv constructs, e.g., mass spectrometry analysis, size exclusion chromatography, and surface plasmon resonance (SPR). Binding affinity and epitope binding (compared to SSI epitope) on the extracellular domain of mesothelin was determined by SPR. The assays and results from this analysis are described below.

Expression of scFv Candidates and Biotinylated Human Mesothelin

[00590] To assess the binding and biophysical characteristics of human anti-mesothelin scFvs identified by phage panning, scFv constructs were transiently produced and purified from HEK293F cells along with human mesothelin extracellular domain (human MSLN ECD). SSI scFv, which is a murine anti-mesothelin scFv, was also produced as a reference. The human anti-mesothelin scFvs tested were M5, Ml 1, Ml2, Ml4, Ml6, Ml7, M21 and M23 (see Table 14). The plasmids encoding the amino acids for the scFv constructs of M5 (SEQ ID NO: 43), Ml 1 (SEQ ID NO: 49), M12 (SEQ ID NO: 50), M14 (SEQ ID NO: 52), M16 (SEQ ID NO: 54), M17 (SEQ ID NO: 55), M21 (SEQ ID NO: 59), M23 (SEQ ID NO: 61) and SSI (SEQ ID NO:

275) and human MSLN ECD (SEQ ID NO: 276) were synthesized externally. ScFvs were produced with a 7x or 8xHis Tag on the C-terminus of the constructs. The human scFv constructs (M5, Ml 1, M12, M14, Ml 6, Ml 7, M21, and M23) had a short linker sequence comprising the sequence GS linking the C-terminus of the scFv to the N-terminus of an 8xHis Tag, e.g., GSHHHHHHHH (SEQ ID NO: 277). Human mesothelin ECD was produced with a C-terminal Avi-Tag (SEQ ID NO: 276) and was site selectively biotinylated *in vitro* with BirA enzyme from Avidity, LLC. Transient expression and purification in HEK293F cells was performed with standard methodology. For scFvs, briefly, 100ml of HEK293F cells at $3x10^6$cells/ml were transfected with 100 μg plasmid and 300 μg polyethylenimine. The cells were incubated at 37°C with 8% $C0_2$ and rotated at 80 rpm. After six days, the cells were harvested by centrifugation at 3500g for 20 minutes. The supernatant was purified by binding the scFv to 200 μΐ Ni-NTA agarose beads (Qiagen) overnight at 4°C. The protein was eluted with 200 μΐ 300mM imidazole, and dialyzed against phosphate buffered saline.

[00591] The sequence of the SSI scFv with the His Tag is provided below:

QVQLQQSGPELEKPGASVKISCKASGYSFTGYTMNWVKQSHGKSLEWIGLITPYNGASS YNQKFRGKATLTVDKSS
STAYMDLLSLTSEDSAVYFCARGGYDGRGFDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIELTQSPAIMSAS PGEK
VTMTCSASSSVS YMHWYQQKSGTS PKRWIYDTSKLASGVPGRFSGSGSGNSYSLTISSVEAEDDATYYCQQWSGYP
LTFGAGTKLEIEFGSHHHHHHH (SEQ ID NO: 275)

[00592] The sequence of the human mesothelin ECD with the C-terminal Avi-Tag is provided below:

DAAQPAASEVEKTACPSGKKAREIDESLIFYKKWELEACVDAALLAT QMDRVNAIPFTYEQLDVLKHKLDELYPQG
YPESVIQHLGYLFLKMSPEDIRKWNVTSLETLKALLEVNKGHEMSPQVATLIDRFVKGRGQLDKDTLDTLTAFYPG
YLCSLS PEELS SVPPSSIWAVRPQDLDTCDPRQLDVLYPKARLAFQNMNGSEYFVKIQSFLGGAPTEDLKALSQQN
VSMDLAT FMKLRTDAVLPLTVAEVQKLLGPHVEGLKAEERHRPVRDWILRQRQDDLDTLGLG
LQGTRGSHHHHHHEFRHDSGLNDI FEAQKIEWHE (SEQ ID NO: 276)

Mass Spectrometry Analysis

[00593] To confirm identity, purified scFvs were analyzed with high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS). 1 μg each was injected onto a Poros Rl/10 2.1 mm x 100 mm column (Life Technologies) heated to 60°C. Separation was performed on a Waters BioAcquity UPLC. Mobile Phase A was 0.1% formic acid; mobile phase B was 0.1% formic acid in 25% acetonitrile, 75% isopropanol. The scFvs were eluted at 0.5

niL/min using a gradient from 25-50% mobile phase B in 15 minutes. Mass spectrometry detection was performed with a Waters Xevo-Tof instrument operating in electrospray positive ion mode scanning from 600-4000 m/z with a cone voltage ramp of 20-50V. The resulting mass spectra were averaged across the width of the peak and deconvoluted using the MaxEntl algorithm from Waters to determine the masses of the expressed scFvs.

Size Exclusion Chromatography Analysis

[00594] Size exclusion chromatography was performed to determine the oligomerization states of the expressed scFvs. 25 μg each was injected onto a TSKGel Super SW3000 4.6 mm x 300 mm column (Tosoh Bioscience) heated to 35°C. The scFvs were eluted at 0.3 mL/min in 750 mM arginine, 1 mM EDTA, 20 mM sodium phosphate, 250 mM sodium chloride, pH 7.2; the UV absorbance was monitored at 280 nm.

Surface Plasmon Resonance (SPR)

[00595] Binding affinity of purified scFvs was measured on a Biacore T200 system. Briefly, recombinant human biotinylated mesothelin ECD was immobilized on a streptavidin (SA) sensor chip surface at a density of 150 RU. Purified scFv was injected over the chip under constant flow rate at three-fold serial dilutions. Association and dissociation rates of the protein complex were monitored for 270 seconds and 400 seconds, respectively. Double referencing was performed against a blank immobilized flow cell and a buffer blank. Affinity was determined with a Langmuir 1:1 binding model when possible, for those scFVs where accurate fitting was not possible due to fast on and off rates, the steady state model was used.

[00596] To determine the relative binding epitope of each of the scFvs in comparison to SSI, 50 nM SSI was captured by biotinylated mesothelin ECD immobilized on a streptavidin sensor chip at a contact time of 180 seconds resulting in a relative density of 70 RU. Purified scFv at 100 nM (M5, Ml 1, M12, M14, Ml6, Ml7, M21, or M23) was then immediately injected over the chip to minimize dissociation of the SSl/mesothelin complex. Binding of the secondary scFv was monitored for 270 seconds.

Results

[00597] The observed masses for the scFvs, determined by HPLC-MS, were consistent with the theoretical values based on the amino acid sequences. Expressed scFvs ranged from 43% monomer to >98% monomer based upon analytical size exclusion chromatography. The selected scFvs represented a broad range of affinities from 0.9 nM to 114 nM compared to SSI control scFV which had an apparent affinity of 0.1 nM for mesothelin ECD (Table 14). Representative SPR sensograms for SSI, M5, and M11 are shown in Figure 41A, B and C, respectively.

[00598] Relative epitope binning (Figure 42) suggests M12, M14, M16, M17, M21, M23 are competitive binders with SSI to human mesothelin while M5 and M11 appear to bind to a unique epitope, as judged by the increased response in the assay upon their injection.

Table 14. Characteristics of anti-human Mesothelin scFvs

[00599]	Identity		SEC[1]	Affinity[2]			
Sample	**Theor. Mass**	**Obs. Mass**	**% Monomer**	**Fit**	**ka (1/Ms)**	**kd (1/s)**	**KD (nM)**
M5	26866	26865	82%	1:1 Binding	4.20E+04	1.13E-03	26.9
M11[3]	26949	26948	ND[3]	Steady State Affinity	-	-	64.7
M12	27261	27260	70%	Steady State Affinity	-	-	307
M14	27420	27422	74.5%	1:1 Binding	2.75E+06	2.51E-03	0.9
M16	27127	27128	94.5%	1:1 Binding	1.74E+05	3.95E-03	22.7
M17	26890	26891	>98%	1:1 Binding	5.22E+05	1.45E-02	27.8
M21	27412	27411	>98%	Steady State Affinity	-	-	110
M23	27602	27604	91%	Steady State Affinity	-	-	114
SS1	26504	26503	43%	1:1 Binding	5.55E+06	5.60E-04	0.1

[1]High aggregate content/low %monomer may result in less accurate affinity determinations due to potential avidity effects.

[2]Affinity was determined with 1:1 binding model when possible, for those scFvs where accurate fitting was not possible due to fast on and off rates, steady state model was used.

[3]Not determined, low concentration precluded accurate determination of % monomer.

Example 3: Analysis and in vitro activity of human scFv bearing CARTs

[00600] Single chain variable fragments for anti-MSLN antibodies were cloned into lentiviral CAR expression vectors with the CD3zeta chain and the 4-IBB costimulatory molecule and the optimal constructs are selected based on the quantity and quality of the effector T cell responses of MSLN CAR transduced T cells ("CART-MSLN" or "CART-MSLN T cells") in response to MSLN expressing ("MSLN+") targets. Effector T cell responses include, but are not limited to, cellular expansion, proliferation, doubling, cytokine production and target cell killing or cytolytic activity (degranulation).

Generation of CART-MSLN

[00601] The human scFv encoding lentiviral transfer vectors are used to produce the genomic material packaged into the VSVg psuedotyped lentiviral particles. Lentiviral transfer vector DNA is mixed with the three packaging components of VSVg, gag/pol and rev in combination with lipofectamine reagent to transfect them together in to Lenti-X 293T cells (Clontech).

[00602] After 30 hours, the media was collected, filtered and stored at -80C. The therapeutic CART-MSLN were generated by starting with the blood from a normal apheresed donor whose naive T cells are obtained by negative selection for T cells, CD4+ and CD8+ lymphocytes. These cells are activated by CD3x28 beads (Dynabeads® Human T-Expander CD3/CD28, Invitrogen) at a ratio of 1:3 in RPMI1640, 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, lx Penicillin/Streptomycin, 100 μM non-essential amino acids, 1 mM NaPyruvate, 10 mM Hepes, and 55 μM 2-mercaptoethanol) at 37°C, 5% $C0_2$. T cells were cultured at lxl 0^6 T cells in 0,5 mL medium per well of a 24-well plate. After 24 hours, the T cells are blasting and 0.5 mL of non-concentrated, or smaller volumes of concentrated viral supernatant is added. The T cells begin to divide in a logarithmic growth pattern, which is monitored by measuring the cell counts per mL, and T cells are diluted in fresh medium every two days. As the T cells begin to rest down after approximately 10 days, the logarithmic growth wanes. The combination of slowing growth rate and T cell size approaching -300 fl determines the state for T cells to be cryopreserved for later analysis.

[00603] Before cryopreserving, percentage of cells transduced (expressing the mesothelin-specific CAR on the cell surface) and their relative fluorescence intensity of that expression were determined by flow cytometric analysis on a FACS-CantoII or FACS Fortessa. Comparing histogram plots of relative fluorescent intensity from that FACS showed the percentage of transduced T cells. The virus transduction show comparable expression levels correlating with transduction efficiency, percent cells transduced. The results indicate that there is no detectable negative effect of the human scFv bearing CAR-MSLN on the cells ability to expand normally when compared to the untransduced T cells ("UTD") and SSI CART-MSLN.

Evaluating cytolytic activity and cytokine secretion of CART-MSLN redirected T cells.

[00604] To evaluate the functional abilities of CART-MSLN T cells to kill and secrete cytokines, the cells were thawed and allowed to recover overnight. In addition to the human scFv bearing CART-MSLN, the CART-MSLN bearing the murine scFv "SSI" (see WO201 3/040577) was used as a control. The control SSI scFV bearing CART-MSLN (also referred to as SSI CART-MSLN) was used in all assays to compare assay variation and/or act as a control. The SSI scFV bearing CART cells, as well as all the other human scFv bearing MSLN-CARTs, were produced in research grade (i.e., not clinical grade) manufacturing conditions. CD19-BBz or Isol-BBz was used as non-targeting CAR for background CART effect.

[00605] T cell killing was directed towards K562, a chronic myelogenous leukemia cell line. K652 MSLN+ (K562-Meso) cells were generated by transduction. Non-MSLN expressing K5652 were used as a control. For the flow based cytotoxicity assay, the target cells are stained with Carboxyfluorescein succinimidyl ester (CSFE) quantitate their presence. The cytolytic activities of CART-MSLN were measured at a titration of effectortarget cell ratios of 10:1, 3:1, and 1:1 where effectors were defined as T cells expressing the anti-MSLN chimeric receptor. Assays were initiated by mixing an appropriate number of T cells with a constant number of targets cells. After 20 hours, the plates were centrifuged and the total volume of each mixture was removed. The remaining cell pellet in each well was washed and cells were stained with live/dead marker 7AAD. After the final wash, the pelleted cells were re-suspended in a specific volume with a predetermined number of counting beads. Cell staining data was collected by Canton flow cytometry and analyzed with FloJo software using beads to quantitate results.

[00606] Plots from 20 hour flow-based killing assays using titrating Effector to Target (E:T) ratios with effector CART-MSLN cells targeting CSFE labeled K562 (FIG. 3A. non-expressing MSLN controls) and K562-Meso (FIG. 3B, K562 cells transduced to express MSLN). Comparing these killing curves, titrating the amount of effector cells shows that those cells expressing MSLN are destroyed. T cells from the same donor and that were transduced with either human scFv bearing CAR-MSLN cells or SSI CAR-MSLN cells were able to kill selectively MSLN+ targets. The cytolytic activities of all CART-MSLN cells are similar and comparable to the murine SSI CART-MSLN. They show that all CART-MSLN cells become Mesothelin reactive upon transduction and gain the ability to kill target cells expressing high levels of Mesothelin. For measuring cytokine production of human scFv bearing CART-MSLN, cells were thawed and allowed to recover overnight. In addition to the humanized CART-MSLN, the SSI CART-MSLN (murine scFv) was used for comparative purposes. CD19-BBz CART was used as a non-targeting control for background CART cell effect. SSI CART-MSLN was used in all assays to compare assay variation. The T cells were directed towards K562, a chronic myelogenous leukemia cell line (both MSLN+ and MSLN), the MLSN-expressing ovarian cancer cell line Ovcar8, and the pancreatic cancer lines SW1990 and Panc0203. When analyzing MSLN expression by flow cytometry, we detected 10-fold lower expression of MSLN on Ovcar8 compared to MSLN+ K562 cells; SW1990 expressed 10-fold less than Ovcar8. MSLN expression on Panc0203 was not detectable by flow cytometry, but was positive by RNA analysis. In addition, PMA/Ionomycin was used to evaluate the potential of T cells to respond to the endogenous immunological signals. The assay tests only an effector:target ratio of 1:1.. The assay is run for 24 hours after mixing of the cells, when the media is removed for cytokine analysis using the IFN- □ CBA-Flex kit for human cytokine detection.

[00607] Background levels of cytokine produced from CART-MSLN cells after exposure to the control K562 cells not expressing MSLN were analyzed. The potential cytokine secretion from stimulation of the T cells with PMA/Ionomycin indicates that the cell populations have slightly variable potential to secrete cytokine. Differences were dealt with by normalizing the specific IFND release over the maximum release (PMA/Ionomycin).

[00608] Data shows that most human scFv bearing CART-MSLN and the murine SSI CART-MSLN produced IFNy when cultured with K562 overexpressig MSLN. When cultuered with cancer cells endogenously expressing MSLN (at lower levels), only few CARTs responded (M5,

11, 14, 15, 16, 17 and SSI), while the two CARTs M5 and Ml 1 showed superior IFNy levels. The lower endogenous MSLN levels they might better reflect naturally expressed levels of mesothelin on tumor cells, those CART having those scFVs might have an enhanced therapeutic response than CARTs having the SSI construct.

Example 4: Clinical trial for lentiviral generated CARTs

[00609] Patients will be selected from those have mesothelin expressing cancers, such as serious epithelial ovarian cancer, malignant plural mesothelioma, or pancreatic cancer.

[00610] The manufacture and release testing of CART-MSLN cells will be performed by the Clinical Cell and Vaccine Production (CVPF) at the University of Pennsylvania. CART-MSLN product will be manufactured from the patients' apheresis product.

[0061 1] An about 10-liter apheresis procedure will be carried out at the University of Pennsylvania Apheresis center (4 weeks prior to first dose of CAR T cells). PBMCs from a patient are obtained for CAR T cells during this procedure. From a single leukapheresis, the intention is to harvest at least 5×10^9 white blood cells to manufacture CAR T cells. Baseline blood leukocytes for FDA look-back requirements and for research are also obtained and cryopreserved. The mesothelin-modified T cell product is expected to be ready for release approximately 4 weeks later.

[00612] Lentiviral generated CARTs were made as described in Example 2 using cells isolated from each patient, i.e., autologous T cells. The CART-MSLN made from each patients T cells express human scFvs (including M5 and Mil), or SSI.

[00613] At the end of cell cultures, the cells are cryopreserved in infusible cryomedia in bags. The dose is $1\text{-}5 \times 10^7$ CAR T cells for Cohort 1, $1\text{-}5 \times 10^8$ CAR transduced cells for Cohort 2 and 3, calculated as a range of 2-50% transduced cells in total cells. Bags containing autologous modified CAR T cell products will be stored in a controlled and monitored freezer at the CVPF at the Hospital of the University of Pennsylvania. Infusion bags will be stored in the freezer until needed.

[00614] Each dose will be packed and cryopreserved in 1 infusion bag at in a volume dependent on the total cell number, a function of the transduction efficiency; the minimum

volume will be lOmL per bag. Each bag will contain an aliquot (volume dependent upon total cell dose) of cryomedia.

[00615] Cohort 1 subjects (N=3-6) will receive a single flat intravenous dose of autologous 1-5xl0 ^{7} CART-MSLN cells on day 0. Cohort 2 subjects (N=3-6) will receive a single flat intravenous dose of autologous l-5xl0 ^{8} CART-MSLN cells on day 0. Subjects in Cohort 1 and 2 will not receive any lymphodepleting chemotherapy prior to CART-MSLN cells. Cohort 3 (N=6) will receive 1.5 grams/m ^{2} of cyclophosphamide intravenously two days prior to a single flat intravenous dose of autologous 1-5x10^{8} CART-MSLN cells.

[0061 6] It is anticipated that patients receiving cyclophosphamide may experience nausea and vomiting as a side effect of the treatment. Anti-emetic prophylaxis premedication for nausea can be administered prior to infusion of chemotherapy according to the institutional standards. Choice of specific agent will be left to the discretion of the investigator, though corticosteroids should not be used due to their immunosuppressive effects.

[00617] Side effects following T cell infusions may include transient fever, chills, rigors, myalgias/arthralgias, headache, fatigue, and/or nausea. Subjects will be pre-medicated with acetaminophen 650 mg by mouth and diphenhydramine hydrochloride by mouth or IV prior to CAR T cell infusion. If Benadryl is contraindicated, an H2-blocker, such as ranitidine, will be administered. These medications may be repeated every six hours as needed. A course of non-steroidal anti-inflammatory medication may be prescribed if the patient continues to have fever not relieved by acetaminophen. It is recommended that patients do not receive systemic corticosteroids such as hydrocortisone, prednisone, prednisolone (Solu-Medrol), or dexamethasone (Decadron). If corticosteroids are required for an acute infusional reaction, an initial dose of hydrocortisone 100 mg is recommended.

- One bag of CART-MSLN cells will be transported by the study coordinator or CVPF stuff on dry ice from CVPF to the subject's bedside in the CTRC.
- Transfected T cells will be thawed by a member of CVPF staff in a 37°C water bath at subject's bedside. The bag will be gently massaged until the cells have just thawed. If the CAR T cell product appears to have a damaged or leaking bag, or otherwise appears to be compromised, it should not be infused, and should be returned to the CVPF as specified below.
- Cells will be infused to the subject while cold within approximately 10-15 minutes after thaw. The transfected T cells will be infused intravenously rapidly through an 18 gauge latex free Y-type blood set with 3-way stopcock. No leukocyte filter at infusion. Dosing

will take place by gravity infusion. If the infusion rate by gravity is too slow, the transfected T cell drug product may be drawn into a syringe via the stopcock and manually infused at the required rate. There should be no frozen clumps left in the bag.

- Prior to the infusion, two individuals will independently verify the information on the label of each bag in the presence of the subject and confirm that the information correctly matches the participant.
- Patients will be monitored during and after infusion of the transfected T cells. Temperature, blood pressure, heart rate, respiratory rate and pulse oximetry will be obtained and recorded immediately prior to dosing and every 15 minutes for 2 hours following infusion completion.
- Emergency medical equipment (i.e. a crash cart) must be available for an emergency situation during the infusion in the event that the subject has an allergic response, or severe hypotensive crisis, or any other reaction to the infusion.
- If no symptoms occur and subject's vital signs remain normal 2 hours after the infusion, the subject will be discharged home with instructions to return to the hospital should any symptoms develop. If a vital sign measurement is not stable, it will continue to be obtained approximately every 15 minutes until the subject's vital signs stabilize or the physician releases the patient. The subject will be asked not to leave until the physician considers that it is safe for him or her to do so.
- Within 60 minutes (± 5 minutes) following completion of transduced CAR T cell dosing, a blood sample will be obtained for a baseline determination of transduced CAR T cell number and cytokine levels.
- Subjects will be instructed to return to the CTRC or Perelman Center in 24 hours after the first infusion for blood tests and follow-up examination according to the SOE.

[0061 8] For CARTs bearing scFv doses of cells can be repeated as needed.

[00619] Descriptive statistics will be applied to determine the relative persistence and trafficking to blood (and optionally tumor) of the CART-MSLN cells. Data regarding the number of CAR T cells in blood, HAMA levels, and the tracking of soluble biomarker levels will be presented graphically. Correlations with radiographic and other standard measures of tumor burden will be determined. We will compute 95% confidence intervals for proportions and means.

[00620] Adverse events will be collected and evaluated for all patients during the protocol specified AE reporting periods. AEs will be graded for severity using the National Cancer Institute (NCI) - Common Toxicity Criteria (v4). All adverse events will be described and exact 95% confidence intervals will be produced for adverse event rates, both overall and within major categories. The data will be monitored continuously for evidence of excessive toxicity. Results will be tabulated and summarized.

[00621] Rates of clinical responses will be summarized in exact 95% confidence intervals. Distributions of progression-free and overall survival and duration of clinical response will be presented graphically using Kaplan-Meier curves. The two-year survival rates will be presented. Preliminary evidence of efficacy will be determined by monitoring tumor response rates in those subjects with measurable disease. Tumor response will be assessed using radiographic imaging (i.e. CT imaging) and serum biomarkers at Day 28, Months 3 and 6 after infusion.

- Radiographic responses will be measured according to Response Evaluation Criteria in Solid Tumors (RECIST 1.1), modified RECIST criteria for pleural mesothelioma, and Immune-Related Response Criteria (iRRC).
- Serum biomarker responses will be evaluated according to standards for each disease; additionally all subjects will have SMRP measured. For example, subjects with pancreatic cancer will have monitoring in levels of CA19-9, CEA and serum mesothelin related protein [SMRP]) following CAR T cell administration. Subjects with ovarian cancer will have serum levels of CA125 monitored, an also SMRP. Subjects with mesothelioma will have serum levels of SMRP measured. In all cases, serum biomarkers will not be used as the sole measurement of tumor response, given that CART-MSLN cells may affect the assays used in these biomarker measurements.
- Data will be analyzed descriptively for overall response rates, progression-free survival, and overall survival.

[00622] PFS and OS up to 2 years post-infusion or until subjects initiate a cancer-related therapy will be evaluated.

[00623] Safety assessments including blood monitoring of cytokine levels will be performed. Subjects will be enrolled serially. Infusions will be staggered to allow 28 days of safety assessment between enrolled subjects in each cohort.

Example 5: Clinical trial for RNA generated CARTs

Autologous T cells are engineered to express an extracellular a SSI CAR construct that recognized mesothelin, along with transmembrane domain, in addition to human 4-IBB and CD3zeta signaling domains described above. See, e.g., WO2013/040577, Example 1.

Production of CAR-containing Nucleic Acids

[00624] SSl-CARs are constructed according as follows. CARs containing human anti-mesothelin scFvs are also constructed using similar methods.

[00625] Two different plasmids were utilized to clone the ssl.bbz fragment. The mesothelin scFv fragment (ssl) was first cloned by the Translational Research Program (TRP) laboratory from the previously published construct of Dr. Pastan (Chowdhury et al., 1998). The human CD8a hinge and transmembrane domain together with 4IBB and CD3ζ sequence was cloned by PCR from the pELNS.CD19-BB^ plasmid described previously (Milone et al, 2009). Sequences for the hinge, transmembrane, and intracellular signaling domains are disclosed herein. The ssl.bbz fragment was first cloned in pGEM.GFP.64A vector (provided by Eli Gilboa and described in Boczkowsk, D et al., 2000). This vector was modified by addition of two 3'UTR beta globin repeats and 150bp of polyA sequence (SEQ ID NO: 27l)(rep lacing the 64 polyA sequence (SEQ ID NO: 273) in pGEM.GFP.64A) for enhanced transgene expression (Holtkamp 2006). The GMP-compliant plasmid for clinical use was derived by subcloning the ssl.bbz.2bgUTR.150A fragment from pGEM into the pDrive vector. The pDrive cloning vector (Qiagen) is designed for highly efficient cloning of PCR products through UA hybridization. It allows for both ampicillin and kanamycin selection of recombinant clones, and comes with universal sequencing primer sites, and both T7 and SP6 promoters for *in vitro* transcription. First, ssl .bbz.2bgUTR. 150A was cut from pGEM vector by Hind III and Ndel (Fill-in blunt) and subcloned into pDrive cut by Kpnl and Notl (Fill-in blunt). The insert with correct orientation was sequence confirmed to generate pDrive.ssl.bbz.2bgUTR.150A. Ampicillin resistance gene in pDrive vectors was deleted by double digestion with Ahdl and BciVL To eliminate potential aberrant proteins translated from internal open reading frames (ORF) inside the CAR ORFs, all internal ORF that were larger than 60 bp in size were mutated by mutagenesis PCR, while the ORF of ssl CAR was maintained intact. The resulting plasmid was designated pD-A.ssl.bbz.OF.2bg.l50A, as shown in Figure 1.

[00626] Production of CAR nucleic acids for introduction into autologous T cells were prepared as follows. The final pD-A.ssl.bbz.OF.2bg.150A construct was introduced into OneShot TOP10 Chemically Competent *E Coli* cells (Invitrogen) as per CVPF SOP 1188. Up to 10 mg plasmid DNA prepared as one batch was generated using the QIAfilter Plasmid Giga DNA isolation kit as per SOP 1191, from two 1.25 liters of LB-media containing 100 μg/ml kanamycin. 1 mg of DNA at a time was linearized with Spel restriction enzyme overnight at 37° C. Linearization was confirmed by gel electrophoresis prior to large scale purification using the Qiagen Plasmid Maxi Kit.

[00627] RNA was transcribed from the plasmids by utilizing the mScript mRNA system and was isolated using the RNeasy Maxi kit (Qiagen). The *in vitro* transcribed RNA was cryopreserved in aliquots of 0.5 mL at a concentration of 1 mg/mL. RNA quality and quantity was analyzed by 1% agarose gel electrophoresis after 15 min denaturation at 70° C in mRNA denaturation buffer (Invitrogen, Carlsbad, CA) and quantified by UV spectrophotometry (OD260/280).

CAR T Cells Product Manufacturing-

[00628] A 7 to 12-liter apheresis procedure is performed about 4 weeks prior to first dose of CAR T cells. PBMC are obtained for CAR T cells during this procedure. From a single leukapheresis, the intention is to harvest at least 5 x 109 white blood cells to manufacture CAR T cells.

[00629] CD3+ T-cells are enriched from a leukapheresis product by depletion of monocytes via counterflow centrifugal elutriation on the CaridianBCT Elutra, which employs a single use closed system disposable set. On day 0, the T cell manufacturing process is initiated with activation with anti-CD3/CD28 monoclonal antibody-coated magnetic beads, and expansion is initiated in a static tissue culture bag. At day 5, cells will be transferred to a WAVE bioreactor if needed for additional expansion. At the end of the culture, cells are depleted of the magnetic beads, washed, and concentrated using the Haemonetics Cell Saver system. The post-harvest cells are incubated overnight at 37° C for electroporation the next morning. Cells are washed and resuspended in Electroporation Buffer (Maxcyte) and loaded into the Maxcyte processing assembly. Cells are electroporated with the ssl RNA, and allowed to recover for 4 hours and then formulated in infusible cryopreservation media.

[00630] *Packasins*

[00631] lxl 0^8 or lxl 0^9 ±20% modified CAR T cells will be supplied in IV infusion bags maintained on wet ice. Each bag will contain an aliquot (volume dependent upon dose) of cryomedia containing the following infusible grade reagents (% v/v): 31.25 plasmalyte-A, 31.25 dextrose (5%), 0.45 NaCl, up to 7.50 DMSO, 1.00 dextran 40, and 5.00 human serum albumin. Bags can be frozen at -135°C.

[00632] *CAR T Cells Product Stability*

[00633] The ssl CAR T cells will be cryopreserved 4 hours post-electroporation, and thawed and administered within a three month window after T cell manufacturing. We have demonstrated that mesothelin scFv expression of the cryopreserved ssl CAR T cells approximately 30 days at <-130°C was 97.4%, almost identical to time of cryopreservation (96.9%), and other cryopreserved T cell products are stable for at least 6 months. Viability post-thaw, based on Trypan blue counts was 75.2 % as compared to 98.7%. The expression data suggests that the final product is stable during storage for the trial, and that the sentinel vial for additional doses should meet release criteria of 70% viability and >20% CAR expression. Additional vials of ssl CAR T cells will be thawed at 3, 6, 9, and 12 months post cryopreservation, and viability and transgene expression tested to generate further product stability data.

[00634] Clinical trial procedure and results: A Phase I clinical trial of autologous mesothelin re-directed T cells administered to patients with malignant pleural mesothelioma is performed as follows. Similar procedures can be utilized for a study for treating patients with pancreatic cancer.

[00635] *Dosages*

[00636] Cohort 1 patients (n=3) will receive a single infusion of $1x10^8$ using flat dosing with anti-meso RNA CAR T cells on day 0 and one infusion of $1x\ 10^9$ RNA CAR T cells on day 7, providing the patients meet the protocol-specified safety assessments before the day 7 infusion.

[00637] Cohort 2 patients (n=6) will be given 2 cycles of modified CAR T cells. One cycle consists of 3 infusions every other day (Monday, Wednesday, Friday). Cycle 1 consists of 3 doses of $1xlO^8$ CAR T cells dosed on MWF (day 0, 2, 4); cycle 2 consists of 3 doses of $1xlO^9$ CAR T cells dosed on MWF (day 7, 9, 11).

[00638] The target dose per infusion is $1x\ 108$ cells (one dose for Cohort 1 and week 1 doses for Cohort 2) and $1x\ 10^9 \pm 20\%$ cells (one dose for Cohort 1 and week 2 doses for Cohort 2). The minimally acceptable dose is $1xl\ 0^8$. If the total cell expansion is lower than the total acceptable cell dose, the patient may undergo a second apheresis in an attempt to expand more cells and fulfill the total target dose. If there are contraindications for the second apheresis or if the second apheresis and expansion fails to produce the minimally acceptable dose, the dose will be deemed a manufacturing failure. For Cohort 1 patients, as many mesothelin CAR electroporated T cells

as possible will be manufactured/frozen to reduce the probability subjects will undergo a second leukapheresis in the case they will follow Extended Cohort 1 regimen.

[00639] Dose de-escalation will occur if more than one patient develops a DLT from modified CAR T cell infusion. In the event of DLT, we will dose de-escalate by 10-fold. Thus, if toxicity occurs during cycle 1 at 10^8 CAR T cells, then all infusions (doses 1 to 6) would be reduced to 10^7 CAR T cells. In the event of unmanageable toxicity at 10^7 CAR T cells, the trial would be stopped.

[00640] A potential issue that can arise in patients being treated using transiently expressing CAR T cells (particularly with murine scFv bearing CARTs) is anaphylaxis after multiple treatments.

[00641] Without being bound by this theory, it is believed that such an anaphylactic response might be caused by a patient developing humoral anti-CAR response, i.e., anti-CAR antibodies having an anti-IgE isotype. It is thought that a patient's antibody producing cells undergo a class switch from IgG isotype (that does not cause anaphylaxis) to IgE isotype when there is a ten to fourteen day break in exposure to antigen. Therefore, for transient CARTs (such as those generated by RNA electroporation), CART infusion breaks should last more than ten to fourteen days.

[00642] In addition, using CARs with human (instead of murine) scFvs could reduce the likelihood and intensity of a patient having an anti-CAR response.

[00643] *Study Procedure*

[00644] The study consists of 1) a screening phase, 2) an intervention phase consisting of apheresis, CAR T cell infusion, side effect assessment, and optional tumor biopsy, and 3) follow up visits.

[00645] *Screening and Baseline Assessment*

[00646] An investigator must explain the nature of the study protocol and risks associated with the protocol in detail to the subject. The subject must sign and date the written informed consent prior to study participation. Informed consent process and date will be recorded in the subject's medical record and on the CRF. Informed consent must be obtained before protocol

procedures are performed. If a procedure required for screening was performed prior to signing the informed consent and the procedure meets the time limits of the protocol, this procedure may be used for the screening evaluation.

[00647] The screening procedures (not required for determination of eligibility) will be completed within 6 weeks of first dose of CAR T cells (unless otherwise noted in the SOE). In the event that a second apheresis is needed to expand additional T cells and reach the target cell dose, this time window will not cover all the procedures. Thus, in this specific situation, a 6-8 week period is allowed for performing the baseline scans.

[00648] Screening procedures include:

- Informed consent
- Confirm an ECOG performance status ≤ 1
- Determination of manufacturing efficiency (for the first 2 patients): a blood sample is sent to the CVPF to determine T cell manufacturing feasibility. In approximately 1 week, the CVPF will return a result as to whether the subjects PBMC are likely to be adequate for large scale CAR T cell manufacturing process. Results must be known before performing any additional study procedures.
- Complete medical history
- Physical examination including vital signs, height, weight and oxygen saturation
- Review of concomitant medications
- Hematology (WBC with differential, RBC, Hct, Hgb and platelets)
- PT and PTT
- Chemistry (sodium, potassium, chloride, bicarbonate, urea nitrogen, creatinine, glucose, total protein, albumin, calcium, alkaline phosphatase, total bilirubin, ALT, AST)
- Viral screens: HIV, HCV, HBV and serology for CMV
- Autoantibody panel: ANA, ESR
- Urinalysis
- 12 lead Electrocardiogram (ECG)
- Serum PHCG pregnancy (women of childbearing potential)
- Spirometry and DLCO
    - Tumor assessment Chest CT Scan with contrast, other radiographic evaluations as clinically indicated. PET/CT scan is optional.
    - Pathological confirmation of epithelial or mixed (biphasic) mesothelioma and stain for mesothelin by protocol specific pathologist.

[00649] *Leukapheresis*

[00650] A 7 to 12-liter apheresis procedure will be carried out at the University of Pennsylvania Apheresis center (4 weeks prior to first dose of CAR T cells). PBMC are obtained for CAR T cells during this procedure. From a single leukapheresis, the intention is to harvest at least 5 x 109 white blood cells to manufacture CAR T cells. If the harvest is unsuccessful,

patients will have the option to undergo a second leukapheresis. Baseline blood leukocytes for FDA look-back requirements and for research are also obtained and cryopreserved. The mesothelin-modified T cell product is expected to be ready for release approximately 4 weeks later. All patients will have the standard leukapheresis screening prior to the procedure.

[0065 1] *Pre-Infiision Assessment (Study Day -3, prior to first dose)*

[00652] This safety assessment includes physical exam (e.g., vital signs, weight, etc.), review of concomitant medications, performace status, chemistry, CBC with differential, pregnancy test (urin) urinalysis, EKG, CXR, and research blood specimens.

[0065 3] *CAR T cell administration*

[00654] Prior to each CAR T cell infusion patients will have limited problem oriented PE, review of adverse events, ECOG performance status, CBC, chemistry, and urinalysis. Subjects will return to HUP 1 day after CAR T cell infusion (Dl, D3, D8 and D10) to have limited problem oriented PE, review of adverse events, concomitant medications, ECOG performance status, CBC and chemistry. On day 7, Cohort 1 patients will also have an EKG. On Day 14 Cohort 2 patients will also have a CXR and EKG.

[00655] CAR T cells will be administered as described above. Subjects' vital signs will be assessed and pulse oximetry will be done prior to dosing, at the end of the infusion and every 15 minutes thereafter for 2 hours and until these are stable.

[00656] *Post-infusion assessment*

[00657] Cytokine levels in blood will be monitored at lh, 4h, 1 day and 3 days following first infusion and at lh, 4h, 1 day and 3 days, 7 days and 14 days following the second infusion for patients of Cohort 1. Blood collection for engraftment analysis (flow cytometry and RT-PCR) will be collected for 2 1 days following the first infusion.

[00658] For Cohort 2 (and Extended Cohort 1) patients, cytokine levels will be monitored at 1h following each infusion and at additional time points according to the SOE. The primary safety and engraftment data is collected for 35 days following the first (Day 0) CAR T cell administration.

[00659] 1 day after the CAR T cell infusion, blood is drawn for flow cytometry and RT-PCR in order to evaluate for circulating CAR T cells. Peripheral blood samples will be processed to isolate: a) peripheral blood mononuclear cells (PBMC) to evaluate immune cell phenotype and function, b) RNA to quantify short term persistence and distribution of infused CAR T cells and

c) serum to quantify and evaluate immune cell subset and soluble immune and growth factors, evaluate the development of anti-infused cell immune responses, and perform protoarray analyses to evaluate the development of humoral anti-tumor ìmmune responses via immune epitope spreading.

[00660] Subjects will be assessed on month 2 and 3 (Cohort 1), and months 2, 3, and 6 following the last CAR T cell infusion (Cohort 2 and extended Cohort 1) for safety assessment as described above.

[0066 1] *Tumor biopsy and mini-apheresis*

[00662] For Cohort 2 and extended Cohort 1: If tumor is visibly accessible or accessible by image-guided biopsy, eligible subjects will undergo direct tumor biopsy or biopsy under image guidance to evaluate the presence of CAR T cells and mesothelin expression. This is to be scheduled at Day 14 after T cell infusion for Cohort 2 subjects. The frequency of total tumor-infiltrating lymphocytes and T cell subsets as well as the expression of molecular markers of T cell antitumor response will be measured. These will be compared to baseline values on stored surgical tumor specimens, if available. Subjects will be given the option to refuse tumor biopsy. Intracavitary fluid may also be submitted as biopsy.

[00663] A mini-apheresis (2L) is done for research purposes and for FDA "lookback" purposes to archive CAR T cells for safety analysis at day 2 1 post-infusion. A blood sample may be collected in lieu of leukapheresis.

[00664] *Tumor response assessment*

[00665] Tumor response will be assessed at Day 35 and Month 2 for Cohort 1 and Day 35, Months 2 and 6 following the last CAR T cell infusion for Cohort 2 (and Extended Cohort 1), or until the patient requires alternative MPM therapy. Tumor assessment will be done by CT scan with contrast for chest and other procedures as clinically indicated.

[00666] The above example can be done using human bearing scFv CARTs, using for example the scFvs in CAR constructs M5, M1 1, M14, M15, M16 and M17.

Example 6: CART-MSLN Induce Anti-tumor Activity in Solid Malignancies

[00667] This example present two case reports from ongoing clinical trials indicating that adoptive transfer of mRNA CAR T cells (PBMCs were electroporated with mesothelin CAR construct) that target mesothelin (CART-MSLN) is feasible and safe without overt evidence of off-tumor on-target toxicity against normal tissues. These cases also demonstrate that CART-

MSLN can infiltrate and have anti-tumor activity in solid malignancies, such as malignant plural mesothelioma (MPM) and pancreatic cancer.

[00668] Subject 17510-105 had MPM and was given 3 infusions of CARTmeso. Subject 21211-101 had metastatic pancreatic cancer (PDA) and was given 8 doses of CARTmeso by intravenous infusion and 2 intratumoral injections. Both patients were elderly and had advancted chemotherapy-refractory cancer with extensive tumor burden at the time of enrollment.

[00669] *Anti-tumor clinical activity of CART meso cells*

[00670] Both cases were evaluated for tumor response by computed tomography (CT) imagining. In addition, the PDA patient was evaluated by [18F]2-fluoro-2-deoxy-D-glucose (FDG) avidity on positron emission tomography/computed tomography (PET/CT) imaging before and after infusions (FIG. 6) The MPM patient had stable disease after receiving CARTmeso infusions, however, developed a confirmed partial response after reciving one infusion of CARTmeso cells on Schedule 2 (FIG. 6A). The PDA patient had stable disease after 3 weeks of intravenous CARTmeso therapy. By FDG PET/Ct imaging, a decrease in the maximum standardized uptake value (SUVmax) was seen in all sites of disease. To further this metabolic response, changes in the mean volumetric product (MVPmean) for each disease site were determined (FIG. 6B, 6C, and 6D). A decrease in MVPmean was observed only in the peritoneal lesions. To further understand the impact of CARTmeso cell therapy on peritoneal tumor burden, ascites fluid was analysed by flow cytometry. Analysis of the ascitic fluid on days +3 and +15 after beginning therapy revealed a 40% decrease in the concentration of tumor cells that co-expressed mesothelin and c-met. (FIG. 6D). Overall, these findings suggest a role for CARTmeso cell infusions in inducing an anti-tumor effect in these patients.

[00671] Serological tumor markers was also evaluated in both patients. Serum mesothelin-related peptide (SMRP) and CA19-9 levels were measured pre- and post-CARTmeso cell infusions. For the MPM patient, SMRP levels declined afte receiving the first infusion on Schedule 2, coinciding with the reduction of tumor burden seen by CT imaging. For the PDA patient, CA19-9 levels increased slowly over the course of treatment but remained stable for 1 month. After completion of the intra-tumoral injections of CARTmeso cells, CA19-9 levels rose consistent with disease progression.

[00672] *In vivo persistence and trafficking of CARTmeso cells*

[00673] A qPCR assay was develed to detect and quantify the persistence of CARTmeso cells in patients following infusion. Analysis of peripheral blood, ascites, and tumor samples from the two patients are presented in FIG. 7. CARTmeso transgene was detected in both paitent immediately after each infusion.In agreement with the biodegradable nature of the CARTmeso transgene, levels were observed to progressively decrease on successive days.

[00674] Trafficking of CARTmeso cells in tumor tissues was evaluated in the PDA patient by collecting ascites at serial time point and by tumor biopsy.

[00675] *Induction of humoral epitope spreading after CARTmeso cell infusion*

[00676] The hypothesis that CARTmeso cells, if able to recognize and lyse primary tumor cells *in vivo*, might elicit a systemic anti-tumor immune response was tested. High-throughput serological analysis was performed to measure induction of antibody responses to antigens to detect the development of a polyclonal immune response that may have occurred as a result of tumor destruction and epitope spreading. These analysis were accomplished using an unbiased interrogation of treatment-induced igG responses to almost 10,000 independent human proteins. In PDA patient, new antibody responses were detected at day +44 to more than 100 proteins. Similarly, elevated antibody responses to a subset of proteins was observed for the MPM patient. Overall, these antibody immune responses observed in both patients are consistent with CAR T cell-mediated tumor destruction leading to the release of self-proteins that are cross-presented in a classical process of epitope spreading.

[00677] Pre-and post-treatment sera of both patients for the induction of anti-tumor immune responses was also examined by immunoblotting using purified tumor associated proteins or protein lysates from human MPM or PDA cell lines. Anti-tumor immune responses were defined by the presence of new bands or increases in the intensity of pre-existing bands on immunoblots (FIG. 9) In both patients, antibody pattern alterations were seen during treatment, e.g., increases in antibodies detecting proteins present in allogeneic tumor cell lines. These findings along with the protoarray analysis suggest that an antitumor humoral immune response was induced by CARTmeso cell infusion.

Example 7: Combination treatment to improve CART function in solid tumors

[00678] Immunotherapy using adoptively transferred tumor infiltrating lymphocytes (TILs) is limited by T cell functional inactivation within the solid tumor microenvironment. In this study, human T cells expressing meso CAR were injected intravensously into immunodeficient mice bearing large, established human mesothelin-expressing mesothelioma flank tumors. Analysis of the isolated meso-CAR T cells (mesoCART) showed rapid loss of effector functions that appeared to be multifactorial. The results from these experiments demonstrate that inhibitors of mechanisms that cause loss of effector function of mesoCART may be useful to in a combination therapy to enhance efficacy of mesoCART treatment *in vivo.*

[00679] Materials and Methods

[00680] **Generation of mesoCAR and lentivirus vector preparation.** Lentiviral constructs containing mesothelin CAR ssl were prepared as described previously. Human mesothelin CAR constructs and T cells expressing the same can also be generated using methods previously described.

[00681] **Cell lines.** A human mesothelioma cell line derived from a patient's tumor was used - EMP (parental). Since EMP did not have baseline expression of the tumor-associated antigen (TAA) mesothelin, it was transfected with a lentivirus to stably express human mesothelin (the transduced cell line was named EMMESO). Cells were also transduced to stably express firefly luciferase (called EMPffluc, EMMESOffluc).

[00682] **T cell Effector Assays.** Effector T cells were cocultured with firefly luciferase expressing tumor cells at different ratios for a specified period time. At the end of the co-culture incubation period, supernatant was saved for IFN levels by ELISA (Biolegend #430106), wells were washed, and remaining tumor cells were lysed with IX cell lysis buffer for 30 minutes. The luciferase activity in the lysates was analyzed using the Luciferase Assay System on a GloMax Multi Detection System (Promega.) Results are reported as percent killing based on luciferase activity in wells with tumor, but no T cells. (% killing = 100-((RLU from well with effector and target cell coculture)/(RLU from well with target cells)xl00)). Effector-to-target ratios represent total T cells per target cell.

[00683] **Animals.** All animal experiments were approved by the appropriate Institutional Animal Care and Use Committee. NOD/scid/IL2ry-/- (NSG) mice were bred in the Animal

Services Unit of the Wistar Institute and Children's Hospital of Philadelphia. Female mice were used for experiments at 10 to 16 weeks of age.

[00684] **In Vivo Xenograft Experiments.** 5 x 10^6 EMMESO tumor cells were injected in the flanks of NSG mice in a solution of X-Vivo media (Lonza) and Matrigel (BD Biosciences). After tumors were established (100-200mm^3), the mice were randomly assigned to one of three intravenous (tail-vein) treatment groups: 1) 20 x 10^6 non-transduced (NT) T cells (Dynabead® activated T cells), 2) 20 x 10^6 mesoCAR T cells (Dynabead® activated T cells transduced with mesoCAR) 3) saline. Tumors were measured using calipers and tumor volume was calculated using the formula (^6)*(length)*(width) ^{2}. Groups contained 10 mice each. The *in vivo* experiments were repeated three times.

[00685] Results

[00686] *Hypofunction observed in tumor infiltrating CARTs*

[00687] The human mesothelin-expressing mesothelioma tumor cell line EMMESO was injected into the flanks of NSG mice and allowed to grow to a size between 100 and 200 mm3. At that time, tumor- bearing mice were given one intravenous injection of 20 million T cells (mesoCAR expression was approximately 50% (data not shown)). Significant tumor growth slowing was seen after a delay 14 days (FIG. 10), however, unlike our experience with another mesothelioma cell line (Carpenito et al., *Proc Natl Acad Sci USA,* 106(9):3360-65 (2009), Zhao et al., *Cancer Res,* 70(22):9053-61 (2010).) no tumor regression or cures were noted. Injection of non-transduced T cells had minimal anti-tumor effects when compared to the saline treated control (FIG. 10), indicating that the reduction in tumor growth observed in animals treated with T cells expressing mesoCAR was the result of the mesoCAR.

[00688] Various analyses were performed to understand why tumor regressions were not observed after CART treatment. EMMESCO tumors were examined and mesoCARTs were found to be present in large numbers, however, the T cells present had become hypofunctional. Given that the level of CAR expression on the CAR TILs was equal or greater to that of the cells prior to injection (FIG. 11A and 1IB), we compared their functional activity. We isolated and analyzed the mesoCAR TILs from EMMESO tumors 40 days after injection (all studies were started immediately after isolation) and compared them to the same batch of mesoCAR T cells that had been used for the original injection and frozen away ("cryo mesoCAR"). These cells

were studied after thawing and incubating in 37oC/5%C02 for 18hrs. When cryo mesoCAR T cells and flank mesoCAR TILs were added to cultured EMMESO cells expressing firefly luciferase (EMMESOffluc) at a 20: 1 ratio for 18 hours, the cryo mesoCAR T cells were highly efficient in killing tumor cells (>95%), while the mesoCAR TILs killed only about 10% of the tumor cells over this same time period (FIG. 11C, pO.001).

[00689] Additional analysis also demonstrated that the mesoCAR T cells did not produce cytokines, such as IL-2 and IFNg, after exposure to antigen or PMA/Ionomycin. In contrast, the cryo-meso CAR T cells produced cytokines. The mesoCAR T cells also demonstrated reduced signaling, by reduced or absence of phosph-ERK expression by immunoblotting 20 minutes after exposure to anti-CD3/CD28-coated beads. In contrast, cryo-meso CAR T cells retained phosphor-ERK expression after exposure to the beads.

[00690] *Human mesoCAR TILs express increased levels of inhibitory receptors*

[00691] Next, the expression of four inhibitory receptors (IR) that have been previously described in hypofunctional TILs isolated from humans was evaluated using flow cytometry on: i) the cryo mesoCAR T cells that were used for injection, ii) freshly isolated mesoCAR TILs from Day 39 from EMMESO, and iii) "recovered" mesoCAR TILs that had been removed from the tumor for 24 hours (Table 6). CAR TILs expressed high levels of inhibitory receptors. These levels were generally much lower after 24 hours of recovery away from the tumor microenvironment. For the CD4 CAR ms, PD-1 went from 73% to 53%, LAG-3 went from 63% to 3%, and TIM3 went from 24% to 1%. 2B4 expression was high and remained elevated after rest (67% to 88%). For the CD8 CAR TILs, PD-1 went from 26% to 21%, LAG-3 went from 48% to 13%, and TFM3 went from 56% to 1%. 2B4 expression was high and remained elevated after rest (96% to 98%).

[00692] Table 6. Expression of inhibitor receptors (IRs) on TILs

[00693]

	Percent positive CD4 T cells				Percent positive CD8 T cells		
EMMESO TIL	Cryo Meso CAR T cells	Fresh TIL	Recovered TIL		Cryo Meso CAR T cells	Fresh TIL	Recovered TIL
PD-1	37.2 (947)	73.0 (1492)	53.0 (1081)		5.4 (326)	26.0 (420)	21.0 (360)
LAG-3	44.7 (2585)	62.8 (3845)	3.0 (1968)		85.2 (4609)	48.0 (4376)	13.0 (3161)
214	4.5 (215)	67.0 (1102)	88.0 (351)		60.0 (2974)	96.0 (6303)	98.0 (5917)
T1M3	1.9 (945)	24.0 (1253)	1,0 (200)		2.4 (941)	56.0 (1408)	1.0 (435)

[00694] Three of these IR's were also evaluated on the human T cells that could be isolated from the spleens of the EMMESO mice. Interestingly, the expression levels of PD-1, TIM3, and LAG3 were all lower on the splenic T cells compared with the TILs (Table 7), supporting a key role for the tumor microenvironment in the upregulation of IR's.

[00695] Table 7. Comparison of expression of inhibitor receptors on TILs and splenic cells

[00696]

	Percent positive CD4 T cells			Percent positive CD8 T cells	
EMMESO TIL	Fresh TIL	Splenic T cells		Fresh TIL	Splenic T cells
PD-1	73	46 (22)		26	15 (17)
LAG-3	63	32 (132)		48	15 (11)
TIM3	24	17 (9)		56	31 (8)

[00697] Human mesoCAR TILs express increased levels of intracellular inhibitory enzymes.

[00698] The expression levels of two intrinsic inhibitors of T cell function that have been implicated in TIL dysfunction, SHP-1 and DGK, was examined using immunoblotting (FIG. 12). The levels of both isoforms of DGK (alpha and zeta), as well as the phosphorylated form of SHP1 (pSHPl), were significantly elevated in mesoCAR TILs that were freshly isolated from EMMESO flank tumor compared to overnight-rested TILs. This was also confirmed for DGK using flow cytometry where 23% of fresh EMMESO TILs expressed DGK. Expression was undetectable after overnight rest.

[00699] Block of inhibitors in human mesoCAR T cells enhances their ex vivo killing function.

[00700] Given these expression data, the potential functional importance of specific inhibitory pathways in mesoCAR TILs was studied by introducing available blocking agents during the *ex*

vivo killing and cytokine release assays. Addition of an anti-PDLl antibody significantly restored the killing activity and ability to secrete IFNby the mesoCAR TILs (FIG. 13A and 13B). The relatively high dose of lOug/ml anti-PDLl antibody was based on previously published investigations on cancer immunotherapy. Addition of either a type I or type II DGK inhibitor significantly increased the killing ability (FIG. 13C), but without significantly increasing tumor-induced IFN secretion (FIG. 13D). Addition of the SHPl inhibitor, sodium stibogluconate (SSG), slightly inhibited the killing ability of cryo mesoCAR T cells, but significantly increased that of the mesoCAR TILs (FIG. 13E), as well as significantly increasing tumor-induced IFN secretion (FIG. 13F) of the mesoCAR TILs. Dose response curves were performed for both DGK inhibitors and for SSG, and the highest doses which did not induced direct tumor cell killing were used.

[00701] Taken together, the results from these experiments demonstrate that hypofunction of CARTs *in vivo* is reversible. Specifically, modulation of the inhibitor mechanisms that reduce CART function can increase effector function, and therefore, may increase therapeutic efficacy. Inhibitors of inhibitor receptors, such as PD-1, LAG3, and TIM3, may be useful in combination therapy with CARTs for increased efficacy.

[00702] **Example 8: Characterization of human meso CAR**

[00703] *Generation of human CART-MSLN*

[00704] PBMCs from a normal subject were isolated from a blood sample. PBMCs were transduced with lentivirus constructs containing the human mesothelin CARs described herein. Transduction and culturing of the cells to produce CART-MSLN are described in the previous examples. The CARTs produced express mesothelin CARS with scFv constructs including Ml, M2, M3, M4, M5, M6, M7, M8, M9, M10, Mil, M12, M13, M14, M15, M16, M17, M18, M19, M20, M21, M22, M23, and M24. Control CARTs were also generated, including CART expressing CAR-ssl (murine anti-mesothelin scFv) (for positive control), and anti-CD19 CART (for negative control).

[00705] *Cytokine assay*

[00706] Cytokine production after stimulation by tumor cells was assessed. CARTs expressing M5, Ml1, Ml7, M21, ssl, and CAR19 were mixed with different tumor cell lines at a 1:1 effector to target ratio, and plated at 50,000 cell per well. The tumor cell lines were K562-meso (CML cell line expressing mesothelin), Ovcar3 (ovarian cancer), Ovcar8 (ovarian cancer),

and SW1990 (pancreatic adenocarcinoma). After 24 hours, CBA analysis was performed to determine cytokine expression and/or secretion of IFNy, TNF, IL-2, and IL-4.

[00707] As shown in FIG. 15A, CART-MSLN21 (M21) leads to the strongest activation by K562-Meso. CART-MSLN5 and CART-MSLNl 1 were stimulated by SW1990 to produce IFNγ. The production/secretion of IL-2 was similar for the tested CART-MSLN (FIG. 15C). As shown in FIG. 15D, there was hardly any production of IL-4. Other studies also showed very little production of IL-10.

[00708] *Killing assay*

[00709] The ability for the CART-MSLN to target and kill tumor cells was also assessed *in vitro*. A luciferase -based assay was used, with tumor cell lines expressing luciferase reporters. Target cells (tumor cells) were plated at 20,000 targets/well. CART-MSLN were added at varying effector:target ratios, from 10:1, 5:1, 2.5:1, and 1:1. The cells were cultured for 20 hours, and the luminescence of each well was detected to determine the percentage of target cells killed. Luciferase-expressing Ovcar3 (ovarian cancer) and U87mg (glioblastoma) cell lines were used as target cells.

[00710] As shown in FIG. 16A, CARTs expressing M5, Ml 1, M17 and ssl performed similarly well in killing target tumor cells. No killing was observed for CART-MSLN21. In contrast, no killing was observed for any of the CART constructs for glioblastoma cells (FIG. 16B). Similar results were obtained for the panel of human meso CARs, where Ml 1, M5, Ml7, and SSI had the highest specific killing of Ovcar3 cells (FIG. 17A and 17B).

[0071 1] *In vivo mouse model*

[00712] Anti-tumor activity of CART-MSLN was assessed *in vivo* in an Ovcar8 xenograft model. lOxlO^6 Ovcar8 cells were implanted subcutaneously on day 0 in NSG mice. At day 14, 2xl0 ^{6} CART cells (CARTs expressing M5, Ml 1, Ml7, M21, SSI, and CD19, or untransduced cells; lOxlO^6 total T cells) in a 100 μl dose was administered intravenously. Mice and tumors were monitored for about 40 days after tumor cell implantation.

[0071 3] Results from this experiment show that CART expressing M5 and Ml 1 exhibit strong anti-tumor activity in this model (FIG. 18). When compared to the mock treated group, M5 and Ml 1-treated mice exhibited statistically significant tumor reduction ($p<0.05$).

[00714] A second *in vivo* xenograft experiment was performed to assess the anti-tumor activity of a second set of CART-MSLN cells. lOxlO^6 OVCAR8-mcherry cells were injected into NSG mice. Once tumors were 150-250 mm^2, mice were randomized into treatment groups. CARTs expressing M12, M14, M16, M23, ssl, CD19, or untreated cells were injected at a dose of lOxlO^6 T cells in 100 μï. About 40% of T cells were expressing CAR, and therefore at the administered dosage, about 4x10^6 CART cells were delivered to each animal. Results from this experiment show that the CART expressing the human anti-MSLN (Ml2, M14, M16 and M23) did not have an effect on tumor growth (FIG. 19). These results taken together with the results from the first set of CART-MSLN assessed indicate that M5 and M11 have specific anti-tumor activity in comparison with the other CART-MSLN tested, and thereby demonstrate their potential for further investigation for cancer therapy. In addition, CART-SSI did not exhibit any anti-tumor activity in the first xenograft or the second xenograft experiment, further indicating that M5 and M11 containing CART-MSLN are more effective at reducing tumor growth.

Example 9: DGK Inhibition Augments CART Efficacy

[00715] Previous studies, for example, the experiments discussed in Example 6, have suggested that CAR T cells lose efficacy over time in vivo (e.g., in the tumor microenvironment). Specifically, mesoCAR T cells that were injected into a tumor mouse model were isolated from tumors after T cell infusion (e.g., 39 days after, hereinafter referred to as tumor infiltrating lymphocytes, TILs) and were assessed for their functional activity in comparison to freshly thawed mesoCAR T cells. The results showed that in ex vivo killing assays and IFNy release assays, the mesoCAR T cells isolated from the tumor had reduced ability to kill tumor cells (FIG. 20A), reduced IFNg production (FIG. 20B), and reduced ERK signaling (as shown by phosphorylation in western blot analysis, FIG. 20C) in response to antigen or CD3/CD28 stimuli (indicating reduced T cell activation.

[00716] Inhibitory mechanisms that possibly explain the decrease in CAR T cell activity in vivo over time include: soluble factors (TGFb, PGE2, adenosine, IL10, RAGE ligands, etc.), cell to cell contact (PD-1, LAG3, CTLA4, TIM3, CD160, etc.), and intrinsic activation-induced intracellular negative feedback systems (diaglycerol kinases: a and ζ isoforms, Egrs (2 and 3), SHP-1, NFAT2, BLIMP-1, Itch, GRAIL, Cbl-b, Ikaros, etc.). T cell activation can induce factors such as DGK. DGK, in turn, inhibits DAG signaling by phosphorylating DAG. This

limits DAG-induced activation of the RAS-ERK-APl pathway that leads to T cell activation. Previous studies have shown that mice deficient in DGKα or ΌGKζ results in CD4 T cells that demonstrate enhanced signal transduction and appear more resistant to anergy-inducing stimuli.

In vitro Cytotoxicity and Cytokine Release Assays

[00717] To investigate the effect of DGK inhibition on CART cell efficacy, transgenic mice with deletions in DGK genes DGKa, ΌGKζ, or both were utilized. Splenic T cells from wild-type and DGK-deficient mice were isolated, and transduced to express mesoCAR (SSI BBZ) using retrovirus. MIGR1 CAR was used as a control.

[0071 8] A cytotoxicity assay was performed using similar methods to those described in previous Examples. Wild-type and DGK-deficient (KO) mesoCAR expressing cells were incubated at various effectortarget ratios and cytotoxicity (% of target cells killed) was quantified (FIG. 21). As shown in Figure 21, deletion of DGKs markedly enhanced effector function of CAR T cells, especially at low effector: target ratios.

[0071 9] Similarly, IFNy release was examined in response to target cells at varying effectortarget ratios after 18 hours. As shown in Figure 22, deletion of DGKs was found to markedly enhance efefctor function of the mesoCAR T cells, especially at low effectortarget ratios.

[00720] Western blot analysis of DGK-deficient mesoCAR T cells in comparison to wild-type mesoCAR T cells showed increased ERK phosphorylation (FIG. 23), indicating that presence of DGK suppresses ERK signaling, while deletion of DGK results in increased ERK signaling. The increase in ERK signaling in the DGK deleted background suggests that inhibition of DGK results in activation of the Ras-ERK-APl pathway, and therefore, T cell activation.

[00721] Recent studies have shown that TGFP modulates the functionality of tumor-infiltrating CD8 T cells though interfering with RAS/ERK signal transduction, the same signaling molecules by which DGK deficiency confers augmented T cell effects. Sensitivity of the DGK-deficient mesoCAR T cells to TGFp was examined. WT and DGK-deficient mesoCAR T cells was incubated with mesothelin-expressing AE17 tumor cells + or - lOng/ml of TGFP for 18 hours. Cytotoxicity and IFNg production by these T cells was measured. As shown in Figure 24, TGFp inhibited killing by 50% in WT CAR T cells (arrows). However, this TGFp-induced inhibition was not observed in CAR T cells with DGK deletion, demonstrating that

DGK-deficient cells are not sensitive to TGFβ modulation, and are more resistant to inhibitor stimuli such as TGFP, which may contribute to the increase in T cell activity.

Therapeutic efficacy of mesoCAR and DGK inhibition in vivo

[00722] Next, therapeutic efficacy of mesoCAR T cells was examined in the context of DGK inhibition or deficiency. AE17meso tumor cells (mesothelioma cells) were injected subcutaneously into C57BL/6 mice. When tumors reached 100 mm^3 (approximately a week later), 10 million mesoCAR T cells were injected intravenously via tail vein. Tumor volumes were then followed over at least 18 days.

[00723] DGK-deficient mesoCAR T cells demonstrated enhanced and prolonged anti-tumor activity compared to wild-type (WT) mesoCAR and untreated cells (FIG. 25A). Specifically, each of the three DGK-deficient mesoCAR T cells was shown to inhibit tumor growth by volume compared to WT and untreated cells up to 18 days after injection. DGKz-deficient cells expressing mesoCAR were also shown to persist and proliferate better than wild-type meso CAR T cells in mice (FIG. 25B).

[00724] These results taken together show that DGK inhibition in combination with mesoCAR T cell treatment can improve mesoCAR T cell activation and anti-tumor activity in therapy.

Example 10: Inhibition of Ikaros augments anti-tumor capacity of CAR-T cells

[00725] One of the major hurdles in CAR T cell therapy is up-regulation of intrinsic negative regulators of T cell signaling, such as diacylglycerol kinase (DGK). As described in Example 9, CAR T cells have been shown to lose efficacy *in vivo* over time. Inhibition of negative regulators of T cell function such as DGK was shown to enhance activity and function of CAR-expressing T cells.

[00726] Another important negative regulator of T cell function is the transcription factor Ikaros. Unlike DGKs which act mainly in proximal TCR signaling, Ikaros is a zinc finger DNA binding protein that negatively regulates gene expression through the recruitment of chromatin remodeling complexes, such as Sin3A, CtBP, and HDACs. Ikaros plays a role in regulating cytokine production and cytolytic function in CD4+ T cells and CD8+ T cells,

[00727] In this example, anti-tumor efficacy of retrovirally-transduced CAR T cells with reduced Ikaros expression was examined *in vitro* and *in vivo.*

[00728] **Materials and Methods**

[00729] ***Cell lines.*** Mouse AE17 mesothelioma cells were described in Jackman et al, *J Immunol.* 2003;171:5051-63). Human mesothelin were introduced into AE17 cells by lentiviral transduction. 3T3Balb/C cells, were purchased from the American Type Culture Collection. Mouse FAP expressing 3T3BALB/C (3T3.FAP) cells were created by lentiviral transduction of the FAP- 3T3 parental line with murine FAP.

[00730] ***Animals.*** Pathogen-free C57BL/6 mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). Ikaros DN+/- mice contain one wildtype Ikaros allele and one Ikaros allele with a deletion of a DNA binding domain (Winandy et al, *Cell.* 1995; 83:289-99). Ikzfl+/- mice have one wildtype Ikaros allele and one allele with deletion of exon 7 (Avitahl et al., *Immunity.* 1999; 10:333-43). Animals used for all experiments were female mice between 6 and 12 weeks old and were housed in pathogen-free animal facilities.

[0073 1] ***Isolation, Transduction and Expansion of Primary Mouse T lymphocytes.*** Primary murine splenic T cells were isolated using the "Pan T cell Negative Selection" kit as suggested by the manufacturer (Miltenyi Biotec), and activated in 24-well plates ($4*10^6$ cells/well in 2 mL supplemented RPMI-1640 with 100 U/mL IL-2) pre-coated with -CD3 (1 μg/mL) and -CD28 (2 μg/mL). After 48 hours, cells ($lxlO^6$ cells/well) were mixed with retrovirus (1 mL crude viral supernatant) in a 24-well plate coated withRetronectin (50 μg/mL; Clontech) and centrifuged, without braking, at room temperature for 45 minutes at 1200g. After overnight incubation, cells were expanded with 50 U/mL of IL-2 for additional 48 hours.

[00732] ***Antigen- or antibody-coated beads.*** Recombinant mesothelin-extracellular domain protein, bovine serum albumin (Fisher Scientific) or anti-CD3/anti-CD28 antibodies (eBioscience) were chemically crosslinked to tosylactivated 4.5 μη Dynabeads (Invitrogen, #140-13) per manufacturers' instructions.

[00733] ***Immunoblotting.*** Anti-mesothelin-CAR transduced T cells were incubated either with BSA-, mesothelin-, or anti-CD3 antibody-coated beads (at 2:1 bead to T cell ratio) for 5 and 20 min. Total cell lysates were then prepared and immunoblotted for phosphorylated ERK, phosphorylated AKT, phosphorylated IKK, phosphorylated JNK, phosphorylated Lck, phosphorylated PKC, phosphorylated PLC, or phosphorylated ZAP70. All anti-phospho-protein

antibodies were purchased from Cell Signaling, with exception of anti-phospho-Lck, which was purchased from Sigma Aldrich. A C-terminus reactive goat anti-mouse antibody to Ikaros (SC-9861) and a goat anti-mouse actin antibody (SC-1615) were purchased from Santa Cruz. β-actin expression levels were determined to normalize the differences in loading.

[00734] ***Cytotoxicity and IFN ELISA.*** AE17, AE17.meso, 3T3 and 3T3.FAP cells were transduced with luciferase as described (Moon et al., *Clinical Cancer Research.* 2011; 17:4719-30). T cells and target cells were co-cultured at the indicated ratios, in triplicate, in 96-well round bottom plates. After 18 hours, the culture supernatants were collected for IFN analysis using an ELISA (mouse IFN, BDOpEIA). Cytotoxicity of transduced T cells was determined by detecting the remaining luciferase activity from the cell lysate using a previously described assay (Riese et al, *Cancer Res.* 2013; 73:3566-77).

[00735] ***CAR T cell transfer into mice bearing established tumors.*** Mice were injected subcutaneously with 2 χ 10^6 AE17.meso tumor cells into the dorsal-lateral flank of C57BL/6 mice. Mice bearing large established tumors (100-150 mm^3) were randomly assigned to receive either wildtype CAR T cells, Ikaros-deficient CAR T cells or remained untreated (minimum, five mice per group, each experiment repeated at least once). $1x10^7$ T cells were administered through the tail vein. Tumor size was measured by electronic scales and calipers, respectively.

[00736] For Day 9 T cell activity assessment, spleen and tumors were processed into single cell suspensions as previously described (Moon et al, *Clinical Cancer Research.* 2014; 20(16):4262-73) . Splenocytes and tumor single cell suspensions were re-stimulated with soluble anti-CD3/CD28 antibodies (1.0 μg/ml) or with phorbol ester/ionomycin (PMA/I: 30ng/ml, luM) for 4-6 hours in the presence of Golgi Stop (BD Biosciences, 0.66μ1/η11) and then harvested for flow cytometric analysis.

[00737] ***Flow cytometric assays.*** Fluorochrome conjugated antibodies against anti-mouse IFN-γ (XMG1), anti-mouse CD25 (PC61), anti-mouse IL-2 (JES6-1A12), anti-mouse CD8 (53-6.7), anti-mouse CD44 (IM7), and anti-mouse CD4 (GK1.5) were purchased from Biolegend. Fixable, Live/Dead Aqua stain (L34957) was purchased from Invitrogen. Fluorochrome antibody to anti-mouse Granzyme B (NGZB) and FoxP3 (FJK-16s) was purchased from eBioscience. Fluorochrome antibodies to anti-mouse TNF-a (MP6-XT22) and anti-mouse CD69 (H1.2F3) were purchased from BD Biosciences. For intracellular cytokine staining, cells were treated with Golgi Stop (BD Biosciences, 0.66 μg/ml) for 4-6 hours. Following harvesting, cells were fixed

with 1% paraformaldehyde for 30 minutes, spun down and washed once with FACS buffer. Cells were then washed with BD Perm Wash (BD Biosciences) 2 times and then stained with cytokine antibodies for 45 minutes at room temperature. Cells were washed 2 times in BD Perm Wash and then re-suspended in FACS Buffer. For transcription factor staining, cells were surfaced stained with fluorochrome-labeled primary antibodies for 20 minutes on ice. After washing in FACS buffer, cells were fixed with Fix/Perm buffer from eBioscience. Following fixation, cells were permeabilized and stained with APC anti-mouse FoxP3. For Ikaros staining, rabbit anti-mouse Ikaros (Abeam, ab26083, 1:2000) was used following fixation and permeabilization with the eBioscience FoxP3 kit. Following staining with the Ikaros antibody, cells were washed and then stained with a PE-labeled anti-rabbit secondary antibody (1:2000). Following completion of stains, cells were processed on a CyanADP (Beckman Coulter) for flow cytometric analysis.

[00738] ***Statistical Analysis.*** All statistical tests were done with GraphPad Prism. Two-way ANOVA was conducted with post-hoc testing, with * p<0.05, ** pO.01, *** pO.001, and **** p<0.0001. Data are presented as mean +/- SEM.

[00739] **Results**

[00740] ***Cytokine production and cytolytic mediator release in CAR-expressing T cells with reduced levels of Ikaros are augmented***

[00741] Given that cytolytic T lymphocytes (CTLs) with reduced Ikaros have enhanced effector function *in vitro* and *in vivo* (O'Brien, et al., *J Immunol.* 2014; 192:5118-29), experiments were performed to test if depletion of Ikaros could improve the efficacy of CAR T therapy. T cells isolated from wild type C57BL/6 and Ikaros-haplodeficient mice *(Ikzfl+/-)* in the C57BL/6 background were retrovirally-transduced to express a mesothelin CAR. Following *ex vivo* activation, transduction, and expansion in IL2, it was confirmed that, in comparison to wild-type (WT) CAR T cells, *Ikzfl+/-* CAR T cells continued to express less Ikaros protein by flow cytometry and western blot (Fig. 26A).

[00742] Since Ikaros is a transcriptional repressor for multi-cytokine gene loci (Thomas et al, *J Immunol.* 2007; 179:7305-15; Bandyopadhyay et al, *Blood.* 2006; 109:2878-2886; Thomas et al., *J of Biological Chemistry.* 2010; 285:2545-53; and O'Brien et al., *J Immunol.* 2014; 192:51 18-29), it was next examined if reduction of Ikaros resulted in autocrine cytokine production by CAR T cells and also whether or not the Ikaros-haplodeficient CAR T cells responded better than their WT counterparts to their target antigen. Both WT and *Ikzfl+/-* CAR

T cells were stimulated with beads coated with either BSA- (control) or mesothelin (the CAR antigen) at a 2:1 bead:T cell ratio for 6 hours, and analyzed their ability to produce IFNy, TNFβ and IL2 by flow cytometry. At baseline (BSA stimulation), there was an ~3-fold increase in IFN-producing *Ikzfl*+/- CAR T cells compared to WT CAR T cells (4.35% vs 1.4%, Fig. 26B), but there was no significant difference in the % IL2 producing cells (Fig. 26D). Following stimulation with mesothelin-coated beads, there was a dramatic increase in the % IFN-γ cytokine-producing *Ikzfl*+/- CAR T cells (25%) while the response was modest in WT CAR T cells (7%). An increase in TNFa production was also seen (Fig. 26C). To investigate if this augmentation in cytokine production was generalized across different stimuli or limited to CAR antigen, both WT and *Ikzf*+/- CAR T cells were treated with PMA and ionomycin for 6 hours. In this case, more IFN γ, TNF-β and IL-2 cytokine-producing cells were observed in the *Ikzfl*+/- CAR T cell compared to their WT counterparts(Figs. 26B-26D). These data support the hypothesis that Ikaros is one of the limiting factors that suppresses cytokine production of T cells, or CAR T cells.

[00743] An important cytotoxic mediator, granzyme B, was shown to be up-regulated in CD3/CD28-activated Ikaros-deficient OT-I cells, and this increased their cytolytic activity against OVA-expressing EL4 tumor cells (O'Brien et al, *J Immunol*. 2014; 192:5118-29). It was hypothesized that granzyme B production would also be enhanced in *Ikzf*+/- T cells bearing CAR. Both WT and *Ikzfl*+/- CAR T cells were stimulated with either BSA- (baseline) or mesothelin- (CAR antigen) coated beads at 2:1 bead:T cell ratio for 6 hours. PMA/ionomycin was used as the positive control for the assay. Similar to the data above, granzyme B level was higher in *Ikzf*+/- CAR T cells than in WT CAR T cells at baseline (BSA stimulation; Fig. 26E). After stimulation with either mesothelin-coated beads or PMA/ionomycin, granzyme B level increased in both WT and *Ikzf*+/- CAR T cells but the production was much higher in the *Ikzf*+/- CAR T cells (Fig. 26E). To determine if there was also a difference in degranulation of CAR T cells with reduced Ikaros, CD 107a expression after antigen stimulation was assessed. Wild-type transduced T cells had moderate levels of CD 107a expression following antigen re-stimulation, however, the T cells with reduced Ikaros demonstrated enhanced CD 107a up-regulation (Fig. 26F). Thus, in response to re-stimulation, the CTLs with reduced Ikaros degranulate more and release more cytotoxic mediators in comparison to their wild-type counterparts.

[00744] ***Depleting Ikaros with a dominant negative allele enhances CAR T cellfunction***

[00745] In addition to the cells with lower levels of Ikaros, T cells from mice expressing one dominant-negative allele of Ikaros (IkDN) were studied. Transgenic mice expressing IkDN have normal lymphoid development but have peripheral T cells with 90% reduced Ikaros DNA binding activity (Thomas et al., *J Immunol.* 2007; 179:7305-15; and Winandy et al., *Cell.* 1995; 83:289-99). T cells isolated from spleens of WT and IkDN mice were activated with plate-bound anti-CD3/CD28 antibodies, transduced with mesothelin CAR, followed by expansion with IL2. Knockdown of Ikaros in IkDN CAR T cells was confirmed by western. WT and IkDN CAR T cells were re-challenged with either BSA- or mesothelin-coated beads at 2:1 bead:T cell ratio for 6 hours, and analyzed their ability to produce IFN and IL2, as well as to de-granulate in response to CAR antigen. Similar to the *Ikzfl+/-* data above, some autocrine IFNy production at baseline was observed (Fig. 27A), but not with IL2 (BSA stimulation; Fig. 27B). Upon ligation of the CAR with its target antigen, mesothelin, IkDN T cells made more IFNy than WT T cells (Mesothelin stimulation; Fig. 27A). De-granulation, as measured by CD107a up-regulation, was also similar in both wild-type and IkDN CAR T cells (Fig. 27D).

[00746] ***Depletion of Ikaros did not augment activation and signaling of CAR T cells following antigen stimulation***

[00747] Given that depletion in Ikaros augmented cytokine release and increased the Granzyme B levels and CD107a expression of CAR T cells, possible mechanisms were explored. It is plausible that these changes in effector function could be due to differences in the activation of the wild-type *and Ikzfl+/-* transduced T cells. Thus, the levels of CD69, CD25 and 4-1BB (markers of T cell activation) were measured by flow cytometry after stimulating with mesothelin-coated beads for 6 and 24 hours. CD69, an early activation marker, was up-regulated to the same extent by both the wild-type and *Ikzfl+/-* cells (Fig. 28A). With longer stimulation, the wild-type and *Ikzfl+/-* CAR-expressing T cells continued to express similar levels of CD69, but i¾ɻ***H+/-*** transduced T cells exhibited increased CD25 expression (Fig. 28B). This may not directly indicate a difference in T cell activation, however, as increased IL-2 by *Ikzfl+/-* cells (Fig. 28D) can act in a positive feed-forward loop on CD25, the IL-2Ra (Depper et al, *ProcNatl AcadofSci USA.* 1985; 82:4230-4; and Nakajima et al, *Immunity.* 1997; 7:691-701). 4-1BB, a member of the TNF Receptor superfamily is also expressed on activated T cells (Vinay et al, *Seminars in Immunology.* 1998; 10:481-9) and was expressed at similar levels by CAR transduced wild-type *and Ikzf1 +/-* T cells following antigen stimulation (Fig. 28C). Thus,

functional differences between our WT and *Ikzf1*+/- transduced T cells were not due to differences in T cell activation.

[00748] The experiments described in Example 9 and Riese et al., *Cancer Research.* 2013; 73:3566-77', demonstrate that depletion of the enzyme diacylglycerol kinase (DGK) in CAR T cells resulted in an increase in RAS/ERK signaling, which correlated well with enhanced activation of CAR T cells. Some signaling pathways in WT and Ikaros-deficient T cells after TCR stimulation with CD3/CD28 antibodies were examined. Lysates from stimulated T cells were prepared and immunoblotted for various phospho-proteins implicated in proximal (PLC and Lck) and distal (ERKl/2, INK, AKT and IKKa) signaling from the TCR. There was a constitutive low-level baseline activation of some TCR signaling proteins in Ikaros-deficient T cells, including Lck, ERK and AKT (Fig. 28D). With TCR/CD28 stimulation, all proteins studied were phosphorylated to the same level when comparing WT and Ikaros-deficient T cells, with the exception of phospho-IKK, which was slightly higher in Ikaros-deficient T cells 20 minutes after stimulation. To determine if the NFB pathway was enhanced in T cells with reduced Ikaros level, the same blot was re-probed for IB, the downstream target for IKK. There was no difference in IB degradation between both WT and Ikaros-depleted T cells. To study CAR signaling, both WT and IkDN mesothelin CAR transduced T cells were re-stimulated with mesothelin-coated beads. Similar to the data with CD3/CD28 stimulation, no difference was found in phosphorylation of PLC and ERK (Fig. 28E). Together, these data indicate that depletion in Ikaros does not alter TCR/CAR-mediated signaling.

[00749] ***Reduction of Ikaros in CAR T cells augments their response against their target cells.***

[00750] Given the increased production of effector factors by CAR T cells with reduced Ikaros, their efficacy against their target tumor cells *in vitro* was tested. Wild-type, *Ikzf1*+/- and IkDN T cells expressing mesoCAR were mixed at different ratios with the parental tumor cell line, AE17 or the mesothelin-expressing cell line, AE17meso. When mixed with the parental cell line, both the wild-type, *Ikzf1*+/- and IkDN T cells failed to produce IFN-γ or lyse cells in response to AE17 (Figs. 29A, 29B and 29C). In contrast, when reacted with AE17meso, IFN-γ production and cytolysis by wild-type T cells increased as the E:T ratio increased (Figs. 29B and 29C). However, both the *Ikzf1*+/- and IkDN T cells produced significantly more IFN-γ and had

significantly increased tumor lysis than wild-type T cells, even at the lowest E:T ratio 1.3:1 (Figs. 29B, and 29C).

[00751] To study the generalizability of this effect, T cells expressing a different CAR construct, which targets fibroblast activation protein (FAP-CAR) and has the same intracellular signaling domain as the mesothelin CAR used above, were examined. The efficacy of comparably transduced FAP-CAR splenic T cells isolated from WT C57BL/6 was compared to those from *Ikzfl+/-* mice. *Ikzfl+/-* FAP-CAR T cells were more efficient in lysing 3T3.FAP cells (Fig. 29D) and in secreting more IFN (Fig. 29E) than WT FAP CAR T cells, with retention of specificity *in vitro.*

[00752] ***Depletion of Ikaros enhances the efficacy of CAR T cells against established tumors***

[00753] The capability of mesothelin-specific T cells with reduced Ikaros *(Ikzfl+/-* and IkDN) to control growth of established AE17meso tumors in mice was next examined. Two million of AE17meso tumor cells were injected into the flanks of syngenic C57BL/6 mice and allowed to form large established tumors (-100-150 mm^3). Ten million CAR T cells prepared from WT or Ikaros-deficient *(Ikzfl+/-* and IkDN) mice were then adoptively transferred into those tumor-bearing mice, and tumor measurements were followed. Mild tumor growth inhibition was induced by wild-type transduced mesoCAR T cells, while both Ikzfl+/- and IkDN transduced mesoCAR T cells inhibited growth of AE17meso tumors significantly more (Figs. 30A and 30B).

[00754] It was also studied if reduction of Ikaros could enhance the therapeutic potential of FAP-CAR T cells. Mice with established AE17meso tumors (100-150 mm^3) were adoptively transferred with 10 millions wild-type or *Ikzfl+/-* transduced anti-mouse FAP CAR T cells. Mice receiving wild-type transduced cells provided minimal tumor delay and the AE17meso tumors continued to grow (Fig. 30C). In contrast, the *Ikzfl+/-* transduced T cells were able to significantly delay tumor growth.

[00755] ***Ikzfl+/- CAR T cells persist longer and more resistant to immunosuppressive tumor microenvironment than WT CAR T cells***

[00756] Given the enhanced efficacy of the Ikaros-inhibited CAR T cells, the possible mechanisms using the Ikzfl+/- mesoCAR T cells were explored. To further interrogate how these mesoCAR T cells operate in an immunosuppressive tumor microenvironment *in vivo,* tumors at 3 and 9 days post-adoptive transfer were harvested and assessed their number and

functionality. These two time points allowed characterization of their activity at early and late time points during the anti-tumor immune response.

[00757] At Day 3 post-transfer, we observed a similar frequency of wild-type and Ikzfl+/- mesoCAR T cells in both the spleens (Fig. 31A) and tumors (Fig. 3IB). These similar levels indicate that both the wild-type and Ikzfl+/- mesoCAR T cells initially traffic equally well to the tumor. In assessing the Day 9 timepoint, the number of both wild-type mesoCAR T cells *Ikzfl+/-* mesoCAR T cells declined in the spleen. However, when the tumors were examined at this time point, there was a significant increase in the number of *Ikzfl+/-* mesoCAR T cells compared with WT mesoCAR T cells (Fig. 3IB). These data show that the *Ikzfl+/-* mesoCAR T cells either persist or proliferate better than WT mesoCAR T cells in the immunosuppressive microenvironment.

[00758] Tumor infiltrating lymphocytes (TILs) become hypofunctional in response to their cognate antigens within the immunosuppressive tumor microenvironment, and is a key phenomenon associated with tumor progression (Prinz et al., *J Immunol.* 2012; 188:5990-6000; and Kerkar et al, *Cancer Res.* 2012; 72:3 125-30). Although there were more *Ikzfl+/-* mesoCAR T cells in the tumors at Day 9, they could still be adversely affected by the tumor microenvironment. To evaluate functionality, CD3/CD28 antibodies were used to stimulate TILS isolated from wild-type and *Ikzfl+/-* mesoCAR T cells at Day 9 post-transfer and characterized differences in lytic mediator production. At Day 9 post-transfer, the wild-type mesoCAR T cells in the spleen continued to produce some moderate levels of IFN (Fig. 31C). As expected, isolated wild-type T cells from the tumors produced much less of this cytokine in comparison to wild-type T cells isolated from the spleen. This indicates that the wild-type TILs begin to become hypofunctional at Day 9 post transfer. In contrast, splenic *Ikzfl+/-* mesoCAR T cells continued to produce more IFN- at baseline and upon stimulation (Fig. 31C). Compared to the wild-type TILs, the *Ikzfl+/-* TILs produced higher amounts of IFNy. These data indicate that Ikzfl+/- TILs could be less sensitive to the immunosuppressive tumor microenvironment.

[00759] Bypassing the proximal defect of TCR signaling often seen in TILs (Prinz, PU et al., *J. Immunol.* 2012; 188:5990-6000) can be achieved through use of PMA/Ionomycin (PMA/I) (Prinz et al., *J Immunol.* 2012; 188:5990-6000). Wild-type and *Ikzfl+/-* mesoCAR T cells were re-stimulated with PMA/I to determine if TCR-stimulation insensitive wild-type and *Ikzfl+/-* TILs were still capable making cytokines in response to other stimuli. At Day 9 post-transfer, the

wild-type TILs demonstrated a noticeable drop in IFN-γ production (Fig. 31C), and this was partially restored through stimulation with PMA/I (Fig. 3ID). However, stimulation of splenic and tumor isolated *Ikzfl+/-* mesoCAR T cells still resulted in increased levels of IFN-γ in comparison to wild-type transferred cells. Through bypassing any defects in TCR signaling via PMA/I stimulation, these results demonstrate that IFNy production differs at the chromatin level and is likely due to differential Ikaros function.

[00760] In light of the increased anti-tumor activity by the transferred *Ikzfl+/-* T cells, the impacts on the composition of the immunosuppressive tumor microenvironment was also examined and the number of regulatory T cells (Tregs) and Myeloid Derived Suppressor Cells (MDSCs) was evaluated at Day 9. At Day 9 post-transfer, similar levels of Tregs in hosts that received wild-type or *Ikzfl+/-* mesoCAR T cells was observed (Fig. 3IE). The presence of Ly6G-/CDl lb+/CD206+ macrophages was determined, which are typically characterized as immunosuppressive and pro-tumorigenic M2 macrophages. In the Day 9 treated groups, that CD206 expression was similar in all 3 groups (untreated, wild-type, and Ikzfl+/-) (Fig. 31F).

[0076 1] ***T cells with reduced Ikaros are less sensitive to soluble inhibitoryfactors TGF and adenosine***

[00762] To further characterize the interaction of the immunosuppressive tumor microenvironment with the mesoCAR T cells, an *in vitro* culture system was utilized. Soluble inhibitory factors such as IDO, IL-10, Adenosine, and TGF-β (Wang et al., *Oncoimmunology.* 2013; 2:e26492) have been shown to contribute to inhibiting infiltrating tumor lymphocytes. The effects of select inhibitory factors *in vitro* on the Ikaros-deficient CAR T cells was tested to determine if the immunosuppressive environment could impact their lytic function. Wild-type CAR T cells had a 50% reduction in their ability to make IFN-γ and had a reduction in their lytic function in the presence of TGF-β and Adenosine (Fig. 32). CAR T cells with reduced levels of Ikaros *(Ikzfl+/-* and IkDN) continued to produce more IFN γ than their wild-type counterparts in the absence of inhibitors and were only marginally inhibited in the presence of TGF β and Adenosine (Fig. 32A). Increased lytic function in *Ikzfl+/-* and IkDN CAR T cells in comparison to wild-type T cells was observed (Fig. 32B). These data demonstrate that the T cells with reduced Ikaros are less sensitive to TGFp and adenosine inhibition.

[00763] **Discussion**

[00764] In this example, a new approach for enabling CAR-expressing T cells to survive and enhance their effector functions in the tumor environment has been identified: inactivation of the transcriptional repressor Ikaros, which is known to inhibit a diverse array of genes involved in T cell function, e.g., cytokine genes (IL2 and IFNy), cytolytic mediators (granzyme B), and the key T-box transcription factors that influence T cell differentiation (R-Bet and Eomes).

[00765] An important finding from the experiments described above is that CAR T cells that were deficient in Ikaros function were significantly better than wild type CAR T cells in restricting tumor growth (Fig. 30). These results were observed in multiple tumor models and using two different CAR constructs. Due to the high number of genes that Ikaros regulates, it is plausible that Ikaros may regulate many pathways that are normally sensitive to immunosuppression. Increased IFN-g production by lowering Ikaros level in CAR T cells can result in up-regulation of Class I MHC expression on the tumor, and thereby improving its immunogenicity, improving anti-angiogenic activity, and driving STAT1 mediated fuction of Thl cells. A possible effect of increased IFNy could have been an alternation of the macrophage phenotype within the tumors, however, no differences in the total number of macrophages, nor the proportion of M2-like macrophages (as measured by CD206 expression) was observed. The increased IL-2 production could have also increased the formation of CD4 Treg cells, however, no differences were observed when comparing the tumors treated with WT CAR T cells with Ikaros-deficient CAR T cells.

[00766] An increased number of tumor infiltrating lymphocytes nine days after injection was observed. This was not likely due to increased trafficking, since the number of WT versus Ikaros-deficient TIL was similar at Day 3. Instead, these results suggest that Ikaros-deficient TIL showed increased proliferation or decreased antigen-induced cell death (AICD). *In vitro* studies suggest that AICD was similar between the two types of T cells, making it more likely that the difference was due to increased proliferation. This would be consistent with the increased IL-2 produced by these cells. In addition to increased persistence, the Ikaros-deficient TIL appeared to be less hypofunctional. When tumor-infiltrating CAR T cells were re-stimulated with anti-CD3 antibody or PMA and lonomycín, CAR TILs with Ikaros deficiency were able to make more IFNy than their wild-type counterparts (Fig. 31C and 3ID).

[00767] The *in vitro* studies allowed further studies of the mechanistic underpinnings of the observed increased anti-tumor efficacy *in vivo.* Consistent with the known inhibitor functions of

Ikaros, deletion of one Ikaros allele *(Ikzf+/-)* or replacing one of its alleles with an Ikaros dominant negative construct (IkDN) resulted in T cells that had some increased baseline autocrine IFNy and Granzyme B production (Fig. 26), but more importantly, showed markedly augmented cytokine secretion and granule release after TCR or CAR stimulation *in vitro.* This was accompanied by increased tumor cell killing *in vitro.* To test whether or not Ikaros-deficient CAR T cells have lower activation threshold than WT CAR T cells, Dynabeads were coated with 10-fold less mesothelin protein and it was shown *that Ikzf+/-* CAR T cells could still respond to the mesothelin antigen at low-density to make IFNy and TNF-a but the WT CAR T cells could not. The Ikaros-disabled CAR T cells were also more resistant to inhibition by known immunosuppressive factors such as TGF-β and adenosine (Fig. 32). This may be due to the fact that cytokine (i.e. IL2 and IFNy and T cell effector (i.e. granzyme B) genes are more accessible for transcription in Ikaros-deficient T cells following TCR/CAR activation. This appears to help compensate for suboptimal T cell activation within immunosuppressive tumor microenvironment.

[00768] In previous studies, e.g., Example 9, a similar phenotype (i.e. increase in cytolysis and IFN production) has been observed in CAR T cells through depletion of DGKs, enzymes that metabolize the second messenger diacylglycerol and limit RAS/ERK activation. With DGK deletion, however, clear changes were observed in the CAR/TCR signaling pathway. Specifically, RAS/ERK activation was dramatically enhanced after both TCR and CAR activation. This resulted in enhanced activation, as measured by increased CD69 upregulation, however production of effector molecules such as TRAIL, FasL and IFNy. Perforin and Granzyme B were similar between WT and DGK-deficient CAR T cells. A very different phenotype in this study with Ikaros-deficient CAR T cells. In contrast to the DGK-deficient CAR T cells, the Ikaros-deficient CAR T cells had similar CAR/TCR activation as shown by CD69 and CD25 upregulation (Fig. 28), and similar CAR/TCR signaling as measured by phosphorylation of multiple TCR signaling molecules (Fig 34) and calcium signaling. Unlike DGK-deficient T cells, Ikaros-depleted CAR T cells had higher granzyme B and IFN levels at baseline (Figs. 26 and 27), as well as constitutive low level activation of some TCR signaling cascades such as ERK and Akt (Fig. 28). This baseline activation may be due to induction of T-bet (Thomas et al., *J of Biol Chemistry.* 2010; 285:2545-53), which cooperates with other transcription factor like Eomes to transactivate IFN and granzyme B gene expression (Pearce et al, *Science.* 2003; 302:1041-3; and Intelkofer et al, *Nat Immunol.* 2005; 6:1236-44).

[00769] These findings raise the possibility that therapeutically targeting Ikaros in transduced human T cells (in clinical trials) might be beneficial using either genetic or biochemical approaches. Genetic approaches could mimic these results in mice and include knockdown of Ikaros in CAR T cells using shRNA or use of a dominant negative construct to compete with endogenous Ikaros. Another option would be to use a pharmacological inhibitor to lower Ikaros levels transiently. Recent reports have indicated that the immunomodulatory drug, Lenalidomide, targets Ikaros for ubiquitin-mediated degradation by the E3 ligase complex CRL4CRBN (Gandhi et al., *Br J Haematol.* 2013; 164:811-21; Kronke et al, *Science.* 2014; 343:301-5; and Sakamaki et al., *Leukemia.* 2013; 28:329-37). CD3-stimulated human T cells treated with Lenalidomide produce more IL-2 (Gandhi et al, *Br J Haematol.* 2013; 164:811-21), a key trait of T cells with reduced Ikaros levels. In preliminary studies, TCR/CD28 stimulated mesothelin-CAR transduced human PBMCs *in vitro* produce more IL-2 and IFNy after pretreatment with lenalidomide. Thus, the combination of CAR T cell therapy with *in vivo* administration of Lenalidomide may provide a therapeutic strategy for reversal of T cell hypofunction through inhibition of Ikaros.

[00770] In conclusion, this example demonstrates for the first time that targeting a transcriptional repressor can enhance CAR-mediated anti-tumor immunity. The mechanisms involved enhanced cytokine and effector function without alterations in signal transduction. Translating this approach into the clinic can be pursued through the use of shRNA, a dominant negative construct, or a pharmacological inhibitor (like Lenalidomide) to target Ikaros in CAR-expressing T cells.

Example 11: Meso-CART in human ovarian cancer

[00771] In this example, the safety of intravenous infusion of autologous T cells transduced to express a mesothelin CAR was determined in a human patient with advanced recurrent serou ovarian cancer.

[00772] A single patient treatment protocol was modeled after a planned Phase I study (as described in Example 3) to assess the safety and feasibility of infusing autologous T cells expressing mesothelin CAR (mesoCART). T cells from the patient were transduced to express a CAR comprising an extracellular anti-mesothelin single chain variable fragment (scFv) fused to 4-1BB and TCRzeta signaling domains. $3xl0^{7}$ mesoCAR T cells/m^2, for a total of 4.65×10^{7}

meso CAR T cells, were infused into the patient. No lymphodepletion with chemotherapy was given prior to infusion.

[00773] The mesoCART infusion had no acute toxic effects. The patient experienced grade 3 bilateral pleural effusions, dyspnea, fever, hypotension, and aspiration. There were no clinical toxicities due to off tumor/on target effects of the meso CART cells, such as pericarditis or peritonitis. The patient was treated with tocilizumab (anti-IL6) on Day 21 after infusion for suspected cytokine release syndrome (CRS), based on unexplained fevers, hypotension requiring pressors, elevated serum levels of ferritin (>7000 ng/mL) and CRP (>160 mg/L).

[00774] The mesoCAR T cells were detectable in peripheral blood samples, as well as tumor samples from liver and peritoneum, with highest numbers in pericardial and pleural fluid. Cytokine evaluation showed a marked increase in IL-6 within the pleural fluid (>80-fold increase over baseline), peaking at Day 22-23. Evaluation of the blood also revealed elevation of IL-6 levels in the blood (15-fold increase over baseline at Day 22). No elevation in TNFα or IFNy levels was detected.

[00775] Preliminary results showed that CT imaging of abdomen and pelvis performed Day 21 showed peritoneal carcinomatosis minimally improved compared to a CT imaging two weeks prior. Specifically, one tumor decreased from 3.4 x 3.1 cm to 3.Ox 2.6 cm. Cytology from the left sided pleural fluid showed malignancy on Day 8 after infusion, but at Day 21 and Day 26, there was no evidence of malignancy.

[00776] These results showed that infusion of meso CART cells without lymphodepletion was found to be feasible and safe. There was no evidence of toxicity with infusion, or life-threatening pericarditis or peritonitis. Also, there was clinical evidence of treatment efficacy as evidenced by the disappearance of malignant pleural effusions.

Example 12; Transient expression of CARs with varying intracellular signaling domains

[00777] In this example, the use of distinct costimulatory domains in the CAR architecture and its effects on cell longevity, memory differentiation, and cell metabolism characteristics are examined.

[00778] A novel system of *in vitro* T cell stimulation was developed to study the consequences of a single round of CAR-specific stimulation and analyze signaling through various CAR signaling endodomains. *In vitro* transcribed mRNA encoding anti-mesothelin SS1-

CAR constructs with varying intracellular signaling domains of CD3zeta, CD28:z, and 4-lBB:z were electroporated into primary resting human T cells. Greater than 90% CAR-positive T cell populations were achieved by this method. Upon verifying CAR expression, the T cells were stimulated with recombinant mesothelin immobilized on beads and then cultured. Since the RNA is transiently expressed, the CAR disappears from the cell surface after one round of stimulation, allowing the unambiguous analysis of signaling through the CAR alone (i.e., without subsequent signaling from additional mesothelin binding events). This approach thus obviated the requirement for stimulation through the endogenous T cell receptor and permitted for the first time an analysis of the consequences of signaling through the surrogate antigen of CAR T cells. These experiments were conducted on primary peripheral blood T cells, sorted naive T cells and cord blood cells.

[00779] The various CAR constructs (SS-1 with CD3zeta, SSI with CD28:z, and SSI with 4-lBB:z) were expressed at equivalent levels on the surface of T cells. All signaling domains were demonstrated to have comparable and specific cytolytic capabilities when cultured with target cells that expressed mesothelin.

[00780] Primary T cells stimulated through the 4-lBBz-containing CARs showed superior survival and expansion profiles when compared to CD28-based CARs. Both 4-1BB and CD28-based CARs exhibited more than 5 population doublings. However, the 4-lBB-based CAR T celsl proliferated for longer *in vitro.* Phenotypic analysis of the 4-lBB-based CAR T cells revealed that an increased population of cells with central-memory surface markers was generated. In contrast, the CD28-based CARs rapidly differentiated to an effector memory pool of CAR T cells.

[00781] CD28z-containing CARs yielded a significantly higher proportion of effector memory cells, with a modest increase in expression of inhibitory PD-1, TIM3 and LAG3 molecules relative to their 4-lBBz counterparts. These results remained consistent whether starting populations of bulk peripheral blood T cells, naive (CD45RO-CD95-CD62L+CCR7+ sorted) peripheral blood T cells, or cord blood T cells were used.

[00782] Metabolic profiling of the mesothelin-stimulated CAR T cells using the Sea-Horse assay (Seahorse Biosciences)in culture revealed a substantial increase in lipid oxidation in 4-lBBz-CAR stimulated cells compared to their CD28z counterparts. Additionaly, microarray

studies have revealed a unique gene signature in cells that are recovered after stimulation through the different CAR signaling domains.

[00783] Together, these results show that CAR T cells exhibit differential survival and function depending on the costimulatory domains. This system also allows for rapid characterization and functional analysis of new CAR designs.

Example 13: Effects of mTOR Inhibition on Immunosenescence in the Elderly

[00784] One of the pathways most clearly linked to aging is the mTOR pathway. The mTOR inhibitor rapamycin has been shown to extend lifespan in mice and improve a variety of aging-related conditions in old mice (Harrison, DE et al. (2009) *Nature* 460:392-395; Wilkinson JE et al. (2012) *Aging Cell* 11:675-682; and Flynn, JM et al. *{2013) Aging Cell* 12:851-862). Thus, these findings indicate that mTOR inhibitors may have beneficial effects on aging and aging-related conditions in humans.

[00785] An age-related phenotype that can be studied in a short clinical trial timeframe is immunosenescence. Immunosenescence is the decline in immune function that occurs in the elderly, leading to an increased susceptibility to infection and a decreased response to vaccination, including influenza vaccination. The decline in immune function with age is due to an accumulation of immune defects, including a decrease in the ability of hematopoietic stem cells (HSCs) to generate naive lymphocytes, and an increase in the numbers of exhausted PD-1 positive lymphocytes that have defective responses to antigenic stimulation (Boraschi, D et al. (2013) *Sci. Transl. Me4.* 5:185ps8; Lages, CS et al. (2010) *Aging Cell* 9:785-798; and Shimatani, K et al., (2009) *Proc. Natl. Acad. Sci. USA* 106:15807-15812). Studies in elderly mice showed that 6 weeks of treatment with the mTOR inhibitor rapamycin rejuvenated HSC function leading to increased production of naive lymphocytes, improved response to influenza vaccination, and extended lifespan (Chen, C et al. (2009) *Sci. Signal.* 2:ra75).

[00786] To assess the effects of mTOR inhibition on human aging-related phenotypes and whether the mTOR inhibitor RAD001 ameliorates immunosenescence, the response to influenza vaccine in elderly volunteers receiving RADOOl or placebo was evaluated. The findings presented herein suggest that RADOOl enhanced the response to influenza vaccine in elderly volunteers at doses that were well tolerated. RADOOl also reduced the percentage of

programmed death (PD)-l positive CD4 and CD8 T lymphocytes that accumulate with age. These results show that mTOR inhibition has beneficial effects on immunosenescence in elderly volunteers.

[00787] As described herein, a 6 week treatment with the mTOR inhibitor RAD001, an analog of rapamycin, improved the response to influenza vaccination in elderly human volunteers.

Methods

Study population

[00788] Elderly volunteers >= 65 years of age without unstable underlying medical diseases were enrolled at 9 sites in New Zealand and Australia. Exclusion criteria at screening included hemoglobin < 9.0 g/dL, white blood cell count <3,500/mm^3, neutrophil count <2,000/mm^3, or platelet count <125,000/mm^3, uncontrolled diabetes, unstable ischemic heart disease, clinically significant underlying pulmonary disease, history of an immunodeficiency or receiving immunosuppressive therapy, history of coagulopathy or medical condition requiring long-term anticoagulation, estimated glomerular filtration rate < 30 ml/min, presence of severe uncontrolled hypercholesterolemia (>350 mg/dL, 9.1 mmol/L) or hypertriglyceridemia (>500 mg/dL, 5.6 mmol/L).

[00789] Baseline demographics between the treatment arms were similar (Table 8). Of the 218 subjects enrolled, 211 completed the study. Seven subjects withdrew from the study. Five subjects withdrew due to adverse events (AEs), one subject withdrew consent, and one subject left the study as a result of a protocol violation.

Table 8: Demographic and Baseline characteristics of the Study Patients

Population		RAD001 0.5 mg daily N=53	RAD001 5 mg weekly N=53	RAD001 20 mg weekly N=53	Placebo pooled N=59	Total N=218
Age (Years)	**Mean (SD)**	70.8 (5.0)	72.0 (5.3)	71.4 (5.2)	71.1 (5.1)	71.3 (5.2)
Gender	**Male– n (%)**	34 (64%)	27 (51%)	32 (60%)	31 (53%)	124 (57%)
BMI* (kg/m2)	**Mean (SD)**	27.4 (4.2)	28.8 (5.0)	28.0 (4.1)	28.0 (4.2)	28.0 (4.4)

Race - n (%)	**Caucasian**	48 (91%)	50 (94%)	46 (87%)	54 (92%)	198 (91%)
	Other	5(9%)	3 (6%)	7 (13%)	5 (8%)	20 (9%)

*The body-mass index is weight in kilograms divided by the square of the height in meters

Study Design and Conduct

[00790] From December 201 1 to April 2012, 2 18 elderly volunteers were enrolled in a randomized, observer-blind, placebo-controlled trial. The subjects were randomized to treatment arms using a validated automated randomization system with a ratio of RADOOl to placebo of 5:2 in each treatment arm. The treatment arms were:

RADOOl 0.5 mg daily or placebo

RADOOl 5 mg weekly or placebo

RADOOl 20 mg weekly or placebo

[00791] The trial was observer-blind because the placebo in the RADOOl 0.5 mg daily and 20 mg weekly cohorts differed slightly from the RADOOl tablets in those cohorts. The study personnel evaluating the subjects did not see the study medication and therefore were fully blinded. The treatment duration for all cohorts was 6 weeks during which time subjects underwent safety evaluations in the clinic every 2 weeks. After subjects had been dosed for 4 weeks, RADOOl steady state levels were measured pre-dose and at one hour post dose. After completing the 6 week course of study drug, subjects were given a 2 week drug free break to reverse any possible RADOOl-induced immunosuppression, and then were given a 2012 seasonal influenza vaccination (Agrippal®, Novartis Vaccines and Diagnostics, Siena, Italy) containing the strains HlNl A/California/ 07/2009, H3N2 A/Victoria/2 10/2009, B/Brisbane/60/ 2008. Four weeks after influenza vaccination, subjects had serum collected for influenza titer measurements. Antibody titers to the 3 influenza vaccine strains as well as to 2 heterologous strains (A/HINI strain A/New Jersy/8/76 and A/H3N2 strain A/Victoria/361/1 1) were measured by standard hemagglutination inhibition assay (Kendal, AP et al. (1982) *Concepts and procedures for laboratory-based influenza surveillance. Atlanta: Centers for Disease Control and Prevention* B17-B35). Levels of IgG and IgM specific for the A/HlNl/California/07/2009 were measured in serum samples taken before and 4 weeks after influenza vaccination as described previously

(Spensieri, F. et al. *(2013) Proc. Natl. Acad. Sci. USA* 110:14330-14335). Results were expressed as fluorescence intensity.

[00792] All subjects provided written informed consent. The study was conducted in accordance with the principals of Good Clinical Practice and was approved by the appropriate ethics committees and regulatory agencies.

Safety

[00793] Adverse event assessment and blood collection for hematologic and biochemical safety assessments were performed during study visits. Adverse event information was also collected in diaries that subjects filled out at home during the 6 weeks they were on study drug. Data on all adverse events were collected from the time of informed consent until 30 days after the last study visit. Events were classified by the investigators as mild, moderate or severe.

Statistical Analysis

[00794] The primary analysis of geometric mean titer ratios was done using a normal Bayesian regression model with non-informative priors. This model was fitted to each antibody titer on the log scale. The primary outcome in each model was the Day 84 measurement. The Day 63 measurement was included in the outcome vector. The model fitted using SAS 9.2 proc mixed with the prior statement. The covariance structure of the matrix was considered as unstructured (option type=UN). A flat prior was used. For the secondary analysis of seroconversion rates, logistic regression was used.

[00795] The intention to treat population was defined as all subjects who received at least one full dose of study drug and who had no major protocol deviations impacting efficacy data. 199 out of the total of 218 subjects enrolled in the study were in the intention to treat population.

Immunophenotyping

[00796] Peripheral blood mononuclear cells were isolated from whole blood collected at 3 time points: baseline; after 6 weeks of study drug treatment; and at the end of study when subjects had been off study drug for 6 weeks and 4 weeks after influenza vaccination. Seventy-six PBMC subsets were analyzed by flow cytometry using 8-color immunophenotyping panels at the Human Immune Monitoring Center at Stanford University, CA, USA as described previously (Maecker, HT et al. (2012) *Nat Rev Immunol.* 12: 191-200). Seventy-six PBMC subsets were analyzed by flow cytometry using 8-color lyophilized immunophenotyping panels (BD Lyoplate,

BD Biosciences, San Diego, CA). PBMC samples with viability >80% and yield of $2x10^6$ cells or greater were included in the analysis.

[00797] Relative changes of the immunophenotypes from baseline to Week 6 of study drug treatment and from baseline to the end of study (Week 12) were calculated for each of the RADOOl dosing cohorts. Student T test was conducted to examine if the relative change of the immunophenotypes from baseline to the two blood sampling time points was significantly different from zero, respectively, within each dosing group after adjusting for placebo effect. Missing data imputation in treatment effect analysis was not conducted. Therefore if a patient has a missing phenotype data at baseline, this patient was not be included in the analysis for this phenotype. If a patient had a missing phenotype data at 6 or 12 weeks, then this patient did not contribute to the analysis of this phenotype for the affected timepoint.

[00798] 608 tests in 76 phenotypes under 3 dosing groups were conducted to compare the treatment effect against the placebo effect. Stratified false discovery rate (FDR) control methodology was implemented to control the occurrence of false positives associated with multiple testing yet provide considerably better power. The cell type group was taken as the stratification factor and conducted FDR (q-value) calculation within each stratum respectively. All null-hypotheses were rejected at 0.05 significance level with corresponding q-value ≤ 0.1. The multiple testing adjustment strategy with rejecting at 0.05 significance level and corresponding $q<0.1$ ensured that less than 10% of the findings are false.

[00799] In a second analysis, the immunophenotype changes between pooled treatment and placebo groups, where all three RADOOl dosing groups were combined. To determine which immunophenotype changes differed between the treated and placebo groups, within-patient cell count ratios for each measured phenotype were calculated between baseline and Week 6 of study drug treatment and between baseline and the end of study (Week 12). The ratios were log transformed, and analyzed by analysis of covariance at each time point in order to detect a difference between the pooled treatment and placebo groups. 152 tests in 76 phenotypes were performed to compare the pooled treatment effect against the placebo effect. Stratified false discovery rate (FDR) control methodology was implemented to control the occurrence of false positives associated with multiple testing yet provide considerably better power (Benjamini, Y. et al. (1995) *J. Roy. Statist.* 57:289-300; and Sun, L. et al. (2006) *Genet. Epidemiol.* 30:519-530). The cell type group was taken as the stratification factor and FDR (q-value) calculation was

conducted within each stratum respectively. All null-hypotheses at 0.05 significance level and q-value less than 20% were rejected. This can be interpreted as rejecting only those hypotheses with P values less than 0.05 and less than 20% probability that the each observed significant result is due to multiple testing.

Results

[00800] In general, RAD001 was well tolerated, particularly the 0.5 mg daily and 5 mg weekly dosing regimens. No deaths occurred during the study. Three subjects experienced four serious adverse events (SAEs) that were assessed as unrelated to RAD001. The 4 SAEs were retinal hemorrhage of the left eye with subsequent blindness in a subject with normal platelet counts who had completed a 6 week course of 5 mg weekly RAD001 6 weeks previously; severe back pain in a subject treated with placebo and severe gastroenteritis in a subject treated with placebo. A list of treatment-related adverse events (AEs) with an incidence >2% in any treatment group is provided in Table 9. The most common RAD001-related AE was mouth ulcer that, in the majority of cases, was of mild severity. Overall, subjects who received RAD001 had a similar incidence of severe AEs as those treated with placebo. Only one severe AE was assessed as related to RAD001 mouth ulcers in a subject treated with 20 mg weekly RAD001.

Table 9: Incidence of treatment-related AEs >2% in any treatment group by preferred term

	RAD001 0.5 mg daily N=53 n (%)	**RAD001 5 mg weekly N=53 n (%)**	**RAD001 20 mg weekly N=53 n (%)**	**Placebo, pooled N=59 n (%)**	**Total N=218 n (%)**
Total AE(s)	35	46	109	21	211
Patients with AE(s)	22 (41.5%)	20 (37.7%)	27 (50.9%)	12 (20.3%)	81 (37.2%)
Mouth ulceration	6 (11.3%)	2 (3.8%)	9 (17.0%)	3 (5.1%)	20 (9.2%)
Headache	0	2 (3.8%)	9 (17.0%)	1 (1.7%)	12 (5.5%)
Blood cholesterol increased	2 (3.8%)	2 (3.8%)	2 (3.8%)	0	6 (2.8%)
Diarrhea	1 (1.9%)	4 (7.5%)	1 (1.9%)	0	6 (2.8%)
Dyspepsia	0	3 (5.7%)	2 (3.8%)	1 (1.7%)	6 (2.8%)
Fatigue	0	2 (3.8%)	4 (7.5%)	0	6 (2.8%)
Low density lipoprotein increased	2 (3.8%)	1 (1.9%)	2 (3.8%)	0	5 (2.3%)
Tongue ulceration	3 (5.7%)	1 (1.9%)	0	1 (1.7%)	5 (2.3%)
Insomnia	1 (1.9%)	2 (3.8%)	1 (1.9%)	0	4 (1.8%)
Dry mouth	0	0	2 (3.8%)	1 (1.7%)	3 (1.4%)
Neutropenia	0	0	3 (5.7%)	0	3 (1.4%)
Oral pain	0	2 (3.8%)	1 (1.9%)	0	3 (1.4%)
Pruritus	0	2 (3.8%)	1 (1.9%)	0	3 (1.4%)
Conjunctivitis	0	2 (3.8%)	0	0	2 (0.9%)
Erythema	0	2 (3.8%)	0	0	2 (0.9%)
Limb discomfort	0	2 (3.8%)	0	0	2 (0.9%)
Mucosal inflammation	0	0	2 (3.8%)	0	2 (0.9%)
Paresthesia oral	2 (3.8%)	0	0	0	2 (0.9%)
Stomatitis	0	0	2 (3.8%)	0	2 (0.9%)
Thrombocytopenia	0	0	2 (3.8%)	0	2 (0.9%)
Urinary tract infection	0	0	2 (3.8%)	0	2 (0.9%)

[00801] The ability of RAD001 to improve immune function in elderly volunteers was evaluated by measuring the serologic response to the 2012 seasonal influenza vaccine. The hemagglutination inhibition (HI) geometric mean titers (GMT) to each of the 3 influenza vaccine strains at baseline and 4 weeks after influenza vaccination are provided in Table 10. The primary analysis variable was the HI GMT ratio (4 weeks post vaccination/baseline). The study was powered to be able to demonstrate that in at least 2 out of 3 influenza vaccine strains there was 1) a ≥ 1.2-fold GMT increase relative to placebo; and 2) a posterior probability no lower than 80% that the placebo-corrected GMT ratio exceeded 1. This endpoint was chosen because a 1.2-fold increase in the influenza GMT ratio induced by the MF-59 vaccine adjuvant was associated with a decrease in influenza illness (lob, A et al. (2005) *Epidemiol Infect* 133:687-693).

Table 10. HI GMTs for each influenza vaccine strain at baseline and at 4 weeks after influenza vaccination

Influenza Vaccine Strain		Time	RAD001 0.5mg daily N=50	RAD001 5mg weekly N=49	RAD001 20mg weekly N=49	Placebo N=55
A/H1N1	GMT (CV%)	Baseline	102.8 (186.9)	84.2 (236.4)	90.1 (188.4)	103.2 (219.7)
		Week 4	190.2 (236.9)	198.73(195.6)	129.7 (175.9)	169.4 (259.8)
	GMT ratio (CV%)		2.6 (302.5)	2.5 (214.3)	1.8 (201.5)	2.0 (132.7)
A/H3N2	GMT (CV%)	Baseline	106.8 (168.2)	126.04 (162.6)	137.1 (211.5)	131.7 (162.3)
		Week 4	194.4 (129.1)	223.0 (118.8)	223.0 (163.6)	184.3 (153.2)
	GMT ratio (CV%)		2.1 (152.6)	2.0 (189.2)	2.1 (277.3)	1.6 (153.6)

B	**GMT (CV%)**	**Baseline**	**44.2 (96.6)**	**64.8 (87.3)**	**58.0 (156.0)**	**57.0 (112.6)**
		Week 4	**98.4 (94.8)**	**117.3 (99.9)**	**99.2 (124.1)**	**114.6 (136.7)**
	GMT ratio (CV%)		**2.5 (111.2)**	**2.2 (112.8)**	**2.1 (126.5)**	**2.2 (109.2)**

Baseline indicates 2 weeks prior to influenza vaccination
Week 4 indicates 4 weeks after influenza vaccination
N is number of subjects per cohort
GMT is geometric mean titer
GMT ratio is the GMT at week 4 post vaccination/GMT at baseline
CV% indicates coefficient of variation

[00802] In the intent-to-treat (ITT) population, the low, immune enhancing, dose RAD001 (0.5 mg daily or 5 mg weekly) cohorts but not higher dose (20 mg weekly) cohort met the primary endpoint of the study (Figure 33A). This demonstrates that there is a distinct immunomodulatory mechanism of RAD001 at the lower doses, and that at the higher dose the known immunosuppressive effects of mTOR inhibition may come into play. Furthermore, the results suggest a trend toward improved immune function in the elderly after low, immune enhancing, dose RAD001 treatment.

[00803] In a subgroup analysis, the subset of subjects with low baseline influenza titers (< 1:40) experienced a greater RAD001-associated increase in titers than did the ITT population (Figure 33B). These data show that RAD001 is particularly effective at enhancing the influenza vaccine response of subjects who did not have protective (>1:40) titers at baseline, and therefore were at highest risk of influenza illness.

[00804] Scatter plots of RAD001 concentration versus increase in titer to each influenza vaccine strain show an inverse exposure/response relationship (Figure 34). Modeling and simulation based on mTOR mediated phosphorylation of S6 kinase (S6K) predicts that the 20 mg weekly dosing regimen inhibits mTOR-mediated S6K activity almost completely, the 5 mg weekly dosing regimen inhibits S6K activity by over 50%, and the 0.5 mg daily dosing regiment inhibits S6K phosphorylation by approximately 38% during the dosing interval (Tanaka, C et al. (2008) *J. Clin. Oncol* 26:1596-1602). Thus, partial mTOR inhibition, e.g., mTOR-mediated S6K phosphorylation, with low, immune enhancing, dose RAD001 may be as, if not more effective,

than near complete mTOR inhibition with high dose RADOOl at enhancing the immune response of the elderly.

[00805] Rates of seroconversion 4 weeks after influenza vaccination were also evaluated. Seroconversion was defined as the change from a negative pre-vaccination titer (i.e., HI titer < 1:10) to post-vaccination HI titer ≥ 1:40 or at least 4-fold increase from a non-negative (> 1:10) pre-vaccination HI titer. In the intention-to-treat population, seroconversion rates for the H3N2 and B strains were increased in the RADOOl as compared to the placebo cohorts although the increases did not meet statistical significance (Table 11). In the subpopulation of subjects with baseline influenza titers <= 1:40, RADOOl treatment also increased the rates of seroconversion to the H3N2 and B strains, and these results reached statistical significance for the B strain in the 0.5 mg daily dosing cohort. These data further show that RADOOl enhanced the serologic response to influenza vaccination in the elderly.

Table 11: Percent of subjects with seroconversion to influenza 4 weeks after vaccination

	Placebo N=54	**0.5 mg N=48**	**5 mg N=49**	**20 mg N=48**
Intention to Treat Population				
H1N1	24	27	27	17
H3N2	17	27	24	25
B	17	27	22	19
Subjects with Baseline Titers <=40				
H1N1	40	42	45	36
H3N2	42	64	53	71
B	16	40*	33	28

* Odds ratio for seroconversion between RADOOl and Placebo significantly different than 1 (two-sided p-value < 0.05 obtained by logistic regression with treatment as fixed effect)

[00806] Current seasonal influenza vaccines often provide inadequate protection against continuously emerging strains of influenza that present as variants of previously circulating viruses. However, mice vaccinated against influenza in the presence of the mTOR inhibitor rapamycin, as compared to placebo, developed a broader serologic response to influenza. The broader serologic response included antibodies to conserved epitopes expressed by multiple subtypes of influenza that provided protection against infection with heterologous strains of influenza not contained in the vaccine (Keating, R et al. (2013) *Nat Immunology* 14:2166-2178). To determine if RADOOl broadened the serologic response to influenza in the elderly volunteers, HI titers to 2 heterologous strains of influenza not contained in the influenza vaccine (A/HINI

strain A/New Jersey/8/76 and A/H3N2 strain A/Victoria/361/1 1) were measured. The increase in the HI GMT ratios for the heterologous strains was higher in the RADOOl as compared to placebo cohorts (Figure 35). In addition, seroconversion rates for the heterologous strains were higher in the RADOOl as compared to placebo cohorts. The increase in seroconversion rates in the 5 and 20 mg weekly RADOOl dosing cohorts was statistically significant for the H3N2 heterologous strain (Table 12). The H3N2 seroconversion rate for the pooled RADOOl cohorts was 39% versus 20% for the placebo cohort (p=0.007). The results presented herein suggest that mTOR inhibition broadens the serologic response of elderly volunteers to influenza vaccination, and increases antibody titers to heterologous strains of influenza not contained in the seasonal influenza vaccine.

[00807] Broadened serologic response to heterologous strains of influenza in mice treated with rapamycin has been associated with an inhibition of class switching in B cells and an increase in anti-influenza IgM levels (Keating, R. et al. (2013) *Nat Immunol* 14:2166-2178). However, inhibition of class switching may not be involved in the broadened serologic response in humans treated with RADOOl because the post-vaccination anti-influenza IgM and IgG levels did not differ between RADOOl and placebo treated cohorts (Figure 36).

Table 12: Percentage of subjects who seroconvert to heterologous strains of influenza 4 weeks after seasonal influenza vaccination

	Placebo, pooled	**RAD001 0.5mg daily**	**RAD001 5 mg weekly**	**RAD001 20 mg weekly**
A/H1N1 strain: A/NewJersey/8/76	7%	17%	16%	8%
A/H3N2 strain: A/Victoria/361/11	20%	38%	39%*	40% *

* Odds ratio for seroconversion between RAD001 and Placebo significantly different than 1 (two-sided p-value < 0.05 obtained by logistic regression with treatment as fixed effect)

[00808] To address the mechanism by which RADOOl enhanced immune function in elderly volunteers, immunophenotyping was performed on PBMC samples obtained from subjects at baseline, after 6 weeks of study drug treatment and 4 weeks after influenza vaccination (6 weeks

after study drug discontinuation). Although the percentage of most PBMC subsets did not differ between the RADOOl and placebo cohorts, the percentage of PD-1 positive CD4 and CD8 cells was lower in the RADOOl as compared to placebo cohorts (Figure 37). PD-1 positive CD4 and CD8 cells accumulate with age and have defective responses to antigen stimulation because PD-1 inhibits T cell receptor-induced T cell proliferation, cytokine production and cytolytic function (Lages, CS et al. (2010) *Aging Cell* 9:785-798). There was an increase in percentage of PD-1 positive T cells over time in the placebo cohort. At week 12 (4 weeks post-vaccination) this increase may have been due to influenza vaccination since influenza virus has been shown to increase PD-1 positive T cells (Erikson, JJ et al. (2012) *JCI* 122:2967-2982). However the percentage of CD4 PD-1 positive T cells decreased from baseline at week 6 and 12 in all RADOOl cohorts (Figure 37A). The percentage of CD8 PD-1 positive cells also decreased from baseline at both week 6 and 12 in the two lower dose RADOOl cohorts (Figure 37B). The percentage of PD-1 negative CD4 T cells was evaluated and increased in the RADOOl cohorts as compared to the placebo cohorts (Figure 37C).

[00809] Under more stringent statistical analysis, where the results from the RADOOl cohorts were pooled and adjusted for differences in baseline PD-1 expression, there was a statistically significant decrease of 30.2% in PD-1 positive CD4 T cells at week 6 in the pooled RAD cohort (n=84) compared to placebo cohort (n=25) with p=0.03 (q=0.13) (Figure 38A). The decrease in PD-1 positive CD4 T cells at week 12 in the pooled RAD as compared to the placebo cohort is 32.7% with p=0.05 (q=0.19). Figure 38B shows a statistically significant decrease of 37.4% in PD-1 positive CD8 T cells at week 6 in the pooled RADOOl cohort (n=84) compared to placebo cohort (n=25) with p=0.008 (q=0.07). The decrease in PD-1 positive CD8 T cells at week 12 in the pooled RADOOl as compared to the placebo cohort is 41.4% with p=0.066 (q=0.21). Thus, the results from Figures 37 and 38 together suggest that the RADOOl-associated decrease in the percentage of PD-1 positive CD4 and CD8 T cells may contribute to enhanced immune function.

Conclusion

[00810] In conclusion, the data presented herein show that the mTOR inhibitor RADOOl ameliorates the age-related decline in immunological function of the human elderly as assessed by response to influenza vaccination, and that this amelioration is obtained with an acceptable risk/benefit balance. In a study of elderly mice, 6 weeks treatment with the mTOR inhibitor rapamycin not only enhanced the response to influenza vaccination but also extended lifespan,

suggesting that amelioration of immunosenescence may be a marker of a more broad effect on aging-related phenotypes.

[0081 1] Since RAD001 dosing was discontinued 2 weeks prior to vaccination, the immune enhancing effects of RAD001 may be mediated by changes in a relevant cell population that persists after discontinuation of drug treatment. The results presented herein show that RAD001 decreased the percentage of exhausted PD-1 positive CD4 and CD8 T cells as compared to placebo. PD-1 expression is induced by TCR signaling and remains high in the setting of persistent antigen stimulation including chronic viral infection. While not wishing to be bound by theory, is possible that RAD001 reduced chronic immune activation in elderly volunteers and thereby led to a decrease in PD-1 expression. RAD001 may also directly inhibit PD-1 expression as has been reported for the immunophilin cyclosporine A (Oestreich, KJ et al. (2008) *J Immunol.* 181:4832-4839). A RADOOl -induced reduction in the percentage of PD-1 positive T cells is likely to improve the quality of T cell responses. This is consistent with previous studies showing that mTOR inhibition improved the quality of memory CD8 T cell response to vaccination in mice and primates (Araki, K et al. (2009) *Nature* 460: 108-1 12). In aged mice, mTOR inhibition has also been shown to increase the number of hematopoietic stem cells, leading to increased production of naive lymphocytes (Chen, C et al. (2009) *Sci Signal* 2:ra75). Although significant differences in the percentages of naive lymphocytes in the RADOOl versus placebo cohorts were not detected in this example, this possible mechanism may be further investigated.

[00812] The mechanism by which RADOOl broadened the serologic response to heterologous strains of influenza may be further investigated. Rapamycin has also been shown to inhibit class switching in B cells after influenza vaccination. As a result, a unique repertoire of anti-influenza antibodies was generated that promoted cross-strain protection against lethal infection with influenza virus subtypes not contained in the influenza vaccine (Keating, R et al. (2013) *Nat Immunol.* 14:2166-2178). The results described herein did not show that RADOOl altered B cell class switching in the elderly subjects who had discontinued RADOOl 2 weeks prior to influenza vaccination. Although the underlying mechanism requires further elucidation, the increased serologic response to heterologous influenza strains described herein may confer enhanced protection to influenza illness in years when there is a poor match between the seasonal vaccine and circulating strains of influenza in the community.

[00813] The effect of RADOOl on influenza antibody titers was comparable to the effect of the MF59 vaccine adjuvant that is approved to enhance the response of the elderly to influenza vaccination (Podda, A (2001) *Vaccine* 19:2673-2680). Therefore, RADOOl-driven enhancement of the antibody response to influenza vaccination may translate into clinical benefit as demonstrated with MF59-adjuvanted influenza vaccine in the elderly (lob, A et al. (2005) *Epidemiol Infect.* 133:687-693). However, RADOOl is also used to suppress the immune response of organ transplant patients. These seemingly paradoxical findings raise the possibility that the immunomodulatory effects of mTOR inhibitors may be dose and/or antigen-dependent (Ferrer, IR et al. (2010) *J Immunol.* 185:2004-2008). A trend toward an inverse RADOOl exposure/vaccination response relationship was seen herein. It is possible that complete mTOR inhibition suppresses immune function through the normal cyclophilin-rapamycin mechanism, whereas partial mTOR inhibition, at least in the elderly, enhances immune function due to a distinct aging-related phenotype inhibition. Of interest, mTOR activity is increased in a variety of tissues including hematopoietic stem cells in aging animal models (Chen C. et al. (2009) *Sci Signal 2*:ra75 and Barns, M. et al. (2014) *Int J Biochem Cell Biol.* 53:174-185). Thus, turning down mTOR activity to levels seen in young tissue, as opposed to more complete suppression of mTOR activity, may be of clinical benefit in aging indications.

[00814] The safety profile of mTOR inhibitors such as RADOOl in the treatment of aging-related indications has been of concern. The toxicity of RADOOl at doses used in oncology or organ transplant indications includes rates of stomatitis, diarrhea, nausea, cytopenias, hyperlipidemia, and hyperglycemia that would be unacceptable for many aging-related indications. However, these AEs are related to the trough levels of RADOOl in blood. Therefore the RADOOl dosing regimens used in this study were chosen to minimize trough levels. The average RADOOl trough levels of the 0.5 mg daily, 5 mg weekly and 20 mg weekly dosing cohorts were 0.9 ng/ml, below 0.3 ng/ml (the lower limit of quantification), and 0.7 ng/ml, respectively. These trough levels are significantly lower than the trough levels associated with dosing regimens used in organ transplant and cancer patients. In addition, the limited 6 week course of treatment decreased the risk of adverse events. These findings suggest that the dosing regimens used in this study may have an acceptable risk/benefit for some conditions of the elderly. Nonetheless, significant numbers of subjects in the experiments described hereindeveloped mouth ulcers even when dosed as low as 0.5 mg daily. Therefore the safety

profile of low, immune enhancing, dose RADOOl warrants further study. Development of mTOR inhibitors with cleaner safety profiles than currently available rapalogs may provide better therapeutic options in the future for aging-associated conditions.

Example 14: Enhancement of Immune Response to Vaccine in Elderly Subjects

[0081 5] Immune function declines in the elderly, leading to an increase incidence of infection and a decreased response to vaccination. As a first step in determining if mTOR inhibition has anti-aging effects in humans, a randomized placebo-controlled trial was conducted to determine if the mTOR inhibitor RADOOl reverses the aging-related decline in immune function as assessed by response to vaccination in elderly volunteers. In all cases, appropriate patent consents were obtained and the study was approved by national health authorities.

[00816] The following 3 dosing regimens of RADOOl were used in the study:

20 mg weekly (trough level: 0.7 ng/ml)

5 mg weekly (trough level was below detection limits)

0.5 mg daily (trough level: 0.9 ng/ml)

[0081 7] These dosing regimens were chosen because they have lower trough levels than the doses of RADOOl approved for transplant and oncology indications. Trough level is the lowest level of a drug in the body. The trough level of RADOOl associated with the 10 mg daily oncology dosing regimen is approximately 20 ng/ml. The trough level associated with the 0.75-1.5 mg bid transplant dosing regimen is approximately 3 ng/ml. In contrast, the trough level associated with the dosing regimens used in our immunization study were 3-20 fold lower.

[0081 8] Since RADOOl-related AEs are associated with trough levels, the 3 dosing regimens were predicted to have adequate safety for normal volunteers. In addition, the 3 doses were predicted to give a range of mTOR inhibition. P70 S6 Kinase (P70 S6K) is a downstream target that is phosphorylated by mTOR. Levels of P70 S6K phosphorylation serve as a measure of mTOR activity. Based on modeling and simulation of P70 S6K phosphorylation data obtained in preclinical and clinical studies of RADOOl, 20 mg weekly was predicted to almost fully inhibit mTOR activity for a full week, whereas 5 mg weekly and 0.5 mg daily were predicted to partially inhibit mTOR activity.

[00819] Elderly volunteers >= 65 years of age were randomized to one of the 3 RAD001 treatment groups (50 subjects per arm) or placebo (20 subjects per arm). Subjects were treated with study drug for 6 weeks, given a 2 week break, and then received influenza (Aggrippal, Novartis) and pneumoccal (Pneumovax 23, Merck), vaccinations. Response to influenza vaccination was assessed by measuring the geometric mean titers (GMTs) by hemagglutination inhibition assay to the 3 influenza strains (H1N1, H3N2 and B influenza subtypes) in the influenza vaccine 4 weeks after vaccination. The primary endpoints of the study were (1) safety and tolerability and (2) a 1.2 fold increase in influenza titers as compared to placebo in 2/3 of the influenza vaccine strains 4 weeks after vaccination. This endpoint was chosen because a 1.2 fold increase in influenza titers is associated with a decrease in influenza illness post vaccination, and therefore is clinically relevant. The 5 mg weekly and 0.5 mg daily doses were well tolerated and unlike the 20 mg weekly dose, met the GMT primary endpoint (Figure 33A). Not only did RAD001 improve the response to influenza vaccination, it also improved the response to pneumococcal vaccination as compared to placebo in elderly volunteers. The pneumococcal vaccine contains antigens from 23 pneumococcal serotypes. Antibody titers to 7 of the serotypes were measured in our subjects. Antibody titers to 6/7 serotypes were increased in all 3 RAD cohorts compared to placebo.

[00820] The combined influenza and pneumococcal titer data suggest that partial (less than 80-100%) mTOR inhibition is more effective at reversing the aging-related decline in immune function than more complete mTOR inhibition.

Example 15: Low dose mTOR inhibition increases energy and exercise

[00821] In preclinical models, mTOR inhibition with the rapalog rapamycin increases spontaneous physical activity in old mice (Wilkinson et al. Rapamycin slows aging in mice. (2012) Aging Cell; 11:675-82). Of interest, subjects in the 0.5 mg daily dosing cohort described in Example 2 also reported increased energy and exercise ability as compared to placebo in questionnaires administered one year after dosing (Figure 39). These data suggest that partial mTOR inhibition with rapalogs may have beneficial effects on aging-related morbidity beyond just immune function.

Example 16: P70 S6 kinase inhibition with RADOOl

[00822] Modeling and simulation were performed to predict daily and weekly dose ranges of RAD001 that are predicted to partially inhibit mTOR activity. As noted above, P70 S6K is phosphorylated by mTOR and is the downstream target of mTOR that is most closely linked to aging because knockout of P70 S6K increases lifespan. Therefore modeling was done of doses of RADOOl that partially inhibit P70 S6K activity. Weekly dosing in the range of >= 0.1 mg and < 20 mg are predicted to achieve partial inhibition of P70 S6K activity (Figure 40).

[00823] For daily dosing, concentrations of RADOOl from 30 pM to 4 nM partially inhibited P70 S6K activity in cell lines (Table 13). These serum concentrations are predicted to be achieved with doses of RADOOl >= 0.005 mg to < 1.5 mg daily.

Table 13: Percent inhibition of P70 S6K activity in HeLa cells *in vitro*

RAD001 concentration	0	6 pM	32 pM	160 pM	800 pM	4 nM	20 nM
% P70 S6K inhibition	0	0	18	16	62	90	95

Conclusion

[00824] Methods of treating aging-related morbidity, or generally enhancing an immune response, with doses of mTOR inhibitors that only partially inhibit P70 S6K. The efficacy of partial mTOR inhibition with low doses of RADOOl in aging indications is an unexpected finding. RADOOl dose ranges between >= 0.1 mg to < 20 mg weekly and >= 0.005 mg to < 1.5 mg daily will achieve partial mTOR inhibition and therefore are expected to have efficacy in aging-related morbidity or in the enhancement of the immune response.

Example 17: MSLN CART in Ovarian Cancer

[00825] Anti-tumor activity of a single dose of 2 x 10^6 mesothelin CAR T cells was previously assessed in an *in vivo* ovarian tumor mouse model, as described in Example 8. In order to see if the anti-tumor activity with the M5 and M1 1 CAR T cells in this model, as described in Example 8, could be increased, the CAR T cells were administered a higher dose of 4 x 10^6 mesothelin CAR T cells 14 days after tumor implantation when the tumor volumes were an average of 150mm^3, and a follow-up identical dose of CAR T cells was administered. Half of the mice were given the second identical dose of the CAR T cells five days later to see if this would enhance the anti-tumor activity seen previously in this model.

Materials and Methods:

[00826] ***Cell line:*** OVCAR8 is a human ovarian cancer cell line that was derived from a 64 year old female patient with ovarian adenocarcinoma, and has been transduced to express mCherry. Cells were grown in RMPI medium containing 10% fetal bovine serum. This cell line grows adherent in tissue culture flasks. This cell line forms tumors in mice when implanted subcutaneously in the flank and mixed with matrigel. The addition of matrigel to the cells during the implantation allow for a matrix to develop in the flank of the mouse that provides a structure for the tumor cells to start growing on. Over 10-12 days, the matrigel plug degrades and the solid tumor that remains is made up of tumor cells and surrounding stromal cells. The OVCAR8 cells have been modified to express mCherry, so that that tumor cell growth can also be monitored by imaging the mice.

[00827] ***Mice:*** 6 week old NSG (NOD.*Cg-Prkdc*^{sc,d}*Il2rg*^{tmlWjl}*/Sz*J) mice were received from the Jackson Laboratory (stock number 005557). Animals were allowed to acclimate in the Novartis NIBRI animal facility for at least 3 days prior to experimentation. Animals were handled in accordance with Novartis ACUC regulations and guidelines. Electronic transponders for animal identification were implanted on the left flank one day prior to tumor implantation. Mice were shaved on the right flank prior to tumor implantation.

[00828] ***Tumor implantation:*** OVCAR8 cells in log phase were harvested after tyrpsinization with 0.25% trypsin-EDTA. The cells were washed in 50ml falcon tubes at 1200rpm for 5 minutes, once in growth media and then two times in cold sterile PBS. The cells were resuspended in PBS at a concentration of 100x1 0^6 per ml and kept on ice. The PBS solution containing the cells was then mixed 1:1 with matrigel, resulting in a final concentration of 50x1 0^6 cells per ml of PBS-matrigel. The cells were placed on ice and immediately implanted in mice. Tumors were implanted subcutaneously in 200ul on the right flank.

[00829] The OVCAR8 model endogenously expresses mesothelin and thus, can be used to test the in vivo efficacy of mesothelin directed CAR T cells. This model grows well when implanted subcutaneously in the flank of mice and can be calipered for tumor volume measurements. Upon implantation of lOxlO^6 tumor cells in a 50:50 mix of PBS and matrigel, the tumors establish and can be accurately measured in one week. Within 13-15 days, the mean tumor volume is 100-200mm^3, with tumors reaching an endpoint volume (1000- 1200mm^3) by 60-65 days. Anti-tumor activities of therapeutic agents are often tested once tumors are fully

engrafted and matrigel resorbed. Thus, there is a large window with this model during which the anti-tumor activity of the CAR T cells can be observed.

[00830] ***CAR Tcell dosing:*** Mice were dosed with 4xl0 ^{6} CAR T cells (13.3xl0 ^{6} total T cells) 14 days after tumor implantation. Cells were partially thawed in a 37 degree Celsius water bath and then completely thawed by the addition of 1 ml of cold sterile PBS to the tube containing the cells. The thawed cells were transferred to a 15 ml falcon tube and adjusted to a final volume of 10 mis with PBS. The cells were washed twice at lOOOrpm for 10 minutes each time and then counted on a hemocytometer. T cells were then resuspended at a concentration of 133xl0 ^{6} cells per ml of cold PBS and kept on ice until the mice were dosed. The mice were injected intravenously via the tail vein with 100 ul of the T cells for a dose of 4x10^{6} CAR T cells (13.3xl0 ^{6} total T cells) per mouse. Seven mice per group were either treated with 100 ul of PBS alone (PBS) or T cells transduced with an isotype CAR (Isotype). 14 mice per group were treated with either the SSI mesothelin CAR T cells, M5 mesothelin CAR T cells, or Ml 1 mesothelin CAR T cells. Five days later the SSI, M5, and Ml 1 groups were split in half and seven mice in each group were treated with an identical second dose of the CAR T cells. The groups were identified as SSI single (one dose of SSI CAR T cells), SSI double (two doses of SSI CAR T cells), M5 single (one dose of the M5 CAR T cells), M5 double (two doses of the M5 CAR T cells), Ml 1 single (one dose of the Ml 1 CAR T cells), Ml 1 double (two doses of the Ml 1 CAR T cells). The isotype T cells, SSI T cells, M5 T cells, and Ml 1 T cells were all prepared from the same donor in parallel.

[0083 1] ***Animal monitoring:*** The health status of the mice was monitored daily, including twice weekly body weight measurements. The percent change in body weight was calculated as (BWcurrent - BWinitiai)/(BWinitiai) x 100%. Tumors were monitored 2-3 times weekly by calipering and tumor volumes (TV) were calculated using the ellipsoid formula: TV(mm ^{3}) = ((length x width ^{2}) x 3.141 59)) / 6. Tumor volumes are reported as the mean +/- standard error of the mean (SEM). Percent treatment/control (T/C) values were calculated using the following formula:

% T/C = 100 x ΔT/AC if AT $\geq$ 0 ;

% Regression = 100 x ΔT/T_{initia}ι if AT < 0 ;

where T = mean tumor volume of the drug-treated group on the final day of the study; $T_{i\eta itia_1}$ = mean tumor volume of the drug-treated group on initial day of dosing; AT = mean tumor volume of the drug-treated group on the final day of the study - mean tumor volume of the drug treated

group on the initial day of dosing; C = mean tumor volume of the control group on the final day of the study; and AC = mean tumor volume of the control group on the final day of the study - mean tumor volume of the control group on the initial day of dosing. T/C values in the range of 100% - 42% are interpreted to have no or minimal anti-tumor activity; T/C values that are ≤ 42% and > 10% are interpreted to have anti-tumor activity or tumor growth inhibition. T/C values ≤ 10% or regression values ≥ -10% are interpreted to be tumor stasis. Regression values < -10% are reported as regression.

Results:

[00832] The anti-tumor activity of mesothelin CAR T cells were assessed in a subcutaneous ovarian adenocarcinoma xenograft model. Following tumor cell implantation on day 0, tumor bearing mice were randomized into treatment groups and were administered 4xl0 ^{6} CAR T cells (13.3xl0 ^{6} total T cells) intravenously via the lateral tail vein on day 14 after tumor implantation. A second identical dose of T cells was given to half of the mice 5 days later. Tumor growth and animal health were monitored until animals achieved endpoint. The mice in all groups were euthanized between days 57 and 62 when tumors were beginning to show signs of ulceration leading to a change in animal body condition scores.

[00833] The mean +/- SEM tumor volume for all treatment groups is plotted in Figure 43. The PBS treatment group, which did not receive any T cells, demonstrates baseline OVCAR8 tumor growth kinetics in subcutaneously implanted NSG mice. The Isotype treatment group received T cells transduced with a control CAR. These cells serve as a T cell control to show the non-specific response of human donor T cells in this model. Both the PBS and the Isotype treatment groups demonstrate continuous tumor progression throughout this study. The Isotype group shows a slight slower tumor growth, due to the background activity of the donor T cells. Similar to previous studies a single dose of 4x10^{6} CAR+ T cells leads to no changes in tumor growth for the SSI treated group. A double dose of the SSI CAR T cells similarly led to no changes in OVCAR8 tumor growth. The M5 and Ml 1 single dose groups show clear anti-tumor activity, as they did previously when dosed with 2x10^{6} CAR+ T cells. Both the M5 and Ml 1 double dose groups show an increased anti-tumor activity as compared with the single dose groups. The tumors in these groups show regression as opposed to tumor growth inhibition. The M5 double dose group demonstrates a complete regression in four out of seven mice with no tumor measureable for multiple time points.

[00834] Change in tumor volume versus the Isotype group (delta T/C %) was calculated on day 52, which is the last tumor volume measurement before mice were removed due to tumors ulcerating. Table 14 shows the delta T/C values for each group. Both the SSI single and double dose groups show no anti-tumor activity, similar to the PBS control treated group. The M5 single dose group shows tumor growth inhibition with a delta T/C value of 30.28%, while the M5 double dose group shows regression with a regression value of -63.90%. The Ml 1 single dose group shows minimal anti-tumor activity with a delta T/C value of 52.91%, while the Ml 1 double dose group also demonstrates regression with a regression value of -15.80%.

Table 14. Delta T/C (Treatment Tumor Volume/Control)

Group	Day	Delta T/C (%)	Regression (%)
PBS	52	162.23	NA
Isotype	52	100.00	NA
SS1 Single	52	172.05	NA
SS1 Double	52	166.15	NA
M5 Single	52	30.28	NA
M5 Double	52	NA	-63.90
M11 Single	52	52.91	NA
M11 Double	52	NA	-15.80

Discussion:

[00835] This study demonstrated that the mesothelin specific CAR T cells (M5 and Ml 1), when dosed a second time five days after an initial T cell dose, are capable of leading to the regression of OVCAR8 tumors. This response along with the anti-tumor activity following a single dose of both the M5 and Ml 1 CAR T cells are durable. In addition, four out of seven mice in the M5 double dose group demonstrate a complete regression with no tumor measureable by caliper or palpation.

[00836] As seen previously, e.g., in Example 8, a single dose of 2xl0 ^{6} CAR T cells of the M5 or Ml 1 CAR T cells shows anti-tumor activity and what appears to be stasis in the OVCAR8 xenograft model in NSG mice. In this study, a single dose of 4x1 0^{6} M5 or Ml 1 CAR T cells shows the same result. Compared to the Isotype CAR T cells or the SSI CAR T cells, the M5

and the M1 1 groups show clear anti-tumor activity even as a single dose (Figure 43). Administering a second dose of the SSI CAR T cells does not show an increased anti-tumor response. However, administering a second dose of the M5 or M1 1 CAR T cells results in an enhanced anti-tumor response and leads to regression of the tumors in both of these groups.

[00837] The increased anti-tumor activity of the M5 and M1 1 CAR T cells can be attributed to several mechanisms. The first is that a larger dose of CAR T cells achieves regression in the OVCAR8 model. Increasing the dose of CAR T cells, e.g., to $8xl0^6$ CAR T cells, e.g., in a single dose, may increase anti-tumor activity and result in regression. Another mechanism is that additional doses of CAR T cells improve anti-tumor activity and tumor regression. For example, the initial dose ay result in some anti-tumor activity due to the expansion of the CAR T cells and the production of cytokines by the T cells. Giving an additional dose, e.g., a second dose five days later while the cells are still undergoing expansion, may increase the activity of the cells due to the cytokines already being produced by the earlier administered CAR T cells.

[00838] In addition, previous studies have shown the infiltration of CAR T cells into the OVCAR8 tumors. Infiltration of CAR T cells is also studied in the mice that have received a double dose of the CAR T cells to determine if there is a greater infiltration of $CD8^+$ (cytotoxic) T cells. The persistence of the CAR T cells is also evaluated through an analysis of the cells in the spleen and bone marrow of these mice.

Example 18: Epitope Mapping by Hydrogen-Deuterium Exhchange-Mass Spectrometry

[00839] Hydrogen-deuterium exchange (HDX) mass spectrometry was performed to predict the regions of mesothelin that contribute to the epitopes recognized by scFv constructs SSI and M5. In HDX, the hydrogens of the amide backbone of a target protein are exchanged with deuterium. Interaction between the target protein and a binding protein, e.g., an antibody, "protects" regions of the target protein from solvent accessibility, thereby preventing hydrogen exchange at the interface between the target protein and the binding protein. Accordingly, HDX mass spectrometry can be used to probe for mapping protein binding interfaces, e.g., predicting the epitope of an antibody.

[00840] As described in Example 2, analysis of scFv constructs M5, M1 1, M12, M14, M16, M17, M21, and M23 by SPR-based epitope binning against SSI indicated that M5 and M1 1 bind

to a different epitope than SSI. To gain insight into the regions of the human mesothelin that may be bound by SSI and M5, HDX mass spectrometry was performed as follows.

[00841] Cloning of Expression Plasmids

[00842] A DNA fragment corresponding to amino acids Val 297 - Gly 588 of Mesothelin (Uniprot Q13421) was cloned into a mammalian expression vector using the restriction sites HindIII and EcoRI at the 5' and 3' respectively. Subsequently, a secretion signal was inserted at amino terminus corresponding to the amino acids METDTLLLWVLLLWVPGSTGDAAQPAASE (SEQ ID NO: 376). At the carboxyl terminus, a V5-His tag corresponding to the amino acids TRGSGKPIPNPLLGLDSTRTGHHHHHHHHHHHH (SEQ ID NO: 377) was also inserted. The three predicted glycosylation sites were mutated: N388Q, N496Q and N523Q. The final expressed sequence is provided below:

Glycosylation Deficient Mesothelin (296-588; N388Q, N496Q, N523Q)

METDTLLLWVLLLWVPGSTGDAAQPAASEVEKTACPSGKKAREIDESLIFYKKWELEACVDAALLATQMDR VNAIPFTYEQLDVLKHKLDELYPQGYPESVIQHLGYLFLKMSPEDIRKWQVTSLETLKALLEVNKGHEMSP QVATL IDRFVKGRGQLDKDTLDTLTAFYPGYLCSLS PEELS SVPPS S IWAVRPQDLDTCDPRQLDVLYPKA RLAFQNMQGSEYFVKIQS FLGGAPTEDLKALSQQQVSMDLAT FMKLRTDAVLPLTVAEVQKLLGPHVEGLK AEERHRPVRDWILRQRQDDLDTLGLGLQGTRGSGKPIPNPLLGLDSTRTGHHHHHHHHHHH (SEQ ID NO: 378)

[00843] The scFv corresponding to the M5 IgG (e.g., SEQ ID NO: 43) was similarly cloned into a mammalian expression vector with the N-terminal leader MALPVTALLLPLALLLHAARP (SEQ ID NO: 379) and a C-terminal eight histidine purification tag GSHHHHHHHH (SEQ ID NO: 380). The final expressed sequence is provided below:

MALPVTALLLPLALLLHAARPQVQ LVQSGAEVEKPGASVKVSCKASGYTFTDYYMHWVRQAPGQGLEWMGW INPNSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCASGWDFDYWGQGTLVTVSSGGGGSG GGGSGGGGSGGGGSDIVMTQSPSSLSASVGDRVTITCRASQSIRYYLSWYQQKPGKAPKLLI YTASILQNG VPSRFSGSGSGTDFTLTISSLQPEDFATYYCLQTYTTPDFGPGTKVEIKGSHHHHHHHH (SEQ ID NO: 381)

[00844] Protein Expression and Purification

[00845] Both the Mesothelin and scFv M5 were expressed under the control of a CMV promoter in the Expi293 expression system (Life Technologies). The mesothelin and scFv plasmids were co-transfected into 2L of Expi293 cells at a density of 2.3 x 10^6 cells/mL and viability >97% using lmg of each purified plasmid and 5mg of polyethylenimine (PEI) in Opti-MEM media (Life Technologies). Mesothelin was also transfected alone following a similar protocol but into 1L of cells using lmg of plasmid DNA and 2.5mg of PEI. The transfection proceeded for 5 days and then the cells were harvested when the viability reached 80%.

[00846] Once the viability dropped to 80%, the cells and conditioned media were collected and spun down at 4000 x g for 10 minutes. The conditioned media was then filtered through a 0.22μM filter to clear any debris and then stirred overnight with Roche's cOmplete His-Tag Purification Resin at 4°C. A total of 3mL of beads was added to the scFv-M5/Mesothelin conditioned media and 1.5mL of beads was added to the Mesothelin alone media.

[00847] The following morning, the beads were loaded onto a Bio-Rad Econo-Column and washed with 15 column volumes of 50mM Tris.Cl pH=8.0, 300mM NaCl and then 8 column volumes of 50mM Tris.Cl pH=8.0, 300mM NaCl, 20mM Imidazole. The proteins were then eluted from the column with 50mM Tris.Cl pH=8.0, 300mM NaCl, 250mM Imidazole. The eluted sample was collected and concentrated to 6mL and loaded onto a Superdex 75 16/60 column connected to an AKTAxpress (GE) in 20mM HEPES pH=7.4, 150mM NaCl. Elutions corresponding to the expected size of the mesothelin:M5 complex or Mesothelin alone were collected and concentrated to 5mg/mL.

[00848] Epitope mapping by hydrogen-deuterium exchange/mass spectrometry

[00849] Hydrogen-deuterium exchange (HDx) in combination with mass spectrometry (MS) (Woods VL, Hamuro Y (2001) High Resolution, High-Throughput Amide Deuterium Exchange-Mass Spectrometry (DXMS) Determination of Protein Binding Site Structure and Dynamics: Utility in Pharmaceutical Design. *J. Cell. Biochem. Supp.;* 84(37): 89-98.) was used to map the putative binding site of scFv antibodies M5 and SSI on human mesothelin(296-588), referred to herein as $hMSLN_{296-588}$ (SEQ ID No 278). In HDx, exchangeable amide hydrogens of proteins are replaced by deuterium. This process is sensitive to protein structure/dynamics and solvent accessibility and, therefore, able to report on locations that undergo a decrease in deuterium uptake upon ligand binding.

[00850] HDx/MS experiments were performed using methods similar to those described in the literature (Chalmers MJ, Busby SA, Pascal BD, He Y, Hendrickson CL, Marshall AG, Griffin PR (2006) Probing protein ligand interactions by automated hydrogen/deuterium exchange mass spectrometry. *Anal. Chem.;* 78(4): 1005-1014.Chalmers, 2006). The experiments were performed on a Waters HDx/MS platform, which included a LEAP autosampler, nanoACQUITY UPLC, and Synapt G2 mass spectrometer. The deuterium buffer used to label the protein backbone of $hMSLN_{296-588}$ with deuterium was 25 mM HEPES, 150 mM NaCl, pH 7.4; the overall percentage of deuterium in the solution was 94.5%. For $hMSLN_{296-588}$ deuterium labeling experiments in the absence of antibody, 400 pmol of $hMSLN_{296-588,}$ volume of 6.9 µï, was diluted using 100 µï of the deuterium buffer for 15 minutes at 4 °C. The labeling reaction was then quenched with 100 µï of chilled quench buffer at 2 °C for three minutes followed by injected onto the LC-MS system for automated pepsin digestion and peptide analysis.

[00851] For $hMSLN_{296-588}$ deuterium labeling experiments in the presence of scFv M5, 400 pmol of $hMSLN_{296-588}$ co-expressed with M5, volume of 6.9 µï, was diluted using 100 µï of the deuterium buffer for 15 minutes at 4 °C. The labeling reaction was then quenched with 100 µï of chilled quench buffer at 2 °C for three minutes followed by injected onto the LC-MS system for automated pepsin digestion and peptide analysis. For $hMSLN_{296-588}$ deuterium labeling experiments in the presence of scFv SSI, 400 pmol of $hMSLN_{296-588}$ is combined with 480 pmol of SSI scFv. After incubation of SSI with $hMSLN_{296-588}$ for 30 minutes at 4 °C, the total volume of 6.9 µï was diluted using 100 µï of the deuterium buffer for 15 minutes at 4 °C. The labeling reaction was then quenched with 100 µï of chilled quench buffer at 2 °C for three minutes, and then injected onto the LC-MS system for automated pepsin digestion and peptide analysis.

[00852] All deuterium exchange experiments were quenched using 0.5 M TCEP and 3 M urea (pH = 2.6). After quenching, the exchanged antigen was subjected to on-line pepsin digestion using a Poroszyme Immobilized Pepsin column (2.1 x 30 mm) at 12 °C followed by trapping on a Waters Vanguard HSS T3 trapping column. Peptides were eluted from the trapping column and separated on a Waters BEH C18 1x 100 mm column (maintained at 1 °C) at a flow rate of 40 µï/min using a binary 8.4 minute gradient of 2 to 35% B (mobile phase A was 99.9% water and 0.1% formic acid; mobile phase B was 99.9% acetonitrile and 0.1% formic acid).

[00853] Peptides from $hMSLN_{296-588}$ that were monitored by the deuterium exchange experiments are indicated in Figure 44 (each bar represents a peptide). Over 80% coverage of $hMSLN_{296-588}$ was achieved.

[00854] For differential experiments between antibody (M5 or SSI) bound and unbound states it is informative to examine the difference in deuterium uptake between the two states. In Figures 45A and 45B a negative value indicates that the mesothelin-antibody complex undergoes less deuterium uptake relative to mesothelin alone. A decrease in deuterium uptake can be due to protection of the region from exchangeable deuterium or stabilization of the hydrogen bonding network. In contrast, a positive value indicates that the complex undergoes more deuterium uptake relative to mesothelin alone. An increase in deuterium uptake can be due to destabilization of hydrogen bonding networks (i.e. localized unfolding of the protein).

[00855] Differential deuterium exchange between apo mesolethin and mesothelin complexed with either SSI or M5 were considered significant if: 1) if the difference is greater than 0.75 Da or, in cases, where the difference is equal to or less than 0.75 Da, 2) the difference is greater than 0.2 Da and statistically significant ($p<0.01$) when using the Tukey test. See, e.g., Houde D, Berkowitz SA, Engen JR (2010) The Utility of Hydrogen/Deuterium Exchange Mass Spectrometry in Biopharmaceutical Comparability Studies. *J. Pharma. Sci.;* 100(6): 2071-2086; Chalmers, MJ, Pascal BD, Willis S, Zhang J, lturna SJ, Dodge JA, Griffin PR (2011) Methods for the Analysis of High Precision Differential Hydrogen Deuterium Exchange Data *Int. J. Mass Spectrom.;* 302(1-3): 59-68.

[00856] Figure 45A shows the differential deuterium uptake of the M5-mesothelin complex (black bars) and the differential uptake of the SSI-mesothelin complex (grey bars) from amino acid position 297 to 464 on mesothelin. Over this region binding of M5 to mesothelin caused no protection and there is some very slight destabilization observed. In contrast, the binding of SSI to mesothelin caused significant protection in the following peptides: 297-315, 315-322, 316-325, 337-346, 337-349, 350-375, and 369-376. Protection in the peptides 297-315, 315-322, 316-325, 337-346, 337-349 is consistent with the published crystallography structure where residues E313, F317, K319, P343, and Y346 form a significant part of the epitope (Ma J, Tang WK, Esser L, Pastan I, Xia D (2012) Recognition of Mesotehlin by the Therapuetic Antibody MORAb-009 *J. Biol. Chem.;* (287)40: 33123-33131.) Figure 45B shows the differential deuterium uptake for

the amino acid positions 458-586 on mesothelin. Over this region, binding of M5, caused significant protection in the following peptides: 481-490, 483-490, 498-510, 501-507, 531-540, 532-540, 532-546, 537-546, 545-558, 546-569, 547-560, 558-571, 561-572. By comparing the protection of overlapping peptides, we deduce that the regions 485-490, 498-507, 532-537, and 545-572 are significantly protected in the M5-mesothelin complex. In contrast, the SS1-mesothelin complex does not contain any peptides in the region 458-586 that are significantly protected using the criteria stated above.

[00857] Figure 46 provides a summary of the protected regions for both the M5 and SSI complex. These data indicate that M5 protects exclusively towards the C-terminal side of mesothelin, and that amino acid residues 485-490, 498-507, 532-537, and 545-572 may contribute to the M5-mesothelin interaction. In contrast, SSI protects exclusively towards the N-terminal side of mesothelin and there was no observed overlap in the regions protected by SSI and those protected by M5. The observation of two distinct protection patterns for SSI and M5 indicate that M5 likely binds a distinct epitope from that bound by SSI. Ml 1 is expected to bind to a similar region of MSLN as compared to M5? Ml 1 and M5 have high homology I both light and heavy chains including identical CDR3 regions.

EQUIVALENTS

[00858] The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific aspects, it is apparent that other aspects and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such aspects and equivalent variations.

What is claimed is:

1. An isolated nucleic acid molecule encoding a chimeric antigen receptor (CAR), wherein the CAR comprises: i) an antibody or antibody fragment comprising a human anti-mesothelin binding domain, ii) a transmembrane domain, and iii) an intracellular signaling domain comprising a stimulatory domain, and wherein said anti-mesothelin binding domain comprises one or more of light chain complementary determining region 1 (LC CDRl), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) comprising an amino acid sequence listed in Table 5 and one or more of heavy chain complementary determining region 1 (HC CDRl), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) comprising an amino acid sequence listed in Table 4.

2. The isolated nucleic acid molecule of claim 1, comprising a LC CDRl, LC CDR2 and LC CDR3, wherein LC CDRl, LC CDR2 and LC CDR3 comprise an amino acid sequence listed in Table 5.

3. The isolated nucleic acid molecule of claim 1, comprising a HC CDRl, HC CDR2 and HC CDR3, wherein HC CDRl, HC CDR2 and HC CDR3 comprise an amino acid sequence listed in Table 4.

4. The isolated nucleic acid molecule of claim 1, comprising LC CDRl, LC CDR2 and LC CDR3, wherein LC CDRl, LC CDR2 and LC CDR3 comprise an amino acid sequence listed in Table 5, and HC CDRl, HC CDR2, and HC CDR3, wherein HC CDRl, HC CDR2 and HC CDR3 comprise an amino acid sequence listed in Table 4.

5. The isolated nucleic acid molecule of claim 1, wherein the anti-mesothlin binding domain comprises an amino acid sequence of any one of the light chain variable regions listed in Table 2.

6. The isolated nucleic acid molecule of claim 1, wherein the anti-mesothlin binding domain comprises an amino acid sequence of any one of the heavy chain variable region listed in Table 2.

7. The isolated nucleic acid molecule of claim 1, wherein the anti-mesothlin binding domain comprises an amino acid sequence of any light chain variable region listed in Table 2 and any heavy chain variable region listed Table 2.

8. The isolated nucleic acid molecule of any of the preceding claims, wherein the anti-mesothelin binding domain is a scFv.

9. The isolated nucleic acid molecule of any of the preceding claims, wherein the anti-mesothelin binding domain comprises a light chain variable region comprising an amino acid sequence having at least one, two or three modifications but not more than 30, 20 or 10 modifications of an amino acid sequence of a light chain variable region provided in Table 2, or a sequence with 95-99% identity to an amino acid sequence provided in Table 2.

10. The isolated nucleic acid molecule of any of the preceding claims, wherein the anti-mesothelin binding domain comprises a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications but not more than 30, 20 or 10 modifications of an amino acid sequence of a heavy chain variable region provided in Table 2, or a sequence with 95-99% identity to an amino acid sequence provided in Table 2.

11. The isolated nucleic acid molecule of any of the preceding claims, wherein the encoded anti-mesothelin binding domain comprises a sequence selected from a group consisting of SEQ ID NO: 39; SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, and SEQ ID NO: 62, or a sequence with 95-99% identify thereof.

12. The isolated nucleic acid molecule of any of the preceding claims, wherein the nucleic acid sequence encoding the anti-mesothelin binding domain comprises a sequence selected from a group consisting of SEQ ID NO: 87; SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, SEQ ID NO: 102, SEQ ID NO: 103, SEQ ID NO: 104, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 109, and SEQ ID NO: 110, or a sequence with 95-99% identify thereof.

13. The isolated nucleic acid molecule of any of the preceding claims, wherein the encoded CAR includes a transmembrane domain that comprises a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154.

14. The isolated nucleic acid molecule of any of the preceding claims, wherein the encoded transmembrane domain comprises a sequence of SEQ ID NO: 6.

15. The isolated nucleic acid molecule of any of the preceding claims, wherein the encoded transmembrane domain comprises an amino acid sequence that comprises at least one, two or

three modifications but not more than 20, 10 or 5 modifications of an amino acid sequence of SEQ ID NO: 6, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO:6.

16. The isolated nucleic acid molecule of any of the preceding claims, wherein the nucleic acid sequence encoding the transmembrane domain comprises a sequence of SEQ ID NO: 17, or a sequence with 95-99% identify thereof.

17. The isolated nucleic acid molecule of any of the preceding claims, wherein the encoded anti-mesothelin binding domain is connected to the transmembrane domain by a hinge region.

18. The isolated nucleic acid molecule of claim 17, wherein the encoded hinge region comprises SEQ ID NO: 2, or a sequence with 95-99% identity thereof.

19. The isolated nucleic acid molecule of claim 17, wherein the nucleic acid sequence encoding the hinge region comprises a sequence of SEQ ID NO: 13, or a sequence with 95-99% identify thereof.

20. The isolated nucleic acid molecule of any of the preceding claims, further comprising a sequence encoding a costimulatory domain.

21. The isolated nucleic acid molecule of claim 20, wherein the costimulatory domain is a functional signaling domain obtained from a protein selected from the group consisting of OX40, CD2, CD27, CD28, CDS, ICAM-1, LFA-1 (CDlla/CD18), ICOS (CD278), and 4-1BB (CD137).

22. The isolated nucleic acid molecule of claim 20 or 21, wherein the encoded costimulatory domain comprises a sequence of SEQ ID NO: 7.

23. The isolated nucleic acid molecule of claim 20 or claim 21, wherein the encoded costimulatory domain comprises an amino acid sequence having at least one, two or three modifications but not more than 20, 10 or 5 modifications of an amino acid sequence of SEQ ID NO:7, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO:7.

24. The isolated nucleic acid molecule of claim 20 or 21, wherein the nucleic acid sequence encoding the costimulatory domain comprises a sequence of SEQ ID NO: 18, or a sequence with 95-99% identify thereof.

25. The isolated nucleic acid molecule of any of the preceding claims, wherein the encoded intracellular signaling domain comprises a functional signaling domain of 4-1BB and/or a functional signaling domain of CD3 zeta.

26. The isolated nucleic acid molecule of any of the preceding claims, wherein the encoded intracellular signaling domain comprises the sequence of SEQ ID NO: 7 and/or the sequence of SEQ ID NO:9 or SEQ ID NO: 10.

27. The isolated nucleic acid molecule of any of the preceding claims, wherein the intracellular signaling domain comprises an amino acid sequence having at least one, two or three modifications but not more than 20, 10 or 5 modifications of an amino acid sequence of SEQ ID NO: 7 and/or an amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 7 and/or an amino acid sequence of SEQ ID NO:9 or SEQ ID NO: 10.

28. The isolated nucleic acid molecule of any of the preceding claims, wherein the encoded intracellular signaling domain comprises the sequence of SEQ ID NO: 7 and the sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein the sequences comprising the intracellular signaling domain are expressed in the same frame and as a single polypeptide chain.

29. The isolated nucleic acid molecule of any of the preceding claims, wherein the nucleic acid sequence encoding the intracellular signaling domain comprises a sequence of SEQ ID NO: 18, or a sequence with 95-99% identify thereof, and/or a sequence of SEQ ID NO:20 or SEQ ID NO: 21, or a sequence with 95-99% identity thereof.

30. The isolated nucleic acid molecule of any of the preceding claims, further comprising a leader sequence.

31. The isolated nucleic acid molecule of claim 32, wherein the leader sequence comprises SEQ ID NO: 1.

32. An isolated polypeptide molecule encoded by the nucleic acid molecule of any one of claims 1-32.

33. The isolated polypeptide of claim 32, comprising a sequence selected from the group consisting of SEQ ID NO: 63; SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, 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, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, and SEQ ID NO: 86.

34. An isolated chimeric antigen receptor (CAR) molecule comprising a human anti-mesothelin binding domain, a transmembrane domain, and an intracellular signaling domain.

35. The isolated CAR molecule of claim 34, comprising i) an antibody or antibody fragment which includes a human anti-mesothelin binding domain, ii) a transmembrane domain, and iii) an intracellular signaling domain.

36. The isolated CAR molecule of any of claims 34 or 35, wherein the anti-mesothelin binding domain does not compete for binding to human mesothelin with an antigen binding domain comprising a sequence comprising SEQ ID NO: 279.

37. The isolated CAR molecule of any of claims 34-36, wherein the anti-mesothelin binding domain competes with an antigen binding domain comprising:

a) one or more light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of an anti-mesothelin binding domain sequence selected from SEQ ID NO: 43 and SEQ ID NO: 49, and one or more heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of an anti-mesothelin binding domain sequence selected from SEQ ID NO: 43 and SEQ ID NO: 49;

b) a LC CDR1 , LC CDR2 and LC CDR3 of an anti-mesothelin light chain amino acid sequence selected from SEQ ID NO: 43 and SEQ ID NO: 49, and an HC CDR1, HC CDR2, and HC CDR3 of an anti-mesothelin heavy chain amino acid sequence selected from SEQ ID NO: 43 and SEQ ID NO: 49; or

c) a sequence selected from SEQ ID NO: 43 and SEQ ID NO: 49.

38. The isolated CAR molecule of any of claims 34-37, wherein the anti-mesothelin binding domain binds to a different epitope of human mesothelin than the epitope of human mesothelin targeted by the antigen binding domain comprising a sequence comprising SEQ ID NO: 279.

39. The isolated CAR molecule of any of claims 34-38, wherein the anti-mesothelin binding domain binds to the C-terminus of human mesothelin.

40. The isolated CAR molecule of claim 39, wherein the anti-mesothelin binding domain binds an epitope within amino acids 450-588 of SEQ ID NO: 278.

41. The isolated CAR molecule of claim 40, wherein the epitope comprises amino acids 485-490, 498-507, 532-537, or 545-572 of SEQ ID NO: 278, or any subset or a combination thereof.

42. The isolated CAR molecule of any of claims 34-41, wherein the human anti-mesothelin binding domain comprises one or more light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) comprising an amino acid sequence listed in Table 5, and one or more heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) comprising an amino acid sequence listed in Table 4.

43. The isolated CAR molecule of any of claims 34-42, comprising LC CDR1, LC CDR2 and LC CDR3 comprising an amino acid sequence listed in Table 5.

44. The isolated CAR molecule of any of claims 34-43, comprising HC CDR1, HC CDR2, and HC CDR3 comprising an amino acid sequence listed in Table 4.

45. The isolated CAR molecule of any of claims 34-44, comprising LC CDR1, LC CDR2 and LC CDR3 comprising an amino acid sequence listed in Table 5, and HC CDR1, HC CDR2, and HC CDR3 comprising an amino acid sequence listed in Table 4.

46. The isolated CAR molecule of any of claims 59-45, wherein the anti-mesothelin binding domain is a scFv.

47. The isolated CAR molecule of any of claims 34-46, wherein the anti-mesothelin binding domain comprises a light chain and a heavy chain of an amino acid sequence listed in Table 2.

48. The isolated CAR molecule of any of claims 34-47, wherein the anti-mesothelin binding domain comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications but not more than 30, 20 or 10 modifications of an amino acid sequence of a light chain variable region provided in Table 2, or a sequence with 95-99% identity with an amino acid sequence provided in Table 2.

49. The isolated CAR molecule of any of claims 34-48, wherein the anti-mesothelin binding domain comprises a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications but not more than 30, 20 or 10 modifications of an amino acid sequence of a heavy chain variable region provided in Table 2, or a sequence with 95-99% identity to an amino acid sequence provided in Table 2.

50. The isolated CAR molecule of any of claims 34-49, wherein the anti-mesothelin binding domain comprises a sequence selected from a group consisting of SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, and SEQ ID NO: 62, or a sequence with 95-99% identity thereof.

51. The isolated CAR molecule of any of claims 34-50, wherein the transmembrane comprises a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154.

52. The isolated CAR molecule of claim 51, wherein the transmembrane domain comprises a sequence of SEQ ID NO: 6.

53. The isolated CAR molecule of claim 51, wherein the transmembrane domain comprises an amino acid sequence having at least one, two or three modifications but not more than 20, 10 or 5 modifications of an amino acid sequence of SEQ ID NO: 6, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 6.

54. The isolated CAR molecule of any one of claims 34-53, wherein the human anti-mesothelin binding domain is connected to the transmembrane domain by a hinge region.

55. The isolated CAR molecule of claim 54, wherein the hinge region comprises SEQ ID NO:2 or SEQ ID NO:36, or a sequence with 95-99% identity thereof.

56. The isolated CAR molecule of any of claims 34-55, wherein the intracellular signaling domain comprises a sequence encoding a costimulatory domain.

57. The isolated CAR molecule of claim 56, wherein the costimulatory domain comprises a functional signaling domain of a protein selected from the group consisting of OX40, CD2, CD27, CD28, CDS, ICAM-1, LFA-1 (CDlla/CD18), CD278 (also known as "ICOS") and 4-1BB (CD137).

58. The isolated CAR molecule of claim 56 or 57, wherein the costimulatory domain comprises a sequence of SEQ ID NO: 7.

59. The isolated CAR molecule of claim 56 or 57, wherein the costimulatory domain comprises an amino acid sequence having at least one, two or three modifications but not more than 20, 10 or 5 modifications of an amino acid sequence of SEQ ID NO: 7, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 7.

60. The isolated CAR molecule of claim 34-59, wherein the intracellular signaling domain comprises a functional signaling domain of 4-IBB and/or a functional signaling domain of CD3 zeta.

61. The isolated CAR molecule of claim 60, wherein the intracellular signaling domain comprises the sequence of SEQ ID NO: 7 and/or the sequence of SEQ ID NO:9.

62. The isolated CAR molecule of claim 60, wherein the intracellular signaling domain comprises the sequence of SEQ ID NO: 7 and/or the sequence of SEQ ID NO: 10.

63. The isolated CAR molecule of any of claims 34-55, wherein the intracellular signaling domain comprises an amino acid sequence having at least one, two or three modifications but not more than 20, 10 or 5 modifications of an amino acid sequence of SEQ ID NO: 7 and/or an amino acid sequence of SEQ ID NO:9 or SEQ ID NO: 10, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO:7 and/or an amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10.

64. The isolated CAR molecule of any of claims 34-55, wherein the intracellular signaling domain comprises the sequence of SEQ ID NO: 7 and the sequence of SEQ ID NO: 9 or SEQ

ID NO: 10, wherein the sequences comprising the intracellular signaling domain are expressed in the same frame and as a single polypeptide chain.

65. The isolated CAR molecule of any of claims 59-64, further comprising a leader sequence.

66. The isolated CAR molecule of claim 65, wherein the leader sequence comprises an amino acid sequence of SEQ ID NO: 1, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 1.

67. A human anti-mesothelin binding domain comprising one or more light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any anti-mesothelin binding domain in SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, and SEQ ID NO: 62, and one or more heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any human anti-mesothelin binding domain in SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, and SEQ ID NO: 62.

68. The human anti-mesothelin binding domain of claim 67, wherein the human anti-mesothelin binding domain is a scFv comprising a light chain and a heavy chain of an amino acid sequence of SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ

ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, and SEQ ID NO: 62.

69. The human anti-mesothelin binding domain of claim 67 or 68, wherein the human anti-mesothelin binding domain comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications but not more than 30, 20 or 10 modifications of an amino acid sequence of a light chain variable region provided in SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, or SEQ ID NO: 62, or a sequence with 95-99% identity with an amino acid sequence of SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, or SEQ ID NO: 62; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications but not more than 30, 20 or 10 modifications of an amino acid sequence of a heavy chain variable region provided in SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, or SEQ ID NO: 62; or a sequence with 95-99% identity to an amino acid sequence in SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO:

56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, or SEQ ID NO: 62.

70. An human anti-mesothelin binding domain, wherein the anti-mesothelin binding domain does not compete for binding to human mesothelin with an antigen binding domain comprising a sequence comprising SEQ ID NO: 279.

71. The human anti-mesothelin binding domain of claim 70, wherein the anti-mesothelin binding domain competes with an antigen binding domain comprising:

a) one or more light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of an anti-mesothelin binding domain sequence selected from SEQ ID NO: 43 and SEQ ID NO: 49 and one or more heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of an anti-mesothelin binding domain sequence selected from SEQ ID NO: 43 and SEQ ID NO: 49

b) a LC CDR1 , LC CDR2 and LC CDR3 of an anti-mesothelin light chain amino acid sequence selected from SEQ ID NO: 43 and SEQ ID NO: 49 and an HC CDR1, HC CDR2, and HC CDR3 of an anti-mesothelin heavy chain amino acid sequence selected from SEQ ID NO: 43 and SEQ ID NO: 49; or

c) a sequence selected from SEQ ID NO: 43 and SEQ ID NO: 49.

72. The human anti-mesothelin binding domain any of claims 70 or 71, wherein the anti-mesothelin binding domain binds to a different epitope of human mesothelin than the epitope of human mesothelin targeted by an antigen binding domain comprising a sequence comprising SEQ ID NO: 279.

73. The human anti-mesothelin binding domain of any of claims 70-72, wherein the anti-mesothelin binding domain binds to the C-terminus of human mesothelin.

74. The human anti-mesothelin binding domain of claim 73, wherein the anti-mesothelin binding domain binds an epitope within amino acids 450-588 of SEQ ID NO: 278.

75. The human anti-mesothelin binding domain of claim 74, wherein the epitope comprises amino acids 485-490, 498-507, 532-537, or 545-572 of SEQ ID NO: 278, or any subset or a combination thereof.

76. A vector comprising a nucleic acid molecule encoding a CAR of any of the preceding claims.

77. The vector of claim 76, wherein the vector is selected from the group consisting of a DNA, a RNA, a plasmid, a lentivirus vector, adenoviral vector, or a retrovirus vector.

78. The vector of claim 76 or 77, further comprising a promoter.

79. The vector of claim 78, wherein the promoter is an EF-1 promoter.

80. The vector of claim 79, wherein the EF-1 promoter comprises a sequence of SEQ ID NO: 11.

81. The vector of any of claims 76-78, wherin the vector is an in vitro transcribed vector.

82. The vector of claim 81, wherein the, the nucleic acid sequence in the vector further comprises a poly(A) tail.

83. The vector of any of claims 81, wwherein the nucleic acid sequence in the vector further comprises a 3'UTR.

84. A cell comprising the vector of any of claims 76-83.

85. The cell of claim 84, wherein the cell is a human T cell.

86. The cell of claim 85, wherein the T cell is a CD8+ T cell.

87. A method of making a cell comprising transducing a T cell with a vector of any of claims 76-83.

88. A method of generating a population of RNA-engineered cells comprising introducing an in vitro transcribed RNA or synthetic RNA into a cell, where the RNA comprises a nucleic acid encoding a CAR molecule of any of the preceding claims.

89. A method of providing an anti-cancer immunity in a mammal comprising administering to the mammal an effective amount of a cell expressing a CAR molecule of any of the preceding claims.

90. The method of claim 89, wherein the cell is an autologous T cell.

91. The method of claim 89 or 90, wherein the cell is an allogeneic T cell.

92. The method of any of claims 89-91, wherein the mammal is a human.

93. A method of treating a mammal having a disease associated with expression of mesothelin comprising administering to the mammal an effective amount of a cell comprising a CAR moleculeof any of the preceding claims.

94. The method of claim 93, wherein the disease associated with mesothelin expression is selected from a proliferative disease such as a cancer or malignancy or a precancerous condition, or is a non-cancer related indication associated with expression of mesothelin.

95. The method of claim 93-94, wherein the disease is a cancer associated with mesothelin selected from the group consisting of mesothelioma, malignant pleural mesothelioma, non-small cell lung cancer, small cell lung cancer, squamous cell lung cancer, or large cell lung cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, pancreatic metatstatic, ovarian cancer, colorectal cancer and bladder cancer, or any combination thereof.

96. The method of any of claims 93-95, wherein the cells expressing a CAR molecule are administered in combination with an agent that increases the efficacy of a cell expressing a CAR molecule.

97. The method of any of claims 93-96, wherein the cells expressing a CAR molecule are administered in combination with an agent that ameliorates one or more side effects associated with administration of a cell expressing a CAR molecule.

98. The method of any of claims 93-97, wherein the cells expressing a CAR molecule are administered in combination with an agent that treats the disease associated with mesothelin

99. The isolated nucleic acid molecule of any of claims 1-31, the isolated polypeptide molecule of any of claims 32-33, the isolated CAR of any of claims 34-66, the anti-mesothelin binding domain of any of claims 67-75, the vector of any of claims 76-83 or the cell of any of claims 84-86 for use as a medicament.

100. The isolated nucleic acid molecule of any of claims 1-31, the isolated polypeptide molecule of any of claims 32-33, the isolated CAR of any of claims 34-66, the anti-mesothelin binding domain of any of claims 67-75, the vector of any of claims 76-83 or the cell of any of claims 84-86 for use in the treatment of a disease expressing mesothelin.

101. A cell of any of claims 84-86, further expressing an inhibitory molecule that comprises a first polypeptide that comprises at least a portion of an inhibitory molecule, associated with a second polypeptide that comprises a positive signal from an intracellular signaling domain.

102. The cell of claim 101, wherein the first polypeptide comprises at least a portion of PD1 and the second polypeptide comprises a costimulatory domain and a intracellular signaling domain.

103. The method of claim 96, wherein the agent is an mTOR inhibitor and the subject is administered a low, immune enhnacing, dose of an mTOR inhibitor, e.g., RADOOl or rapamycin.

104. The method of claim 103, wherein the mTOR inhibitor is a RADOOl.

105. The method of claim 103, wherein the dose comprises an allosteric and a catalytic mTOR inhibitor.

106. The method of 103, wherein the mTOR inhibitor is administered for an amount of time sufficient to decrease the proportion of PD-1 positive T cells, increase the proportion of PD-

1 negative T cells, or increase the ratio of PD-1 negative T cells/ PD-1 positive T cells, in the peripheral blood of the subject, or in a preparation of T cells isolated from the subject.

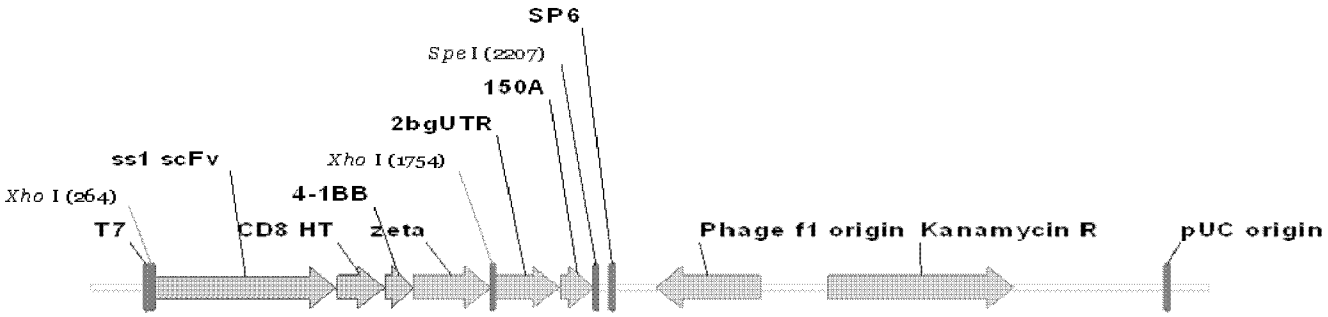
107. The method of claim 103, wherein the immune effector cell, e.g., T cell, to be engineered to express a CAR, is harvested after a sufficient time, or after sufficient dosing of the low, immune enhancing, dose of an mTOR inhibitor, such that the level of PDl negative immune effector cells, e.g., T cells, or the ratio of PDl negative immune effector cells, e.g., T cells/ PDl positive immune effector cells, e.g., T cells, in the subject or harvested from the subject has been, at least transiently, increased.

108. The method of claim 103, wherein the dose of an mTOR inhibitor is associated with mTOR inhibition of at least 5 but no more than 90%, e.g., as measured by p70 S6 kinase inhibition.

109. The method of claim 103, wherein the dose of an mTOR inhibitor is associated with mTOR inhibition of at least 10% but no more than 40%, e.g., as measured by p70 S6 kinase inhibition.

110. A method of treating a subject having a disease associated with expression of mesothelin comprising administering to the subject an effective amount of cells comprising a nucleic acid of any of claims 1-31, wherein the nucleic acid is introduced into T cells or NK cells using in vitro transcription, and the subject receives an initial administration of the cells comprising the nucleic acid, and one or more subsequent administrations of cells comprising the nucleic acid, wherein the one or more subsequent administrations are administered less than 15 days, e.g., 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 days after the previous administration.

FIG. 1

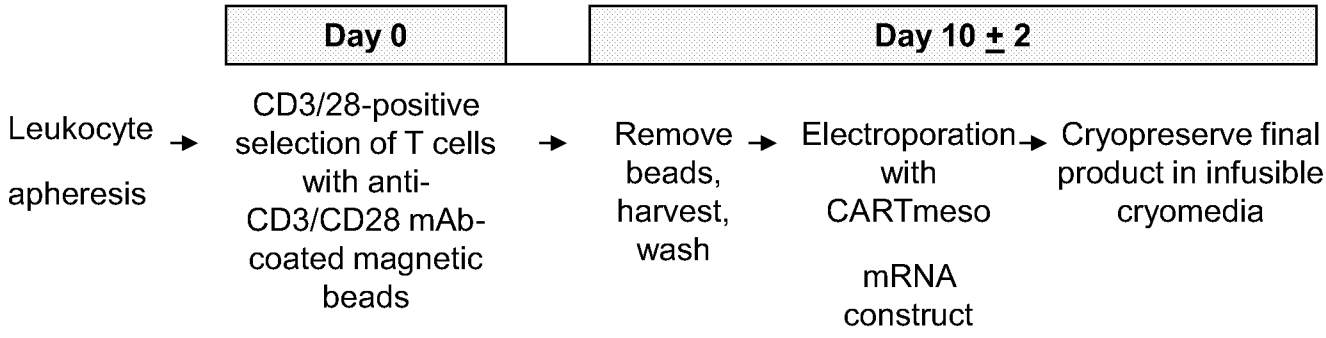


pD-A. ss1.OF.2bg.150A

4893bp

FIG. 2

A


Day 0
Day 10 ± 2
Leukocyte apheresis
CD3/28-positive selection of T cells with anti-CD3/CD28 mAb-coated magnetic beads
Remove beads, harvest, wash
Electroporation with CARTmeso mRNA construct
Cryopreserve final product in infusible cryomedia

B

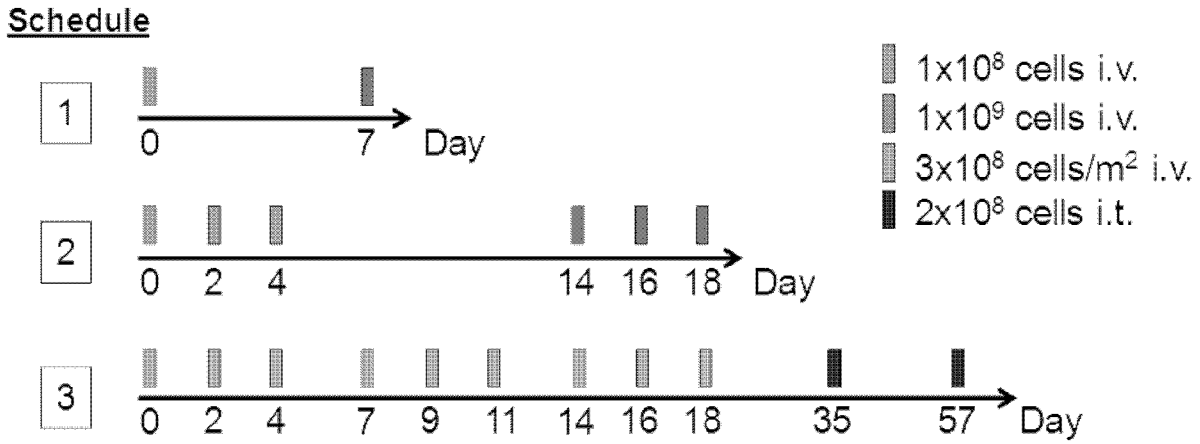
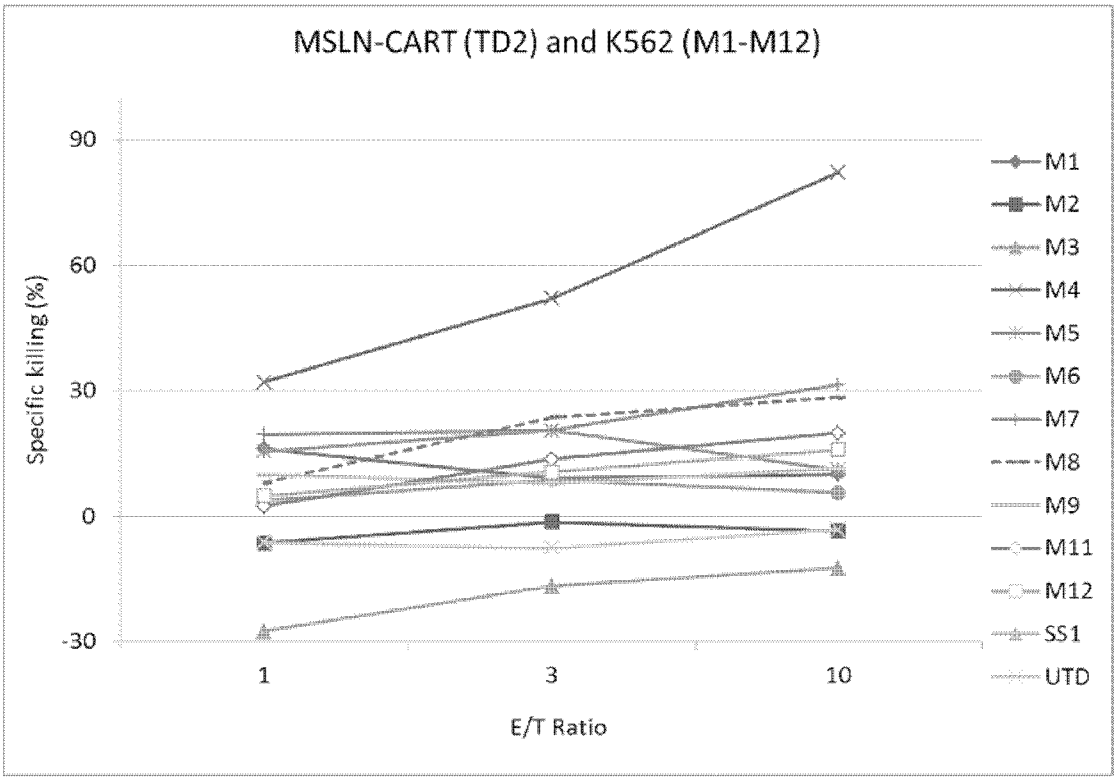

Schedule
1
0
7
Day
2
0 2 4 14 16 18
Day
3
0 2 4 7 9 11 14 16 18 35 57
Day
1x10^8 cells i.v.
1x10^9 cells i.v.
3x10^8 cells/m^2 i.v.
2x10^8 cells i.t.

FIG. 3

A


MSLN-CART (TD2) and K562 (M1-M12)
Specific killing (%)
90
60
30
0
-30
1
3
10
E/T Ratio
M1
M2
M3
M4
M5
M6
M7
M8
M9
M11
M12
SS1
UTD

B

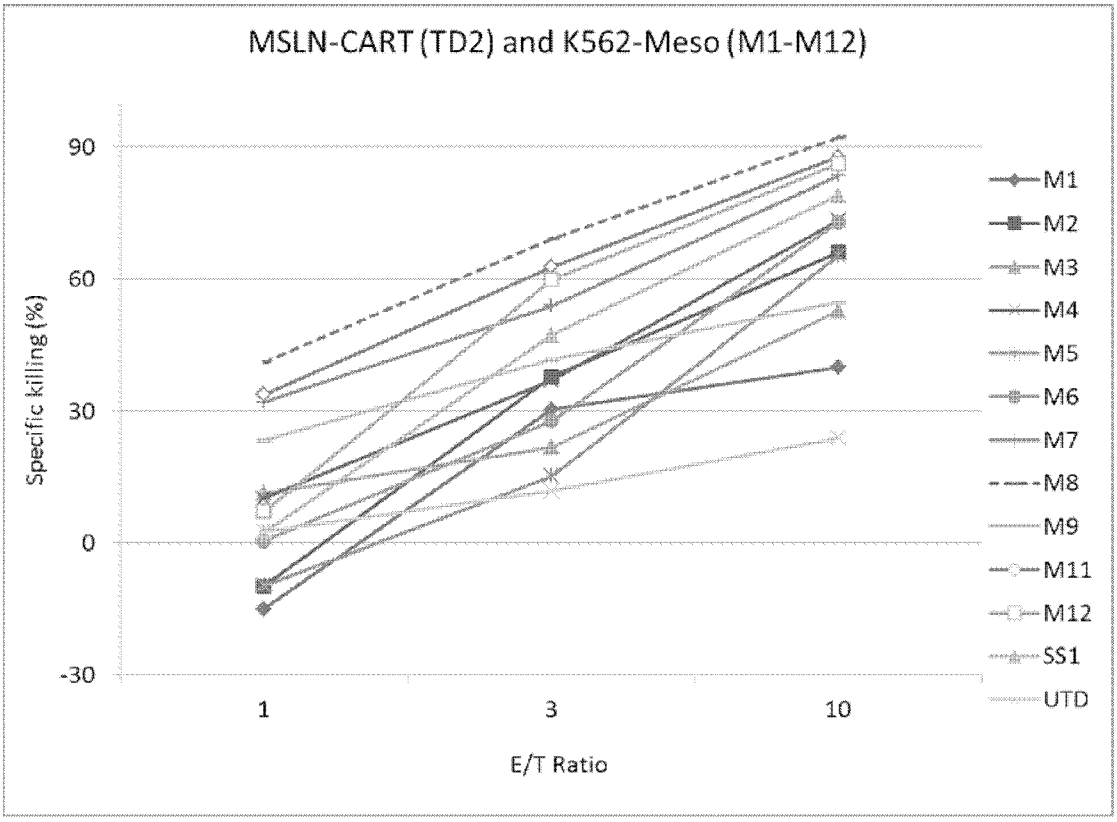
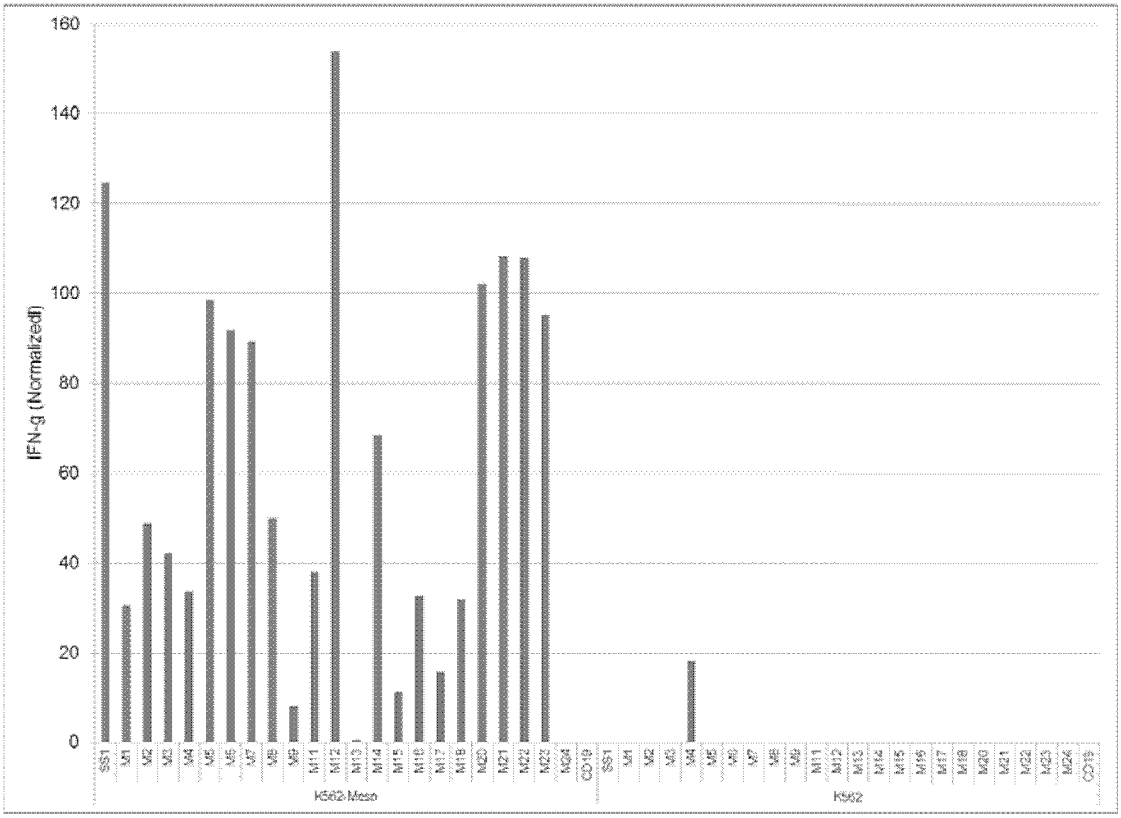

MSLN-CART (TD2) and K562-Meso (M1-M12)
Specific killing (%)
90
60
30
0
-30
1
3
10
E/T Ratio
M1
M2
M3
M4
M5
M6
M7
M8
M9
M11
M12
SS1
UTD

FIG. 4

A


IFN-g (Normalized)
160
140
120
100
80
60
40
20
0
SS1
M1
M2
M3
M4
M5
M6
M7
M8
M9
M11
M12
M13
M14
M15
M16
M17
M18
M20
M21
M22
M24
CD19
K562-Meso
K562

B

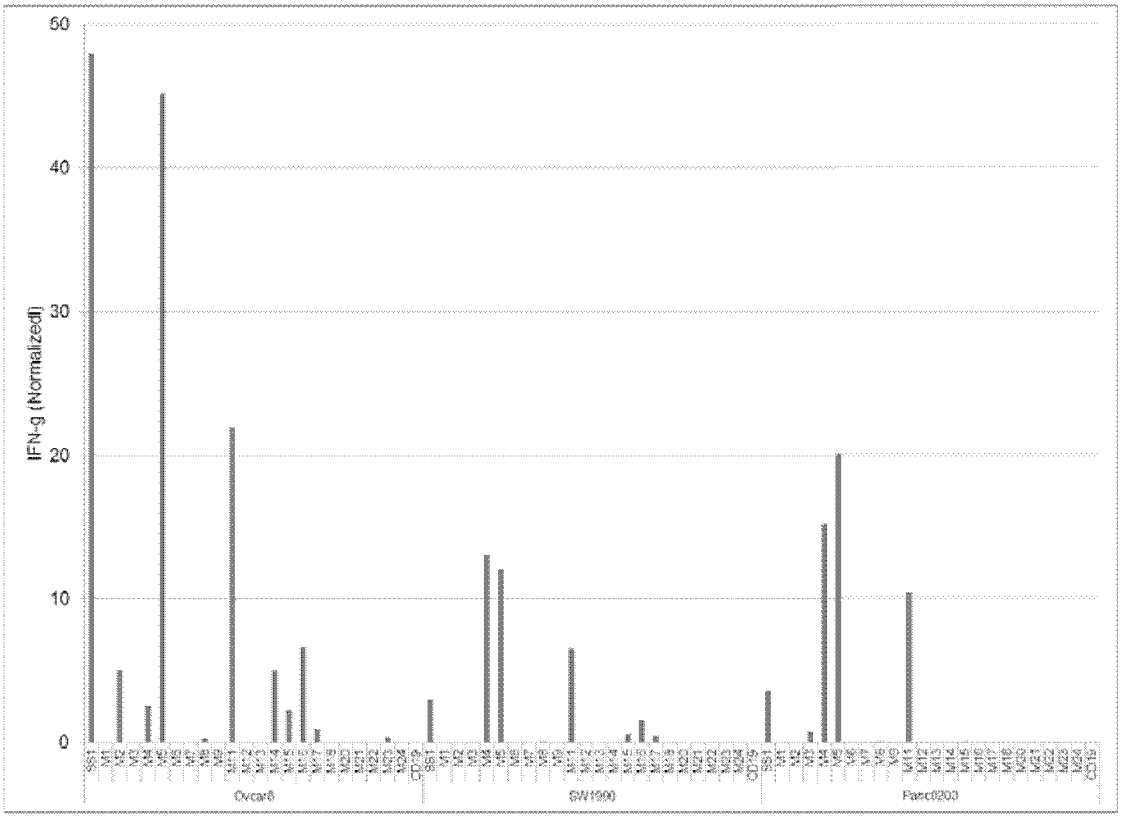

IFN-g (Normalized)
50
40
30
20
10
0
Ovcar8
SW1990
Panc0203

FIG. 5

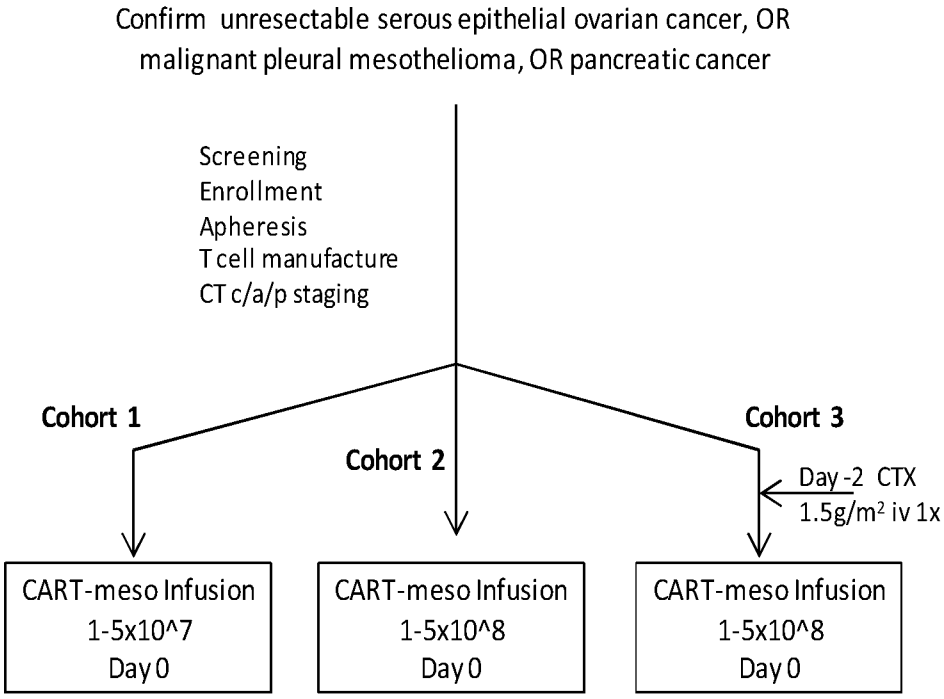

Confirm unresectable serous epithelial ovarian cancer, OR malignant pleural mesothelioma, OR pancreatic cancer
Screening
Enrollment
Apheresis
T cell manufacture
CT c/a/p staging
Cohort 1
Cohort 2
Cohort 3
Day -2 CTX
1.5g/m2 iv 1x
CART-meso Infusion
1-5x10^7
Day 0
CART-meso Infusion
1-5x10^8
Day 0
CART-meso Infusion
1-5x10^8
Day 0

FIG. 6

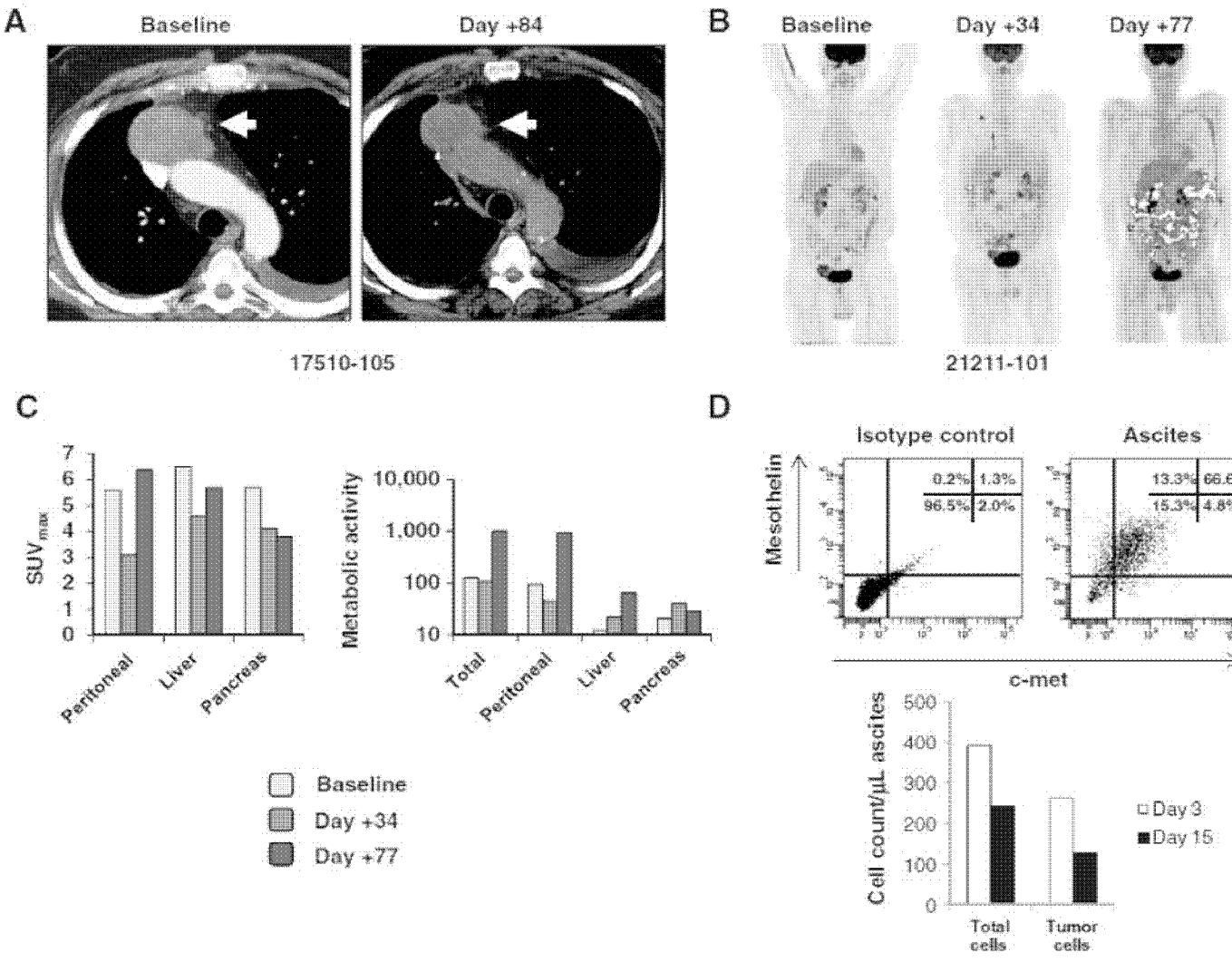

A
Baseline
Day +84
17510-105
B
Baseline
Day +34
Day +77
21211-101
C
SUV max
Peritoneal
Liver
Pancreas
Metabolic activity
Total
Peritoneal
Liver
Pancreas
Baseline
Day +34
Day +77
D
Isotype control
Ascites
Mesothelin
c-met
0.2%
1.3%
96.5%
2.0%
13.3%
66.6%
15.3%
4.8%
Cell count/μL ascites
Total cells
Tumor cells
Day 3
Day 15

FIG. 7

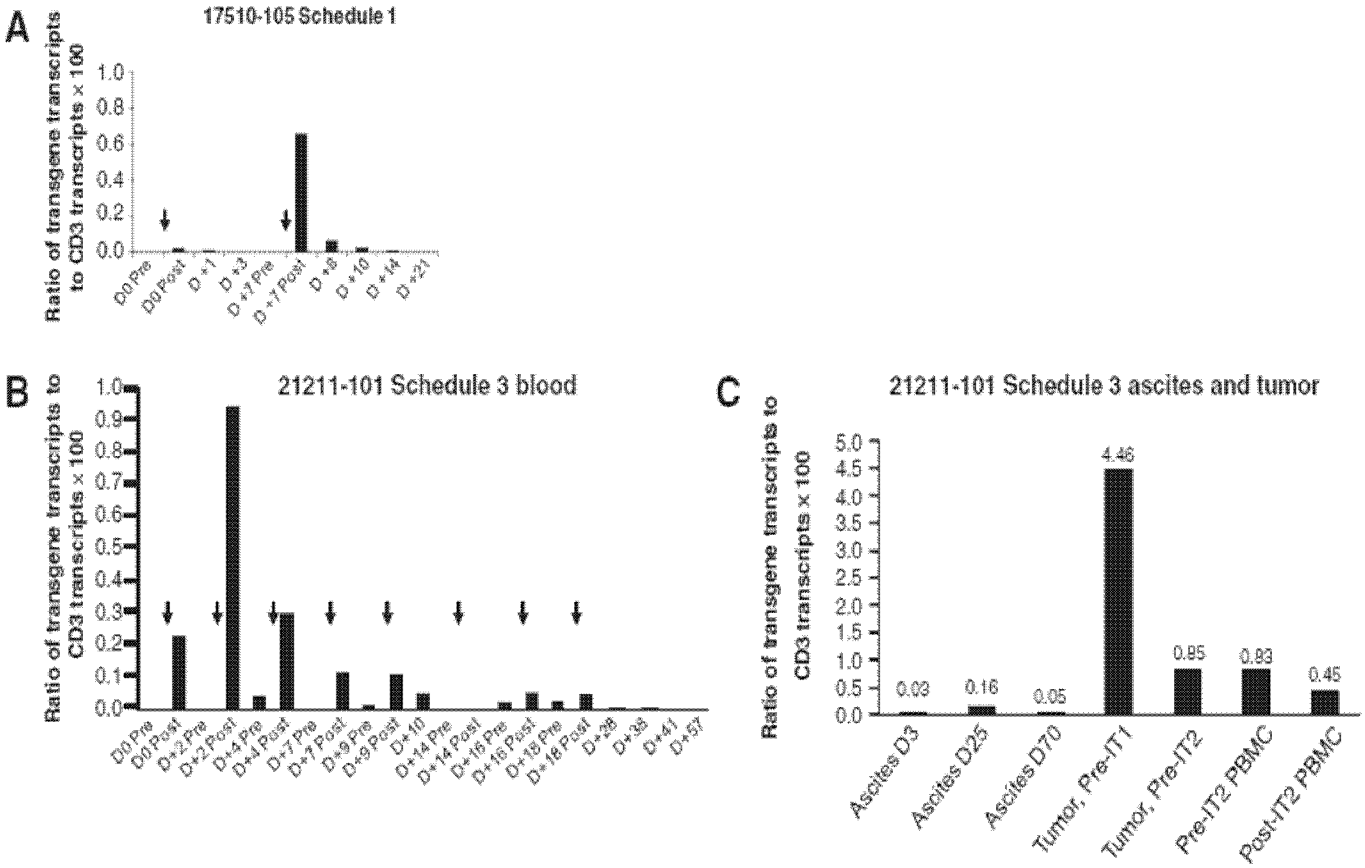

A
17510-105 Schedule 1
Ratio of transgene transcripts to CD3 transcripts x 100
1.0
0.8
0.6
0.4
0.2
0.0
D0 Pre
D0 Post
D +1
D +3
D +7 Pre
D +7 Post
D +8
D +10
D +14
D +21
B
21211-101 Schedule 3 blood
Ratio of transgene transcripts to CD3 transcripts x 100
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
D0 Pre
D0 Post
D+2 Pre
D+2 Post
D+4 Pre
D+4 Post
D+7 Pre
D+7 Post
D+9 Pre
D+9 Post
D+10
D+14 Pre
D+14 Post
D+16 Pre
D+16 Post
D+18 Pre
D+18 Post
D+28
D+35
D+41
D+57
C
21211-101 Schedule 3 ascites and tumor
Ratio of transgene transcripts to CD3 transcripts x 100
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.03
0.16
0.05
4.46
0.95
0.93
0.45
Ascites D3
Ascites D25
Ascites D70
Tumor, Pre-IT1
Tumor, Pre-IT2
Pre-IT2 PBMC
Post-IT2 PBMC

FIG. 8

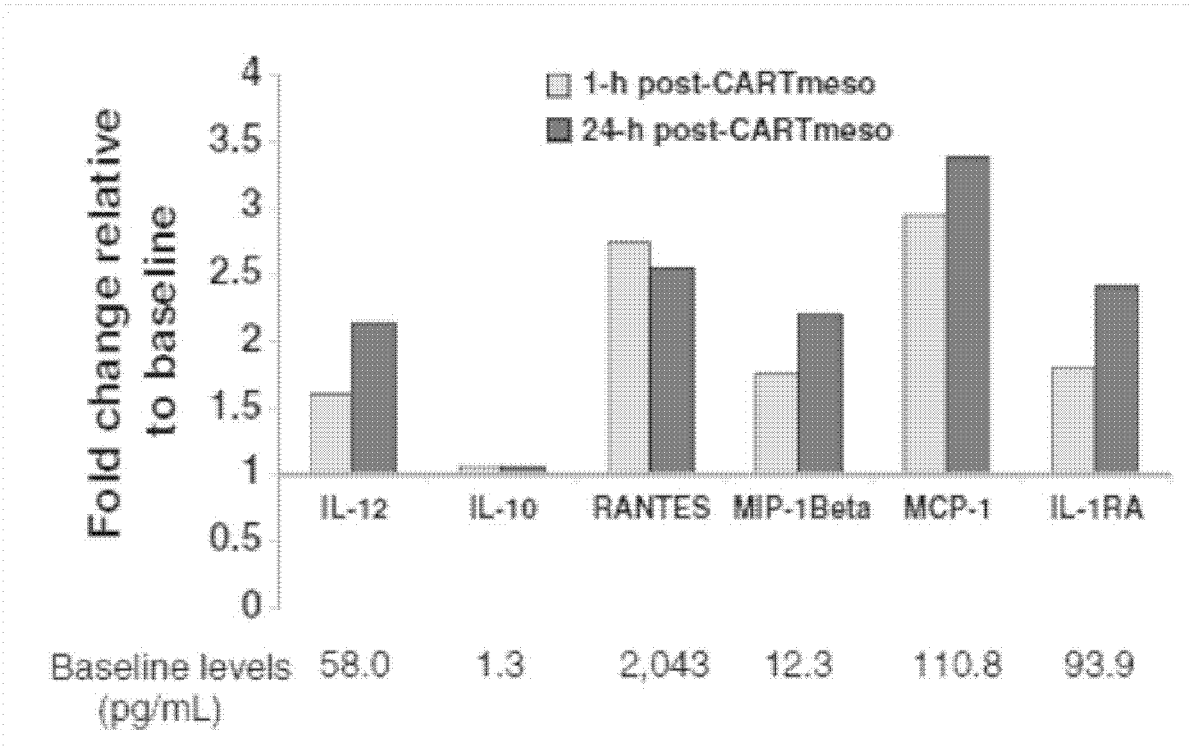

Fold change relative to baseline
4
3.5
3
2.5
2
1.5
1
0.5
0
1-h post-CARTmeso
24-h post-CARTmeso
IL-12
IL-10
RANTES
MIP-1Beta
MCP-1
IL-1RA
Baseline levels (pg/mL)
58.0
1.3
2,043
12.3
110.8
93.9

FIG. 9

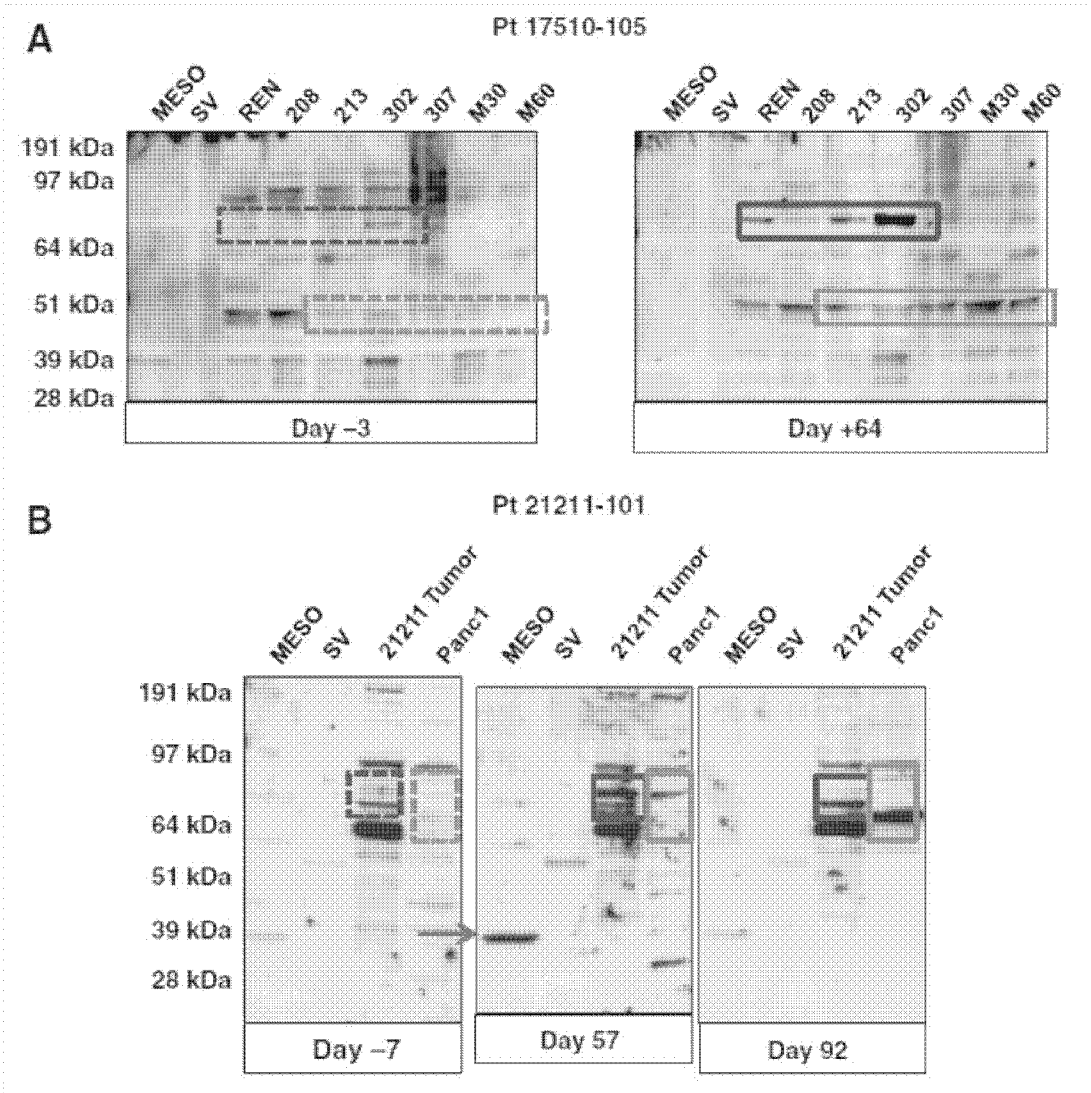

A
Pt 17510-105
MESO
SV
REN
208
213
302
307
M30
M60
191 kDa
97 kDa
64 kDa
51 kDa
39 kDa
28 kDa
Day −3
Day +64
B
Pt 21211-101
MESO
SV
21211 Tumor
Panc1
191 kDa
97 kDa
64 kDa
51 kDa
39 kDa
28 kDa
Day −7
Day 57
Day 92

FIG. 10

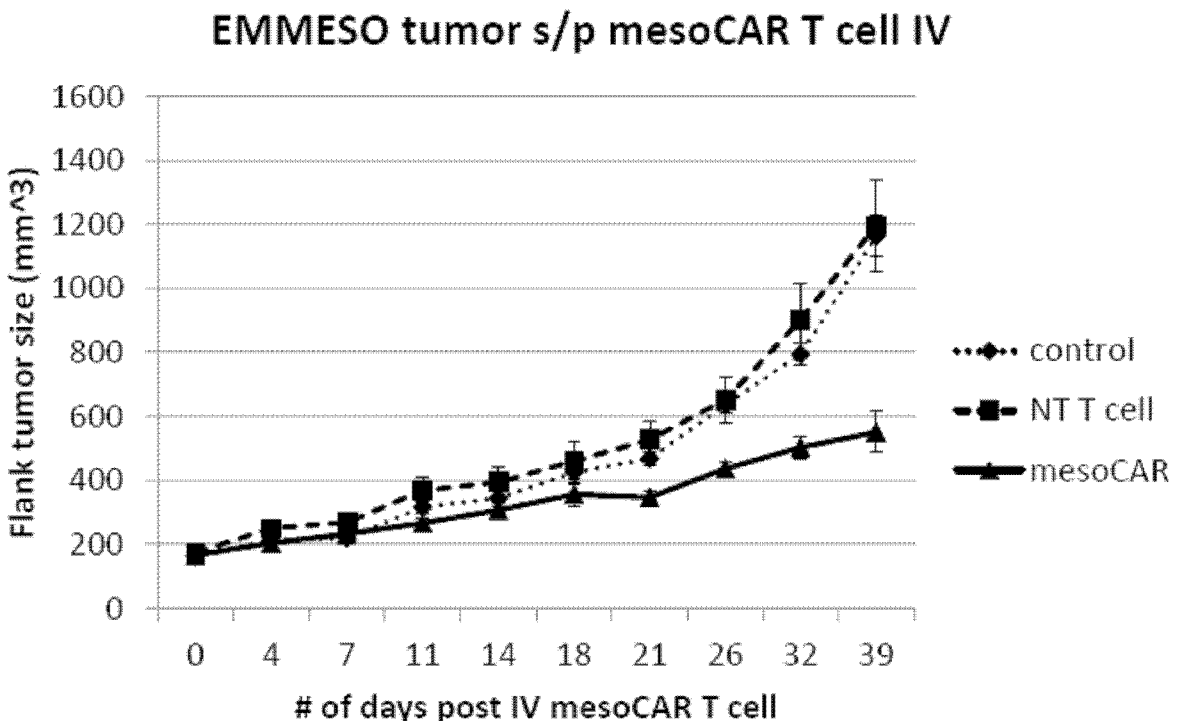

EMMESO tumor s/p mesoCAR T cell IV
Flank tumor size (mm^3)
1600
1400
1200
1000
800
600
400
200
0
0
4
7
11
14
18
21
26
32
39
# of days post IV mesoCAR T cell
control
NT T cell
mesoCAR

FIG. 11

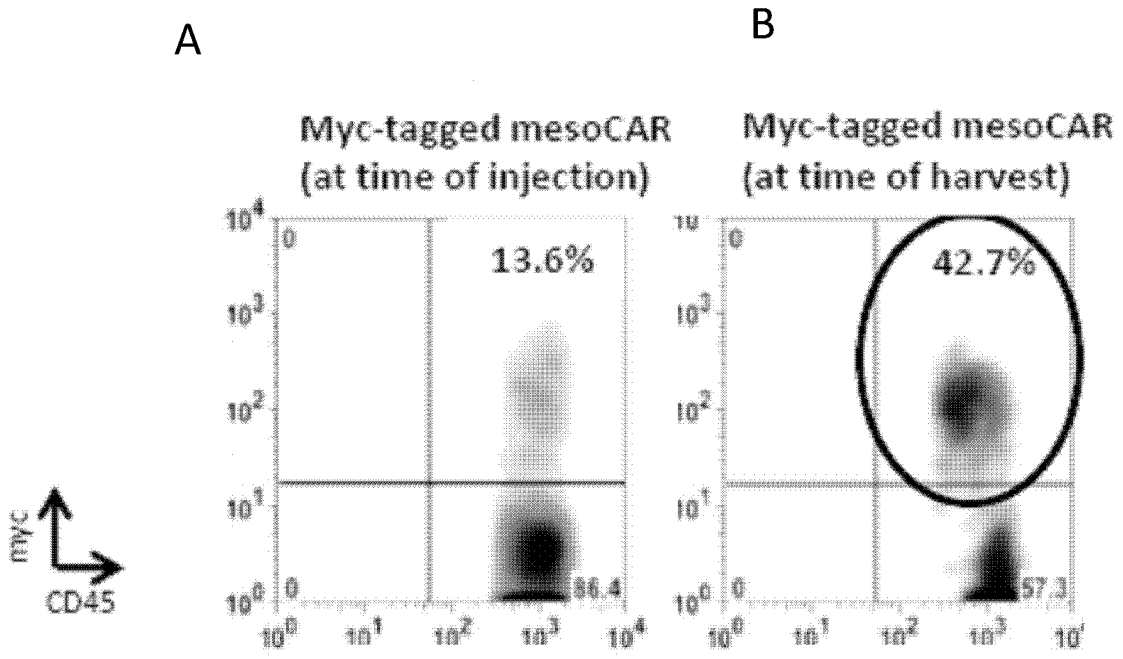

A
B
Myc-tagged mesoCAR
(at time of injection)
Myc-tagged mesoCAR
(at time of harvest)
13.6%
42.7%
86.4
57.3
myc
CD45

FIG. 12

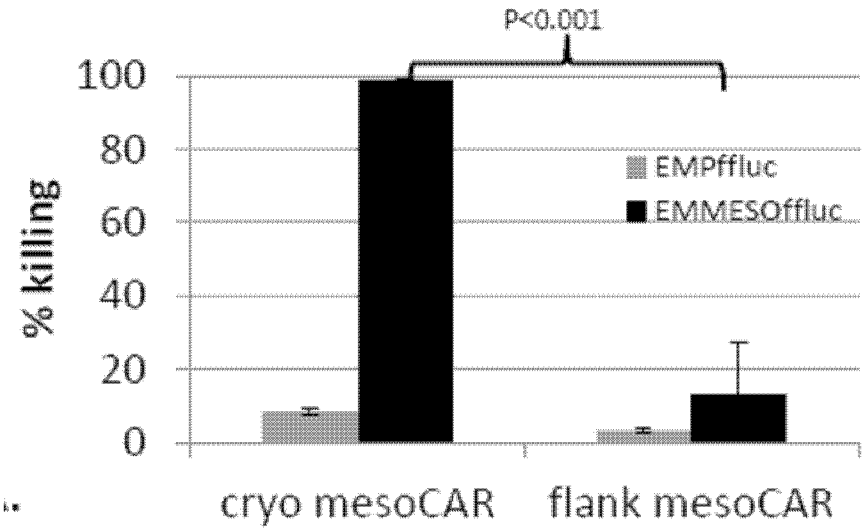

P<0.001
100
80
60
40
20
0
% killing
EMPffluc
EMMESOffluc
cryo mesoCAR
flank mesoCAR

FIG. 13

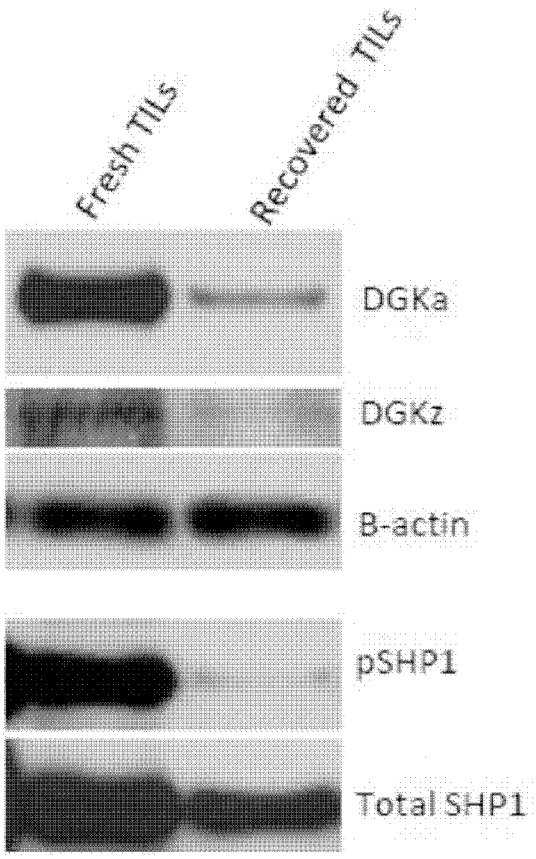

Fresh TILs
Recovered TILs
DGKa
DGKz
B-actin
pSHP1
Total SHP1

FIG. 14

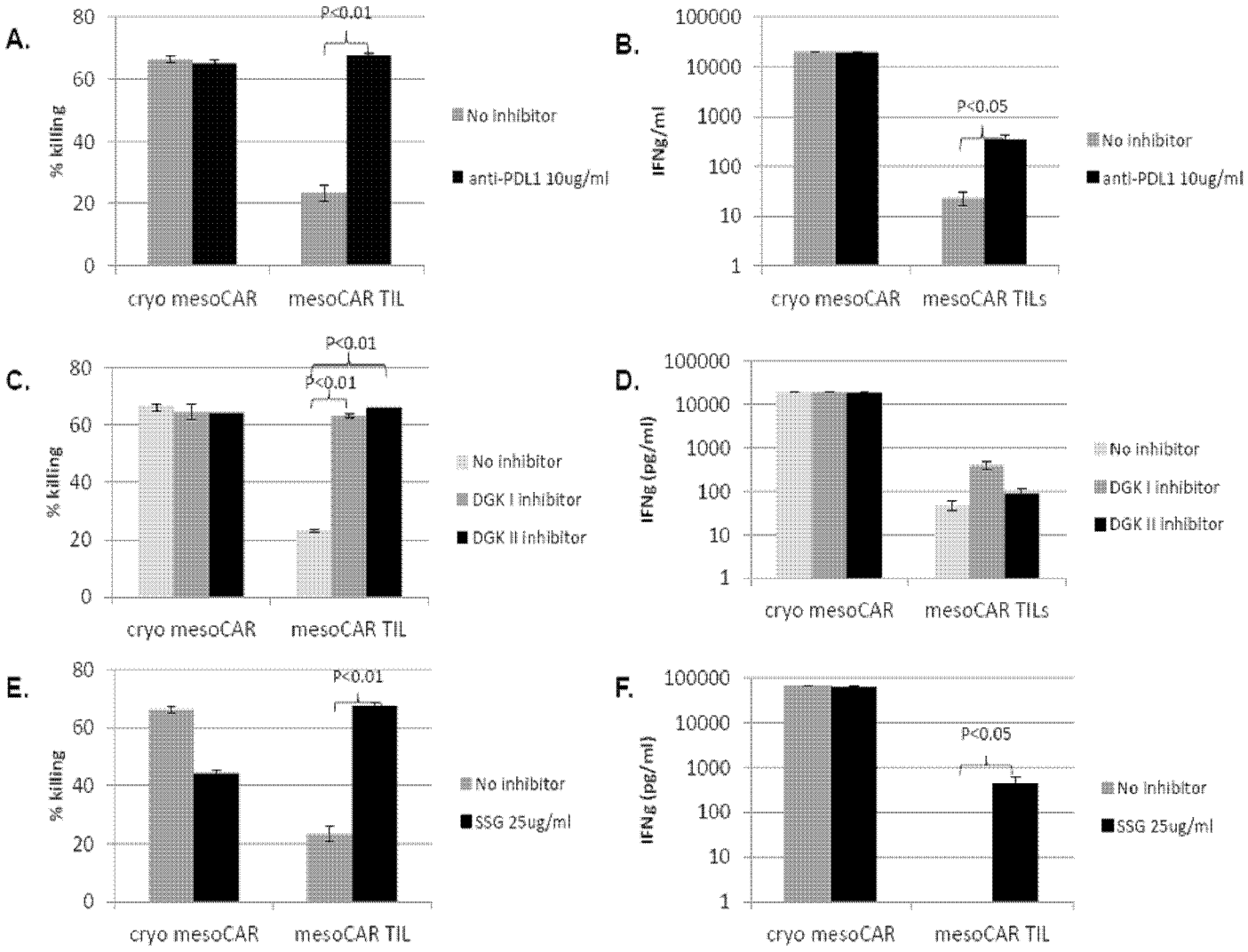
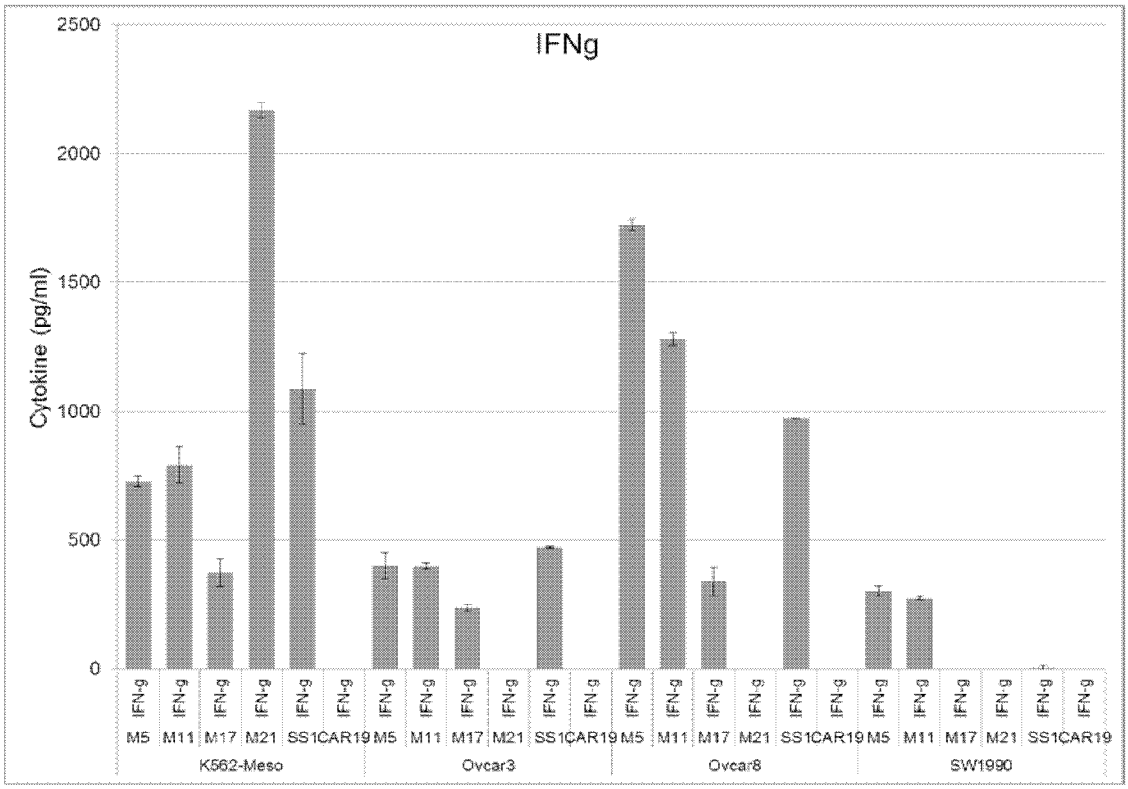

A.
% killing
80
60
40
20
0
P<0.01
cryo mesoCAR
mesoCAR TIL
No inhibitor
anti-PDL1 10ug/ml
B.
IFNg/ml
100000
10000
1000
100
10
1
P<0.05
cryo mesoCAR
mesoCAR TILs
No inhibitor
anti-PDL1 10ug/ml
C.
% killing
P<0.01
P<0.01
cryo mesoCAR
mesoCAR TIL
No inhibitor
DGK I inhibitor
DGK II inhibitor
D.
IFNg (pg/ml)
cryo mesoCAR
mesoCAR TILs
No inhibitor
DGK I inhibitor
DGK II inhibitor
E.
% killing
P<0.01
cryo mesoCAR
mesoCAR TIL
No inhibitor
SSG 25ug/ml
F.
IFNg (pg/ml)
P<0.05
cryo mesoCAR
mesoCAR TIL
No inhibitor
SSG 25ug/ml

FIG. 15

A


IFNg
Cytokine (pg/ml)
2500
2000
1500
1000
500
0
IFN-g
M5 M11 M17 M21 SS1CAR19
K562-Meso
Ovcar3
Ovcar8
SW1990

B

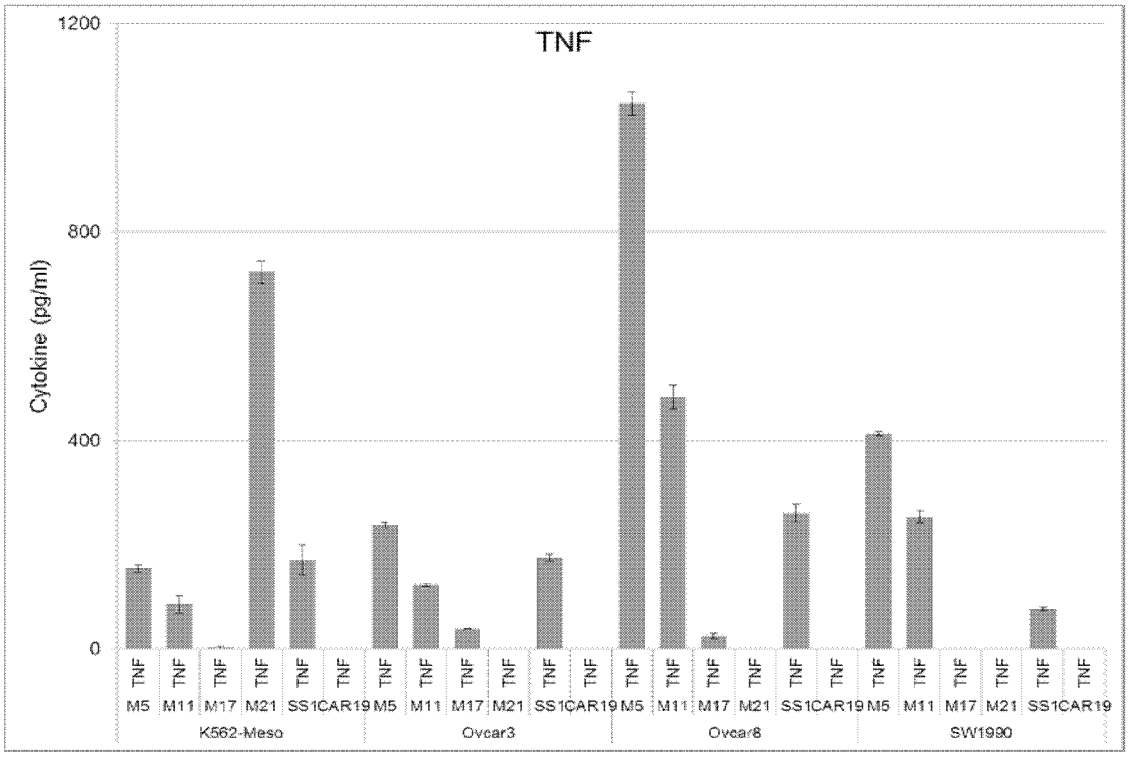
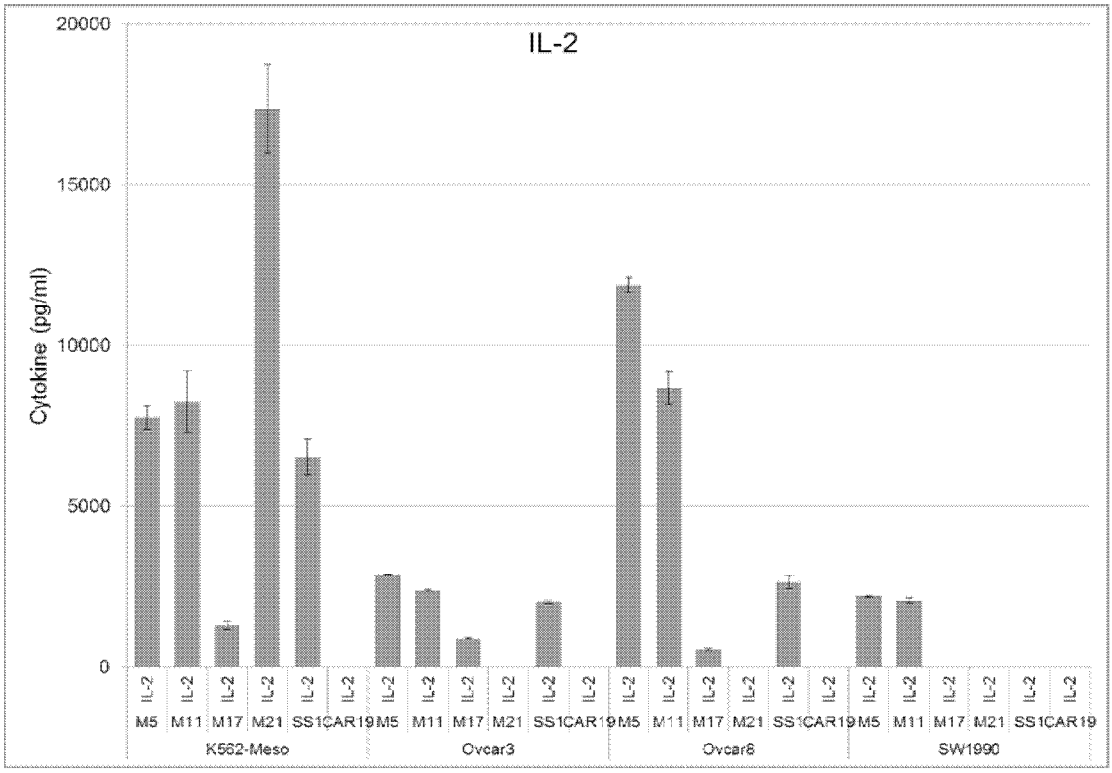

TNF
Cytokine (pg/ml)
1200
800
400
0
TNF
M5 M11 M17 M21 SS1CAR19
K562-Meso
Ovcar3
Ovcar8
SW1990

FIG. 15

C


IL-2
Cytokine (pg/ml)
20000
15000
10000
5000
0
IL-2
M5
M11
M17
M21
SS1CAR19
K562-Meso
Ovcar3
Ovcar8
SW1990

D

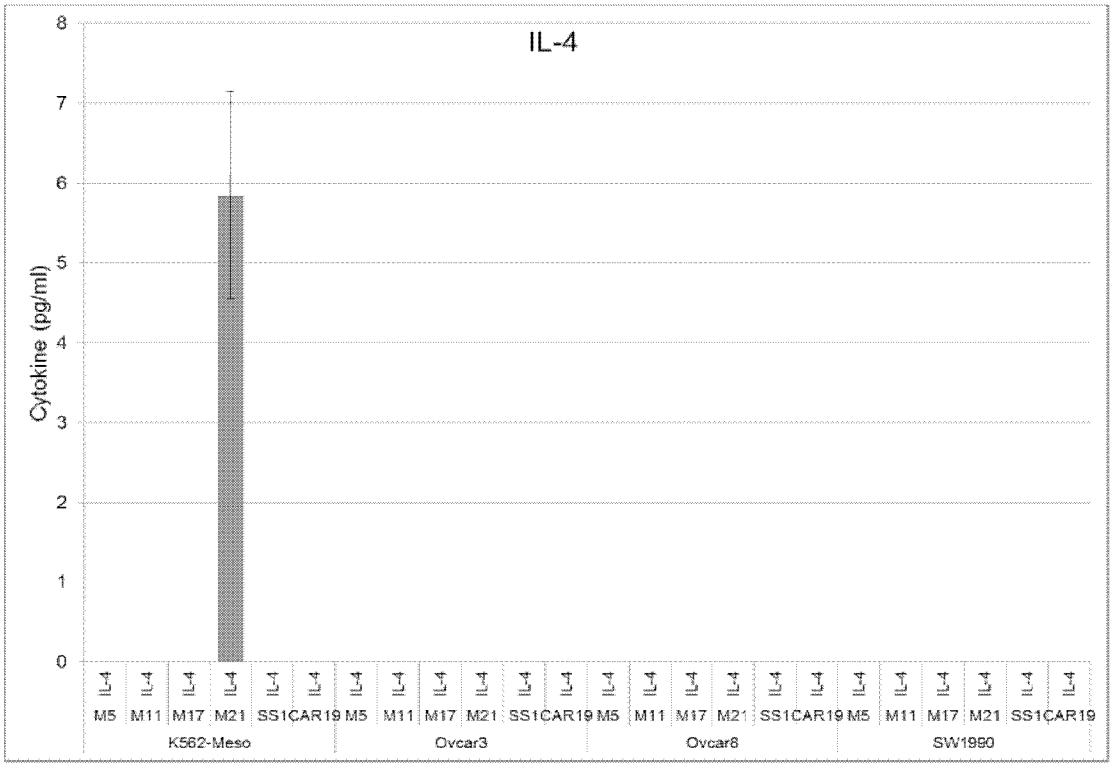
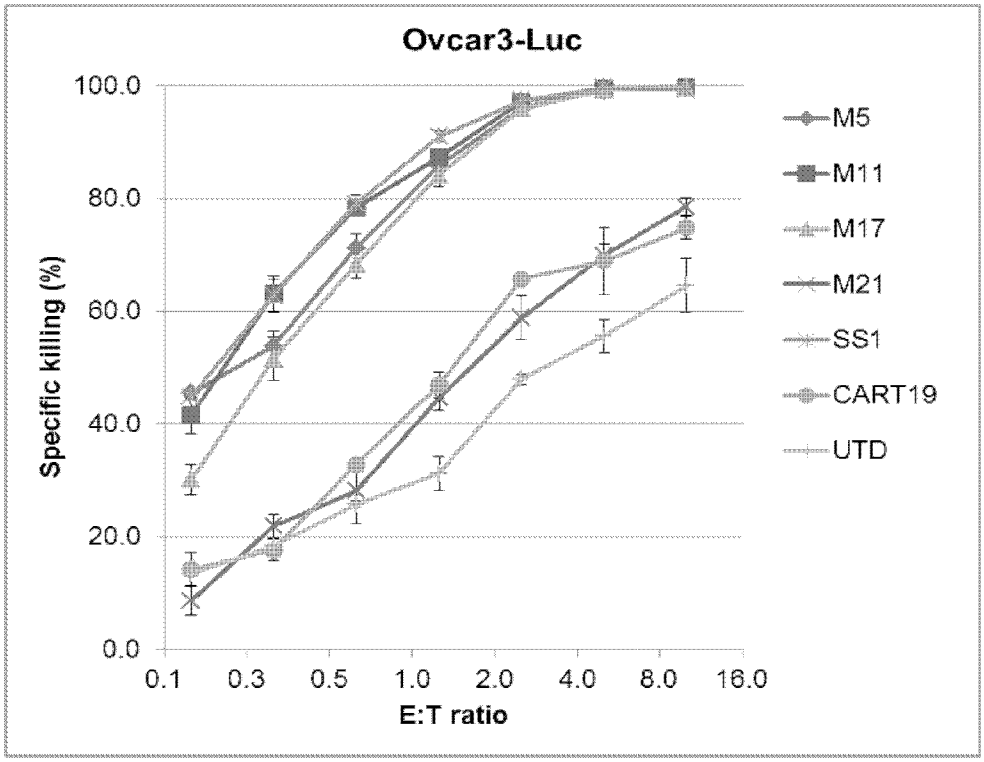

IL-4
Cytokine (pg/ml)
8
7
6
5
4
3
2
1
0
M5
M11
M17
M21
SS1CAR19
K562-Meso
Ovcar3
Ovcar8
SW1990

FIG. 16

A


Ovcar3-Luc
100.0
80.0
60.0
40.0
20.0
0.0
Specific killing (%)
0.1
0.3
0.5
1.0
2.0
4.0
8.0
16.0
E:T ratio
M5
M11
M17
M21
SS1
CART19
UTD

B

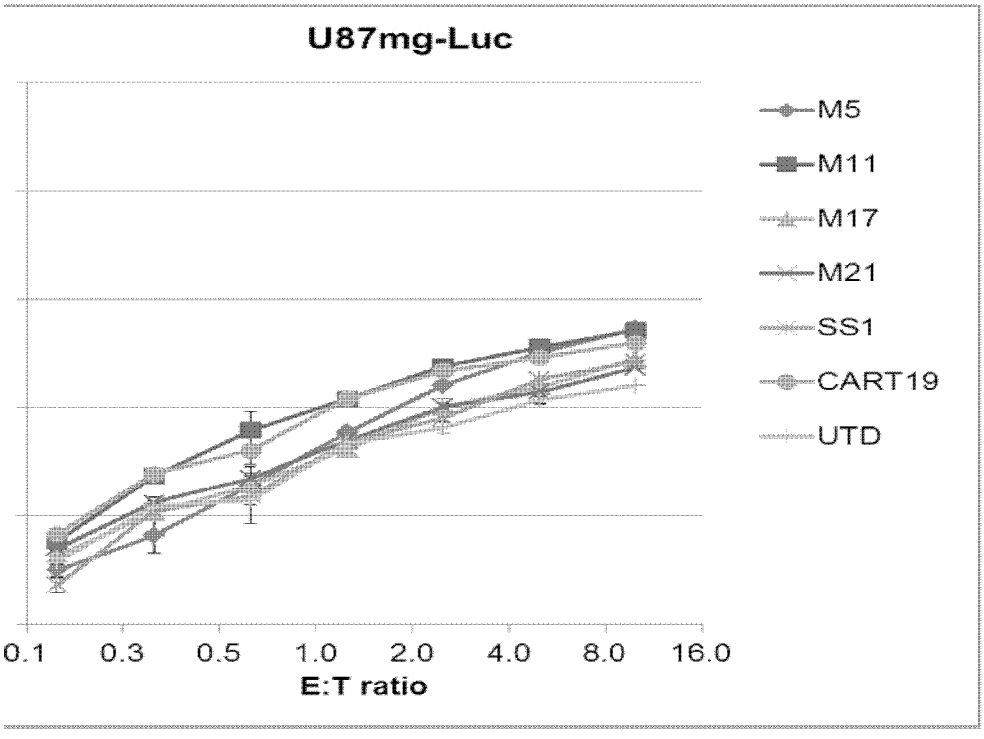
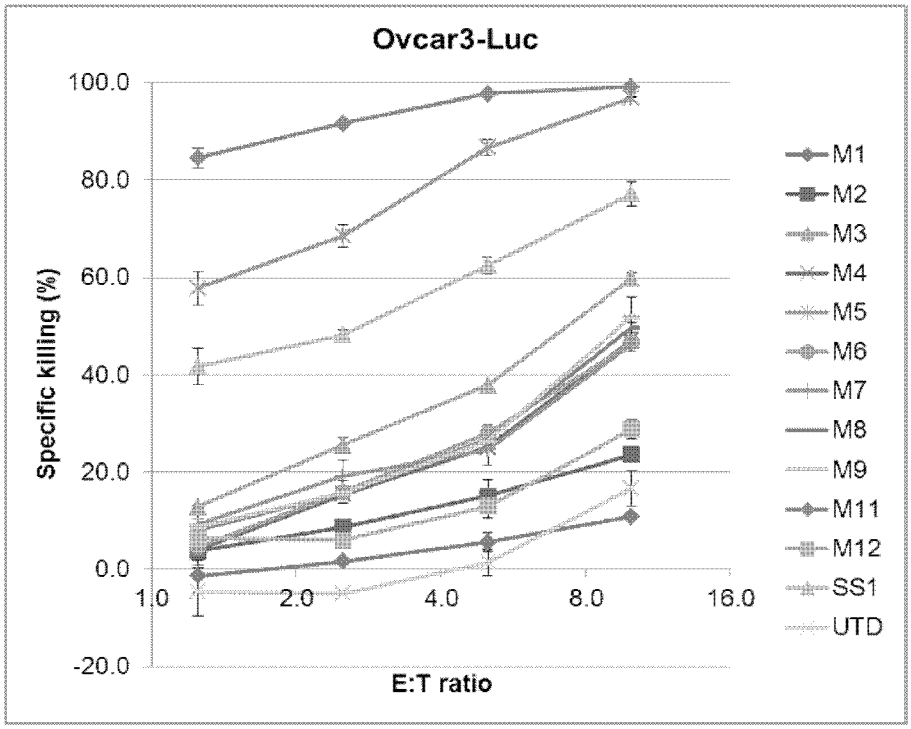

U87mg-Luc
0.1
0.3
0.5
1.0
2.0
4.0
8.0
16.0
E:T ratio
M5
M11
M17
M21
SS1
CART19
UTD

FIG. 17

A


Ovcar3-Luc
100.0
80.0
60.0
40.0
20.0
0.0
-20.0
Specific killing (%)
1.0
2.0
4.0
8.0
16.0
E:T ratio
M1
M2
M3
M4
M5
M6
M7
M8
M9
M11
M12
SS1
UTD

B

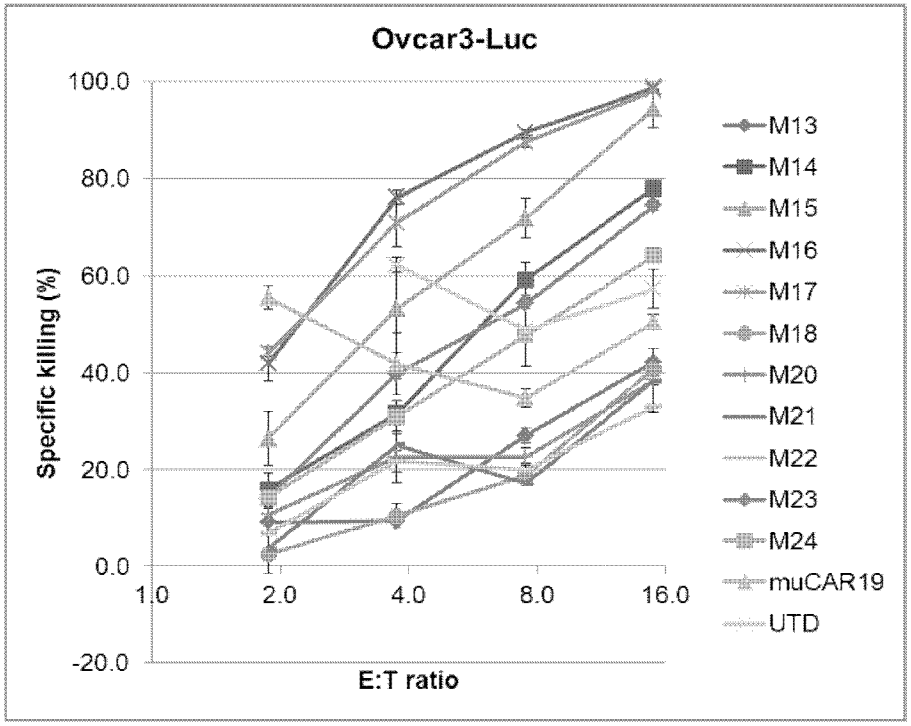

Ovcar3-Luc
100.0
80.0
60.0
40.0
20.0
0.0
-20.0
Specific killing (%)
1.0
2.0
4.0
8.0
16.0
E:T ratio
M13
M14
M15
M16
M17
M18
M20
M21
M22
M23
M24
muCAR19
UTD

FIG. 18

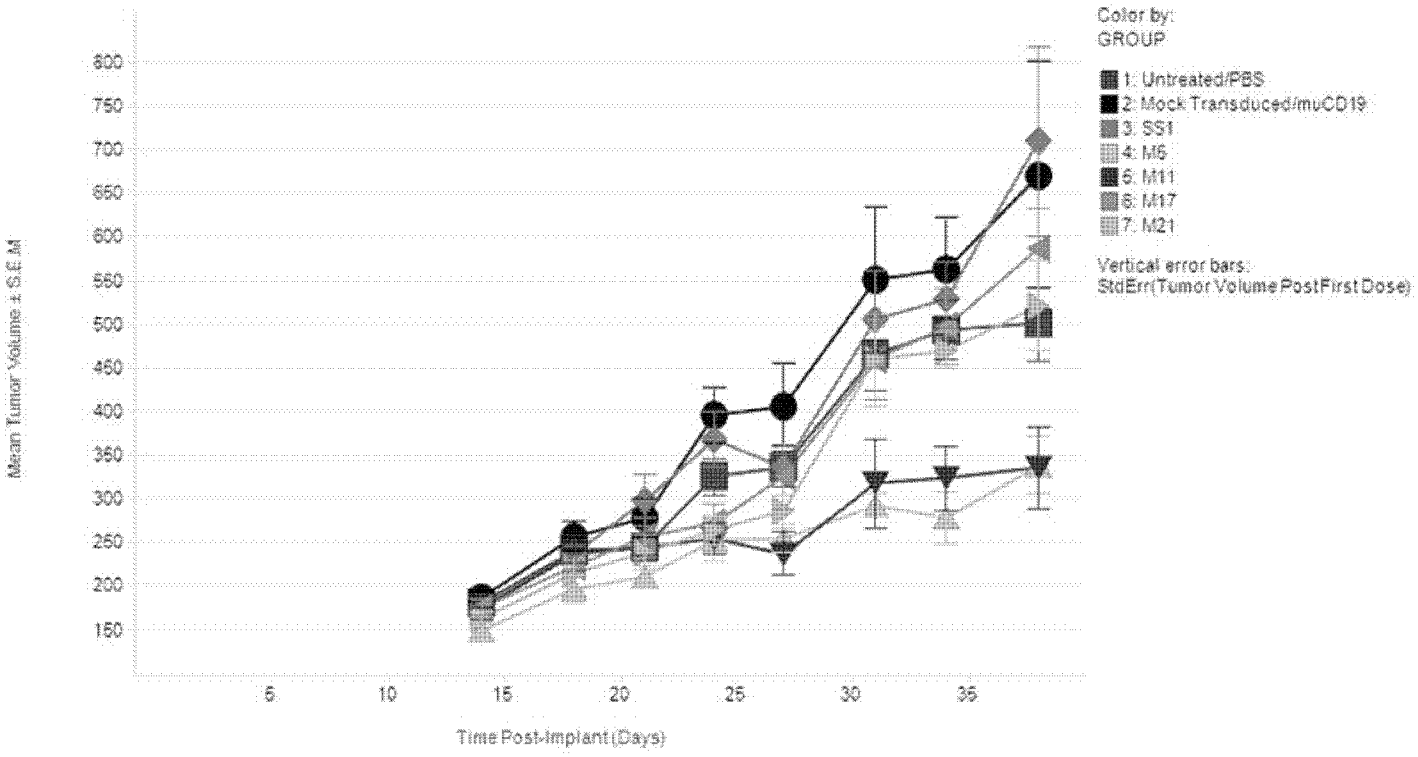

Color by:
GROUP
1: Untreated/PBS
2: Mock Transduced/muCD19
3: SS1
4: M6
6: M11
8: M17
7: M21
Vertical error bars:
StdErr(Tumor Volume PostFirst Dose)
Mean Tumor Volume ± S.E.M
800
750
700
650
600
550
500
450
400
350
300
250
200
150
5
10
15
20
25
30
35
Time Post-Implant (Days)

FIG. 19

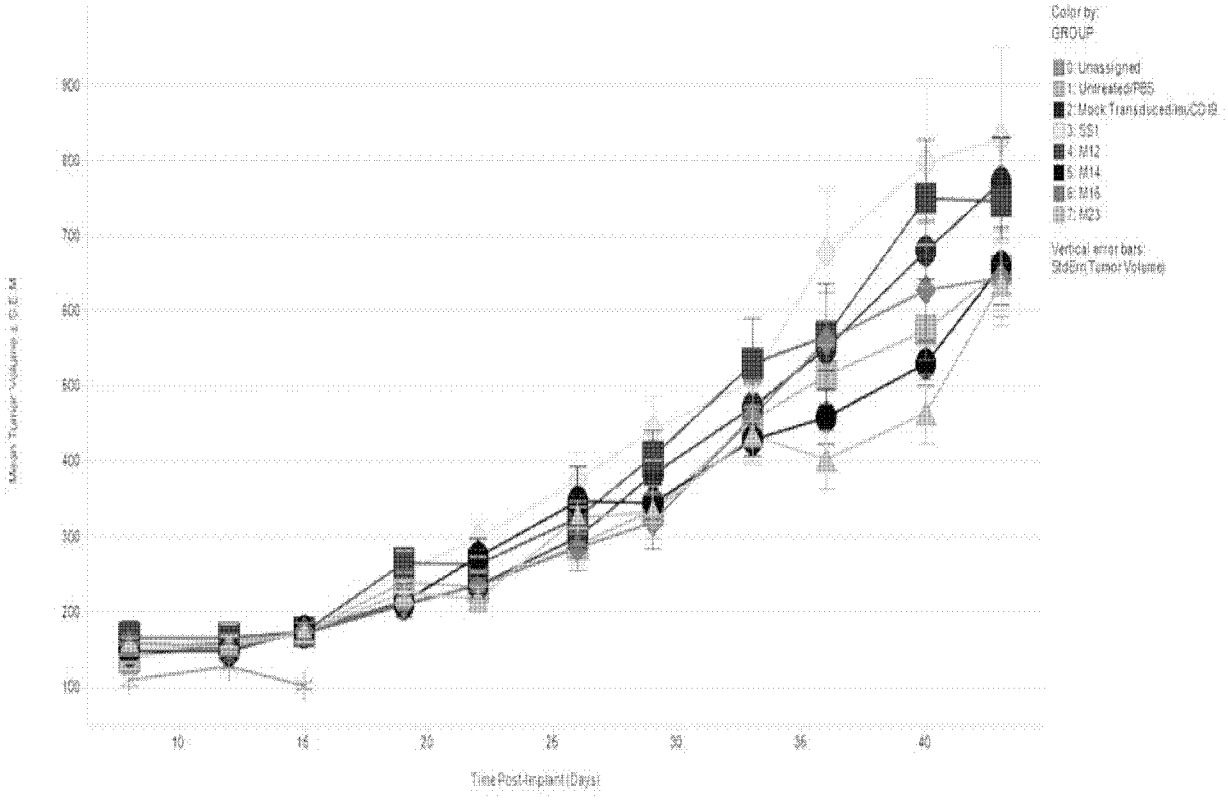

Color by:
GROUP
0: Unassigned
1: Untreated/PBS
2: Mock Transduced/mIgG1
3: SS1
4: M12
5: M14
6: M16
7: M23
Vertical error bars:
StdErr Tumor Volume
900
800
700
600
500
400
300
200
100
10
15
20
25
30
35
40
Time Post-Implant (Days)

FIG. 20A

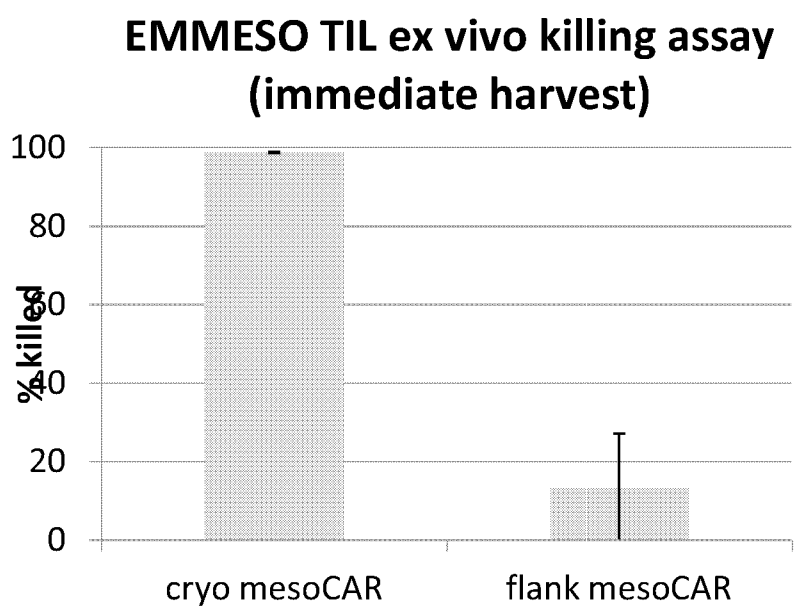


FIG. 20B

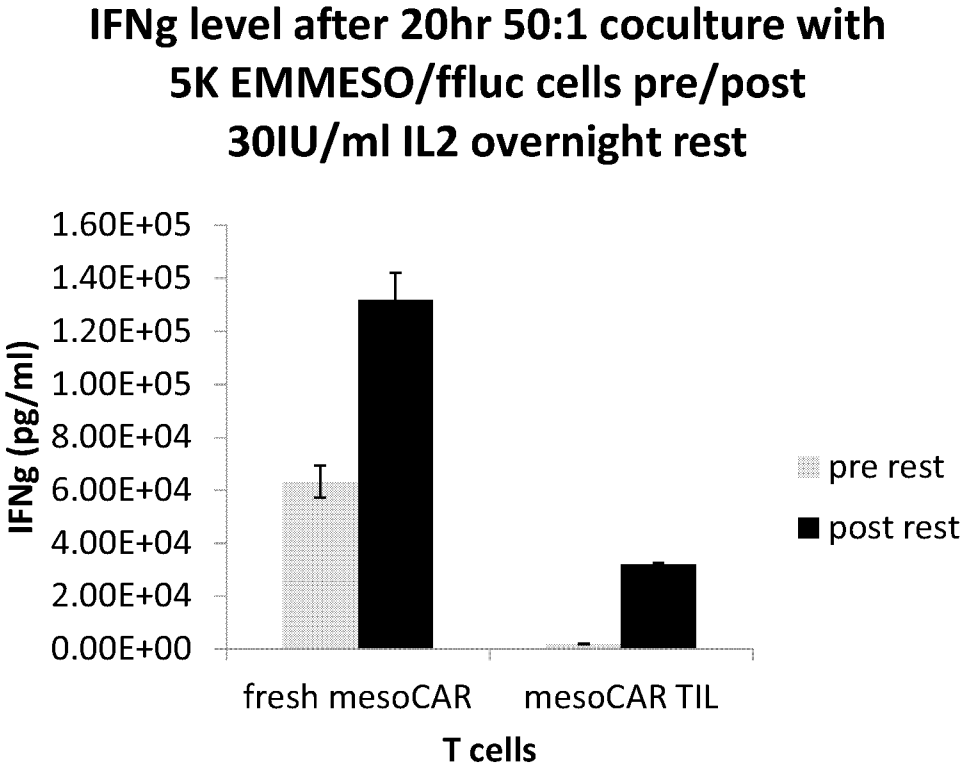


FIG. 20C

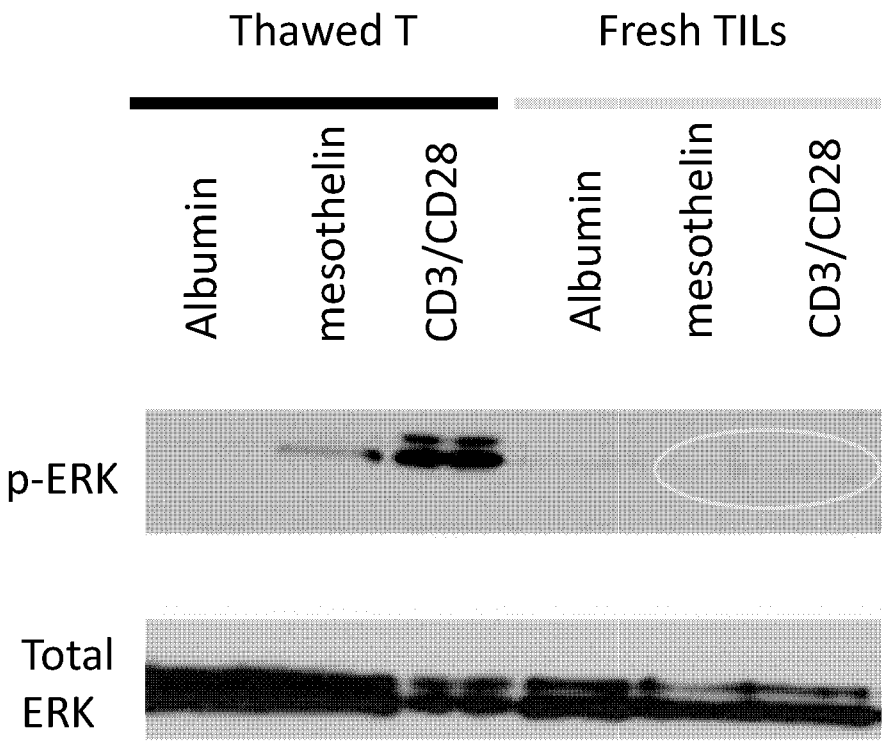

Thawed T
Fresh TILs
Albumin
mesothelin
CD3/CD28
Albumin
mesothelin
CD3/CD28
p-ERK
Total ERK

FIG. 21

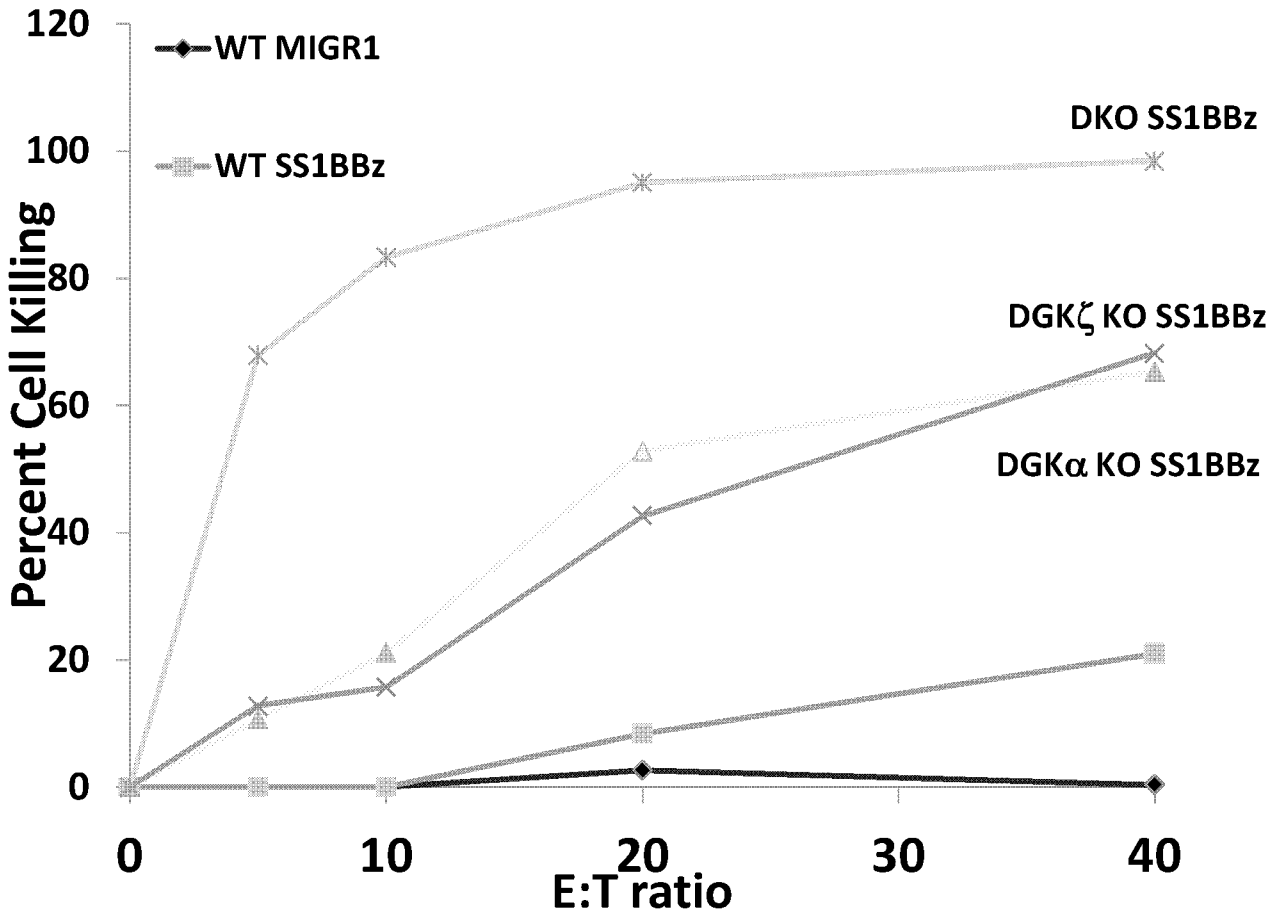

120
100
80
60
40
20
0
Percent Cell Killing
WT MIGR1
WT SS1BBz
DKO SS1BBz
DGKζ KO SS1BBz
DGKα KO SS1BBz
0
10
20
30
40
E:T ratio

FIG. 22

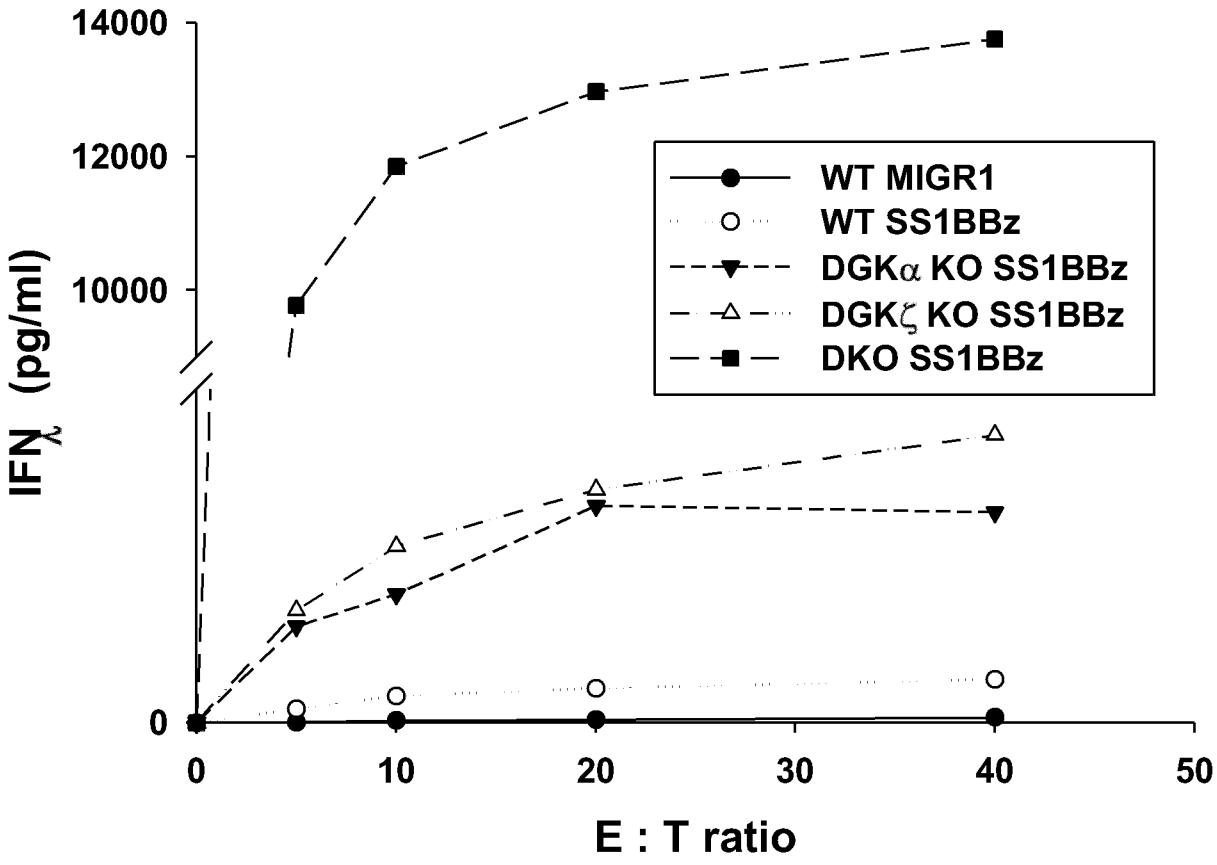

14000
12000
10000
0
IFNγ (pg/ml)
0
10
20
30
40
50
E : T ratio
WT MIGR1
WT SS1BBz
DGKα KO SS1BBz
DGKζ KO SS1BBz
DKO SS1BBz

FIG. 23

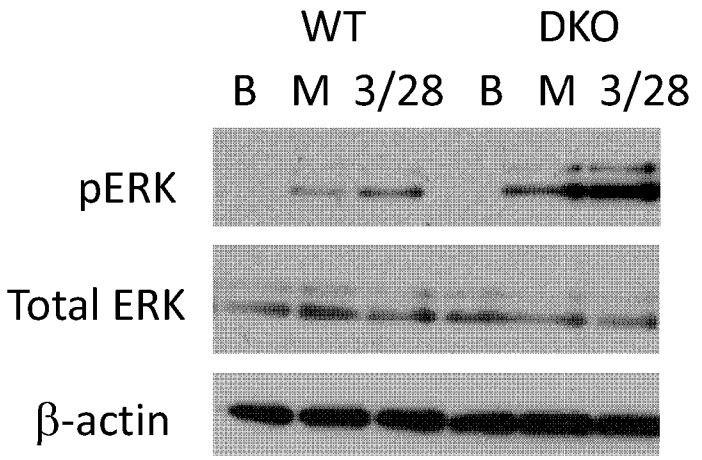

WT
DKO
B M 3/28 B M 3/28
pERK
Total ERK
β-actin

FIG. 24

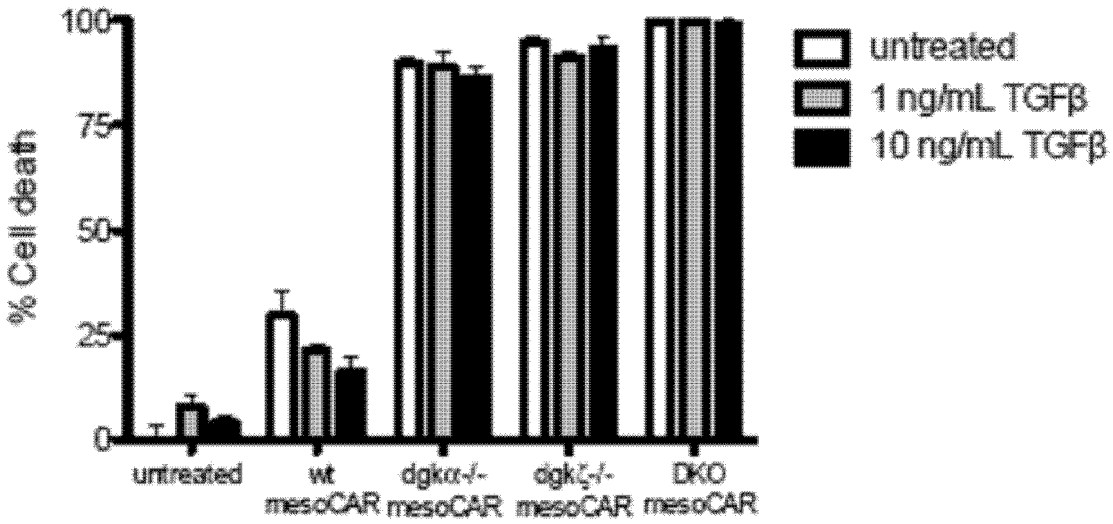

% Cell death
100
75
50
25
0
untreated
wt mesoCAR
dgkα-/- mesoCAR
dgkζ-/- mesoCAR
DKO mesoCAR
untreated
1 ng/mL TGFβ
10 ng/mL TGFβ

FIG. 25A

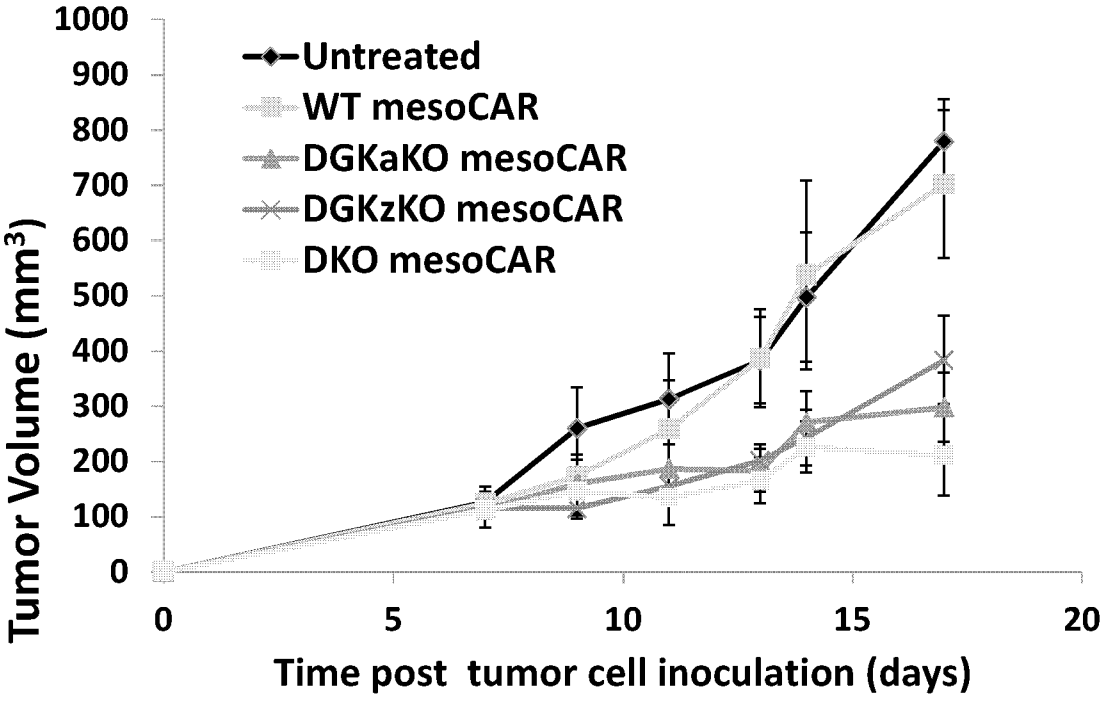


FIG. 25B

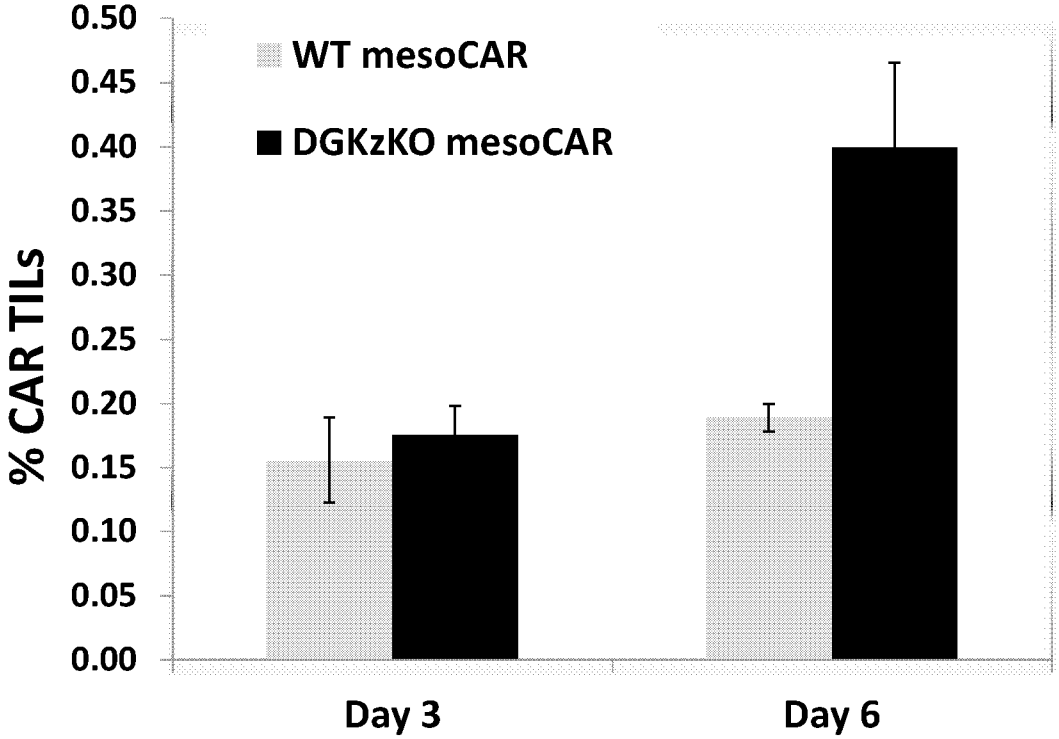


FIG. 26

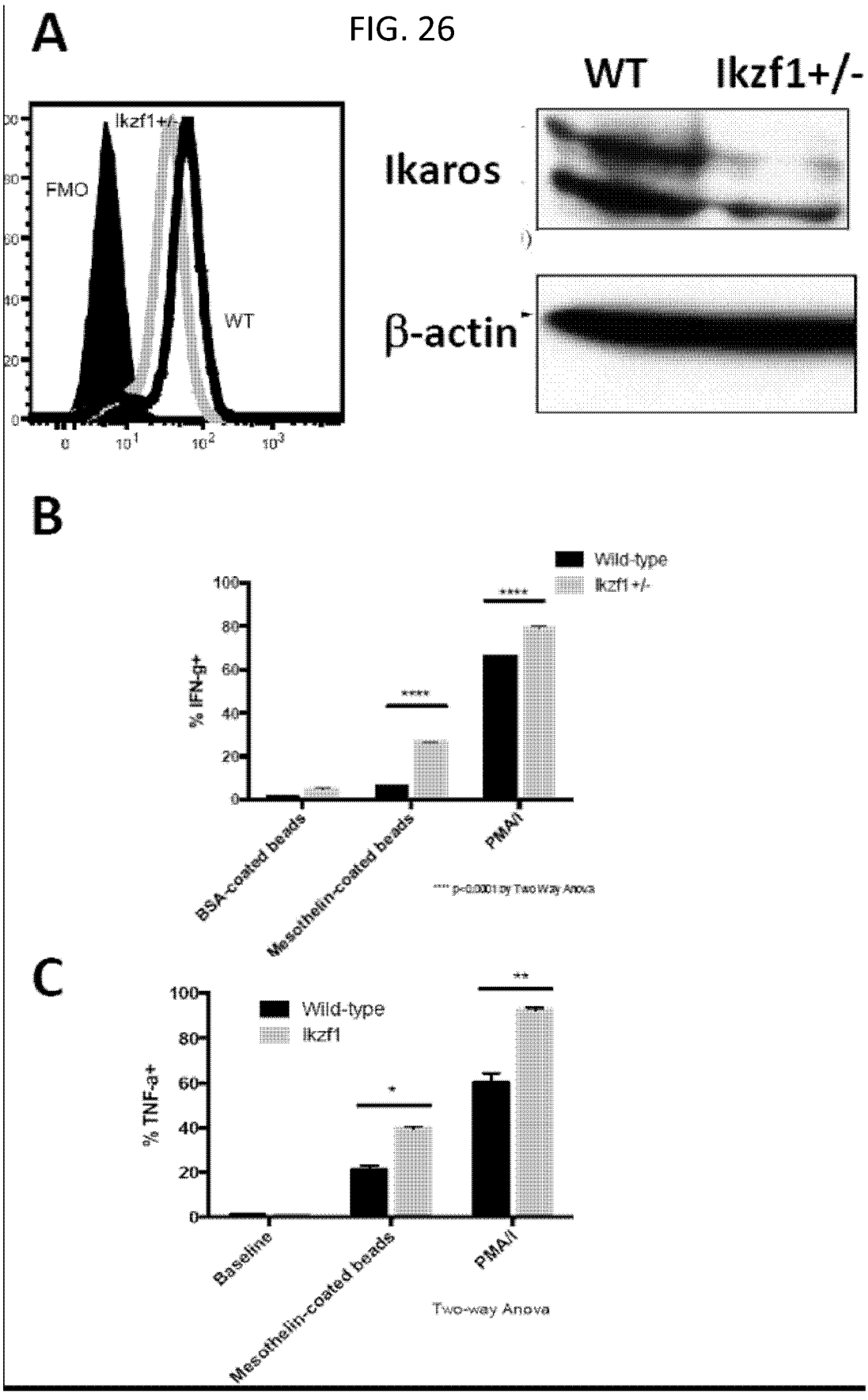

A
WT
Ikzf1+/-
Ikzf1+/-
FMO
WT
Ikaros
β-actin
B
Wild-type
Ikzf1+/-
% IFN-g+
****
****
BSA-coated beads
Mesothelin-coated beads
PMA/I
**** p<0.0001 by Two Way Anova
C
Wild-type
Ikzf1
% TNF-a+
*
**
Baseline
Mesothelin-coated beads
PMA/I
Two-way Anova

FIG. 26

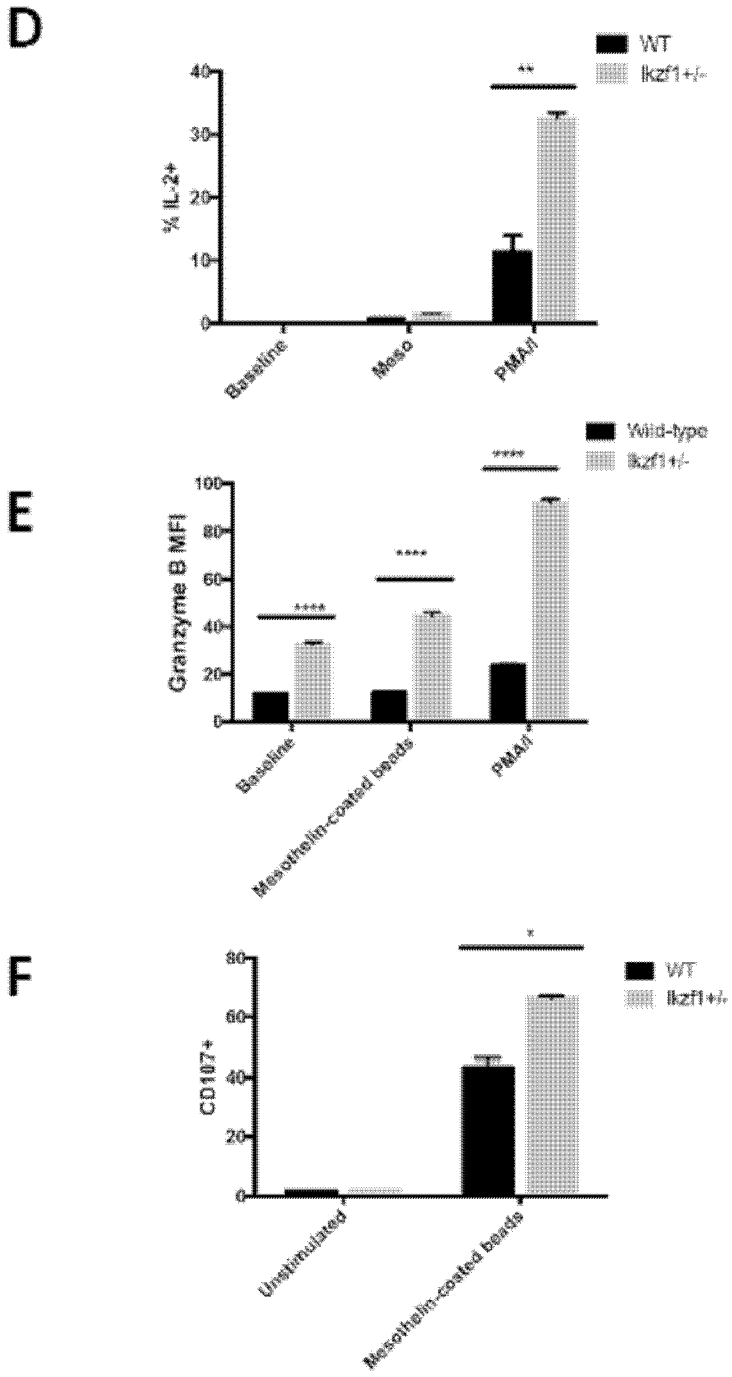
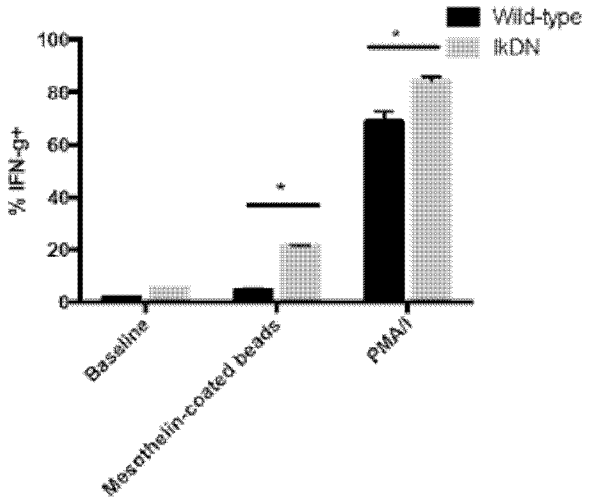
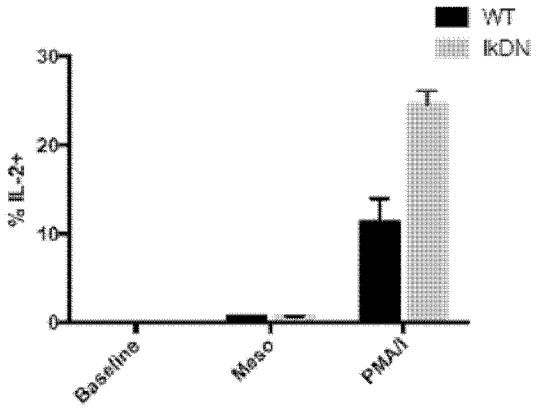

D
WT
Ikzf1+/-
**
% IL-2+
40
30
20
10
0
Baseline
Meso
PMA/I
E
Wild-type
Ikzf1+/-
****
****
****
Granzyme B MFI
100
80
60
40
20
0
Baseline
Mesothelin-coated beads
PMA/I
F
*
WT
Ikzf1+/-
CD107+
80
60
40
20
0
Unstimulated
Mesothelin-coated beads

FIG. 27

A


Wild-type
IkDN
% IFN-g+
100
80
60
40
20
0
*
*
Baseline
Mesothelin-coated beads
PMA/I

B


WT
IkDN
% IL-2+
30
20
10
0
Baseline
Meso
PMA/I

C

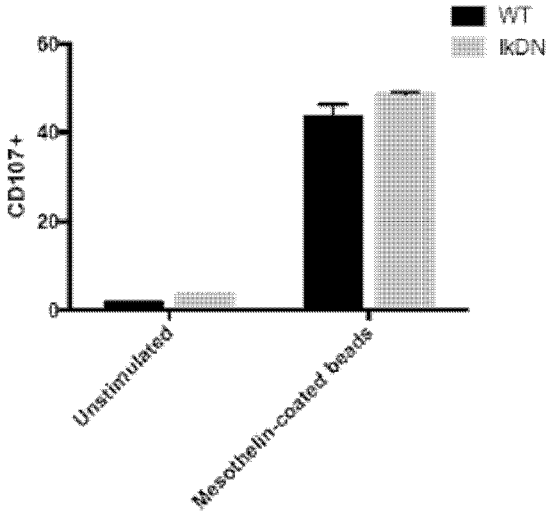

WT
IkDN
CD107+
60
40
20
0
Unstimulated
Mesothelin-coated beads

FIG. 28

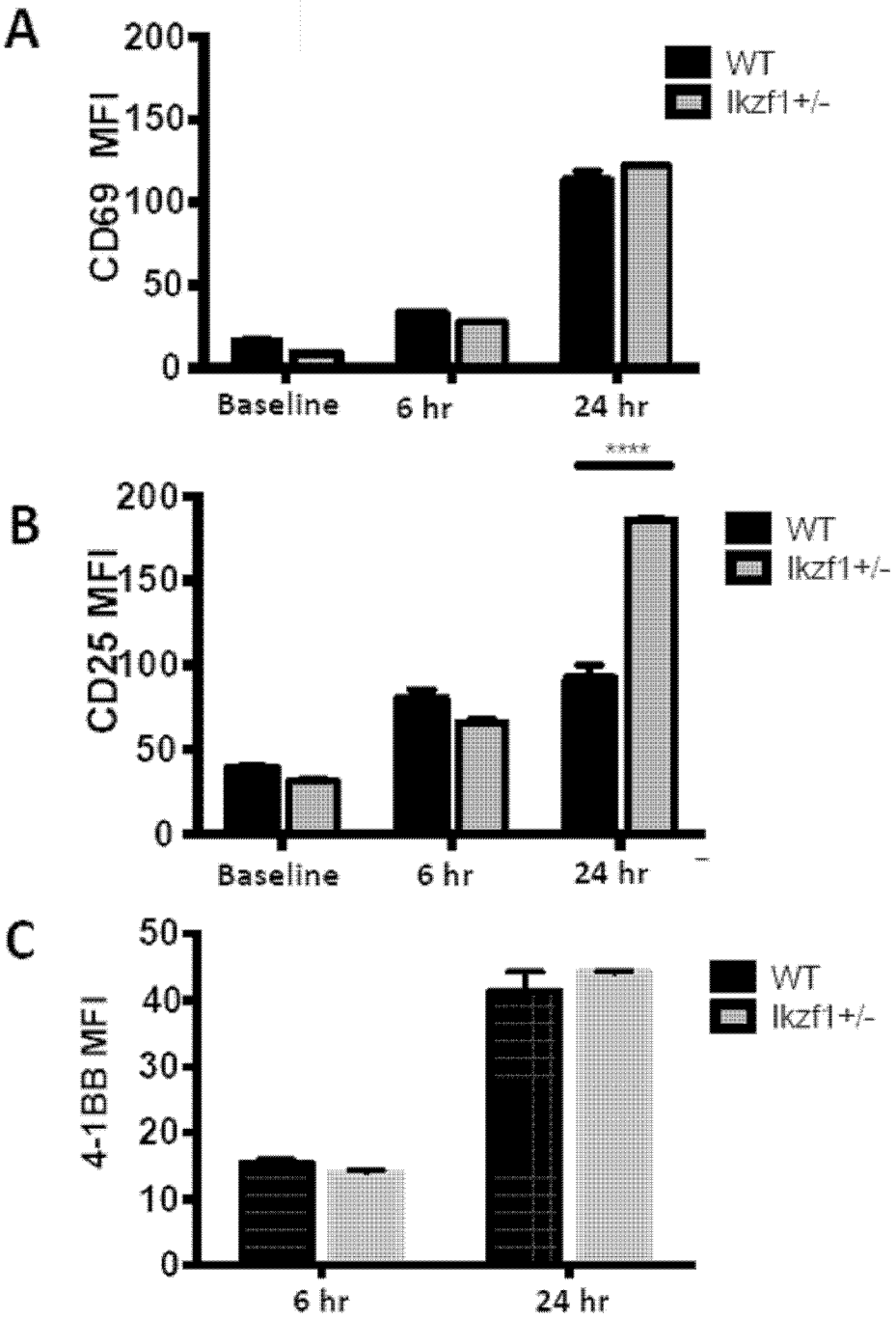
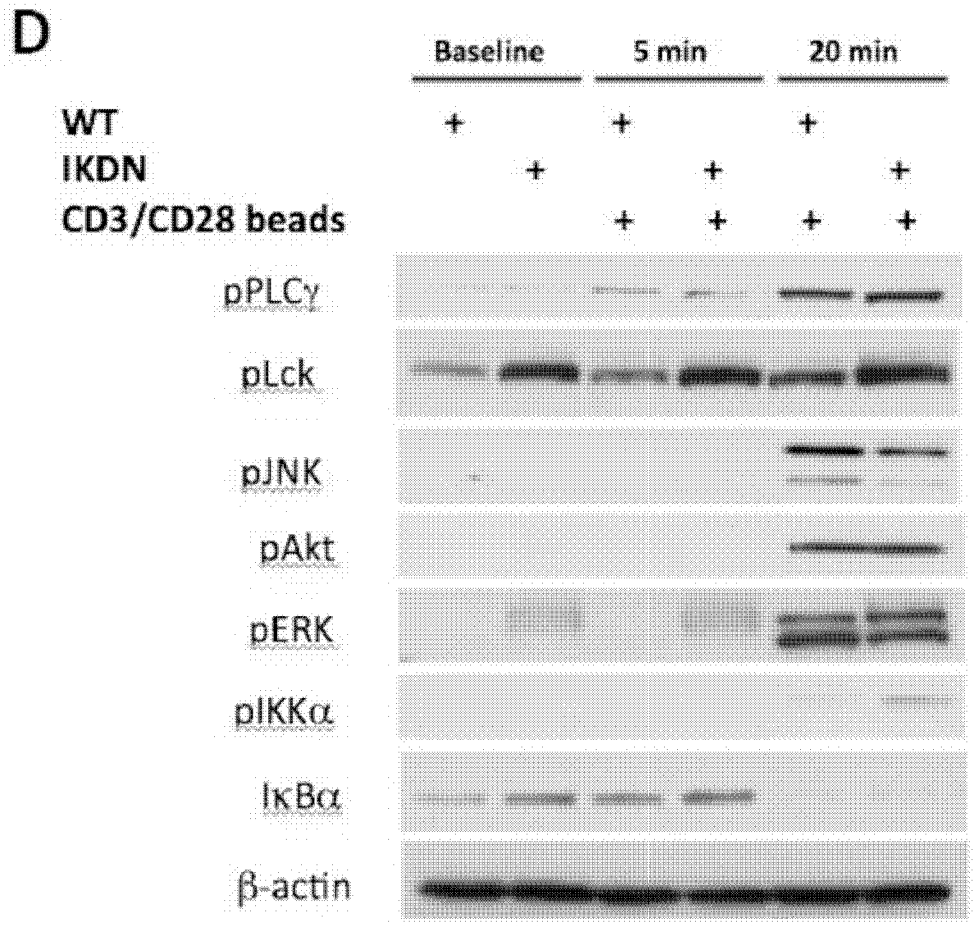

A
CD69 MFI
200
150
100
50
0
Baseline
6 hr
24 hr
WT
Ikzf1+/-
****
B
CD25 MFI
200
150
100
50
0
Baseline
6 hr
24 hr
WT
Ikzf1+/-
C
4-1BB MFI
50
40
30
20
10
0
6 hr
24 hr
WT
Ikzf1+/-

FIG. 28


D
Baseline
5 min
20 min
WT
IKDN
CD3/CD28 beads
pPLCγ
pLck
pJNK
pAkt
pERK
pIKKα
IκBα
β-actin

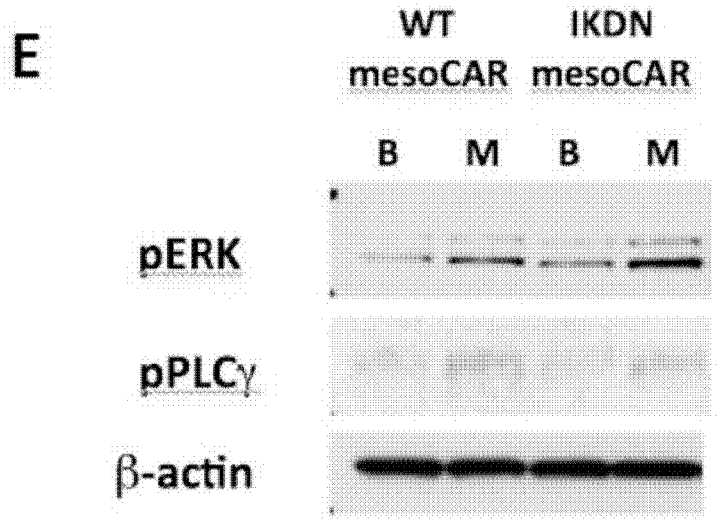

E
WT mesoCAR
IKDN mesoCAR
B
M
B
M
pERK
pPLCγ
β-actin

FIG. 29

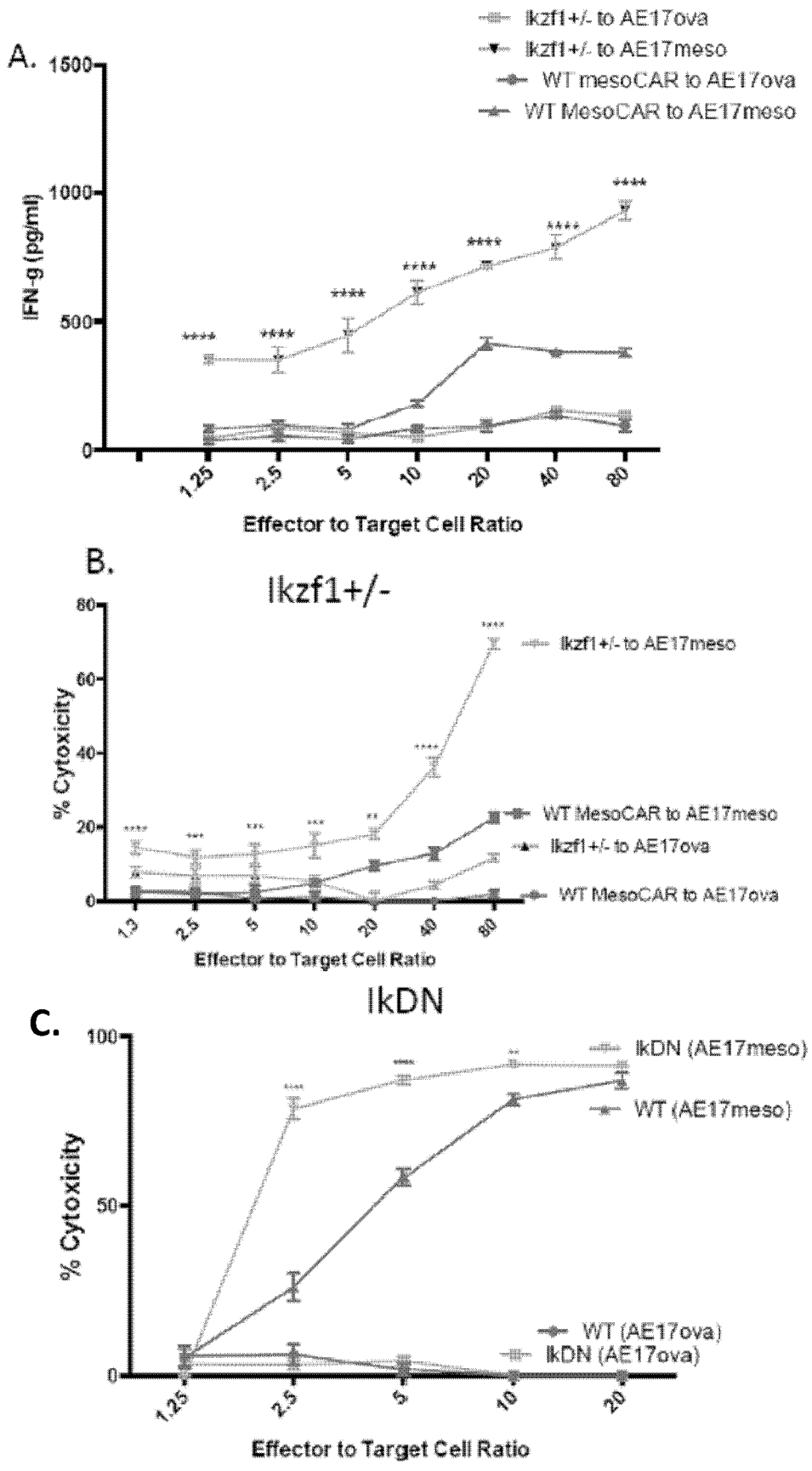
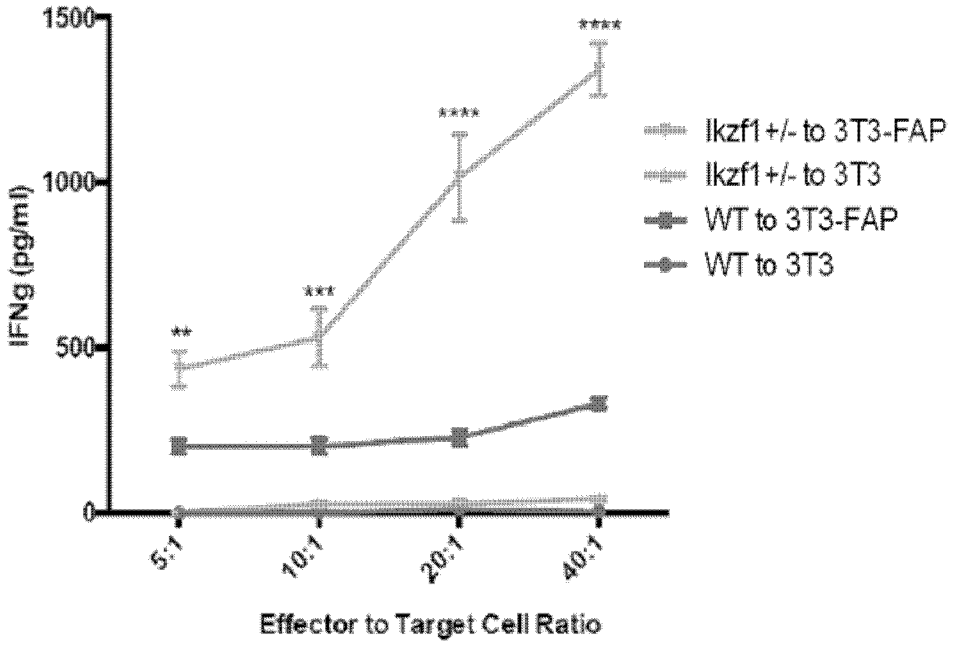

A.
Ikzf1+/- to AE17ova
Ikzf1+/- to AE17meso
WT mesoCAR to AE17ova
WT MesoCAR to AE17meso
IFN-g (pg/ml)
1500
1000
500
0
1.25
2.5
5
10
20
40
80
Effector to Target Cell Ratio
B.
Ikzf1+/-
% Cytoxicity
60
40
20
0
Ikzf1+/- to AE17meso
WT MesoCAR to AE17meso
Ikzf1+/- to AE17ova
WT MesoCAR to AE17ova
1.3
2.5
5
10
20
40
80
Effector to Target Cell Ratio
IkDN
C.
% Cytoxicity
100
50
0
IkDN (AE17meso)
WT (AE17meso)
WT (AE17ova)
IkDN (AE17ova)
1.25
2.5
5
10
20
Effector to Target Cell Ratio

FIG. 29

D.


1500
1000
500
0
IFNg (pg/ml)
**
***
****
****
5:1
10:1
20:1
40:1
Effector to Target Cell Ratio
Ikzf1+/- to 3T3-FAP
Ikzf1+/- to 3T3
WT to 3T3-FAP
WT to 3T3

E.

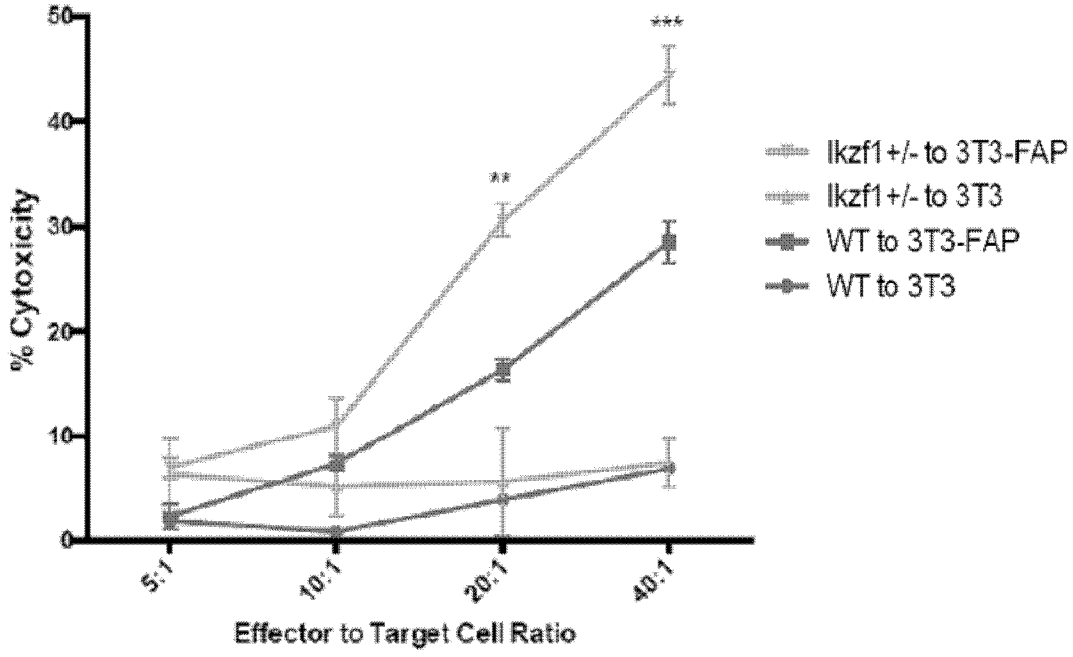

50
40
30
20
10
0
% Cytoxicity
**
***
5:1
10:1
20:1
40:1
Effector to Target Cell Ratio
Ikzf1+/- to 3T3-FAP
Ikzf1+/- to 3T3
WT to 3T3-FAP
WT to 3T3

FIG. 30

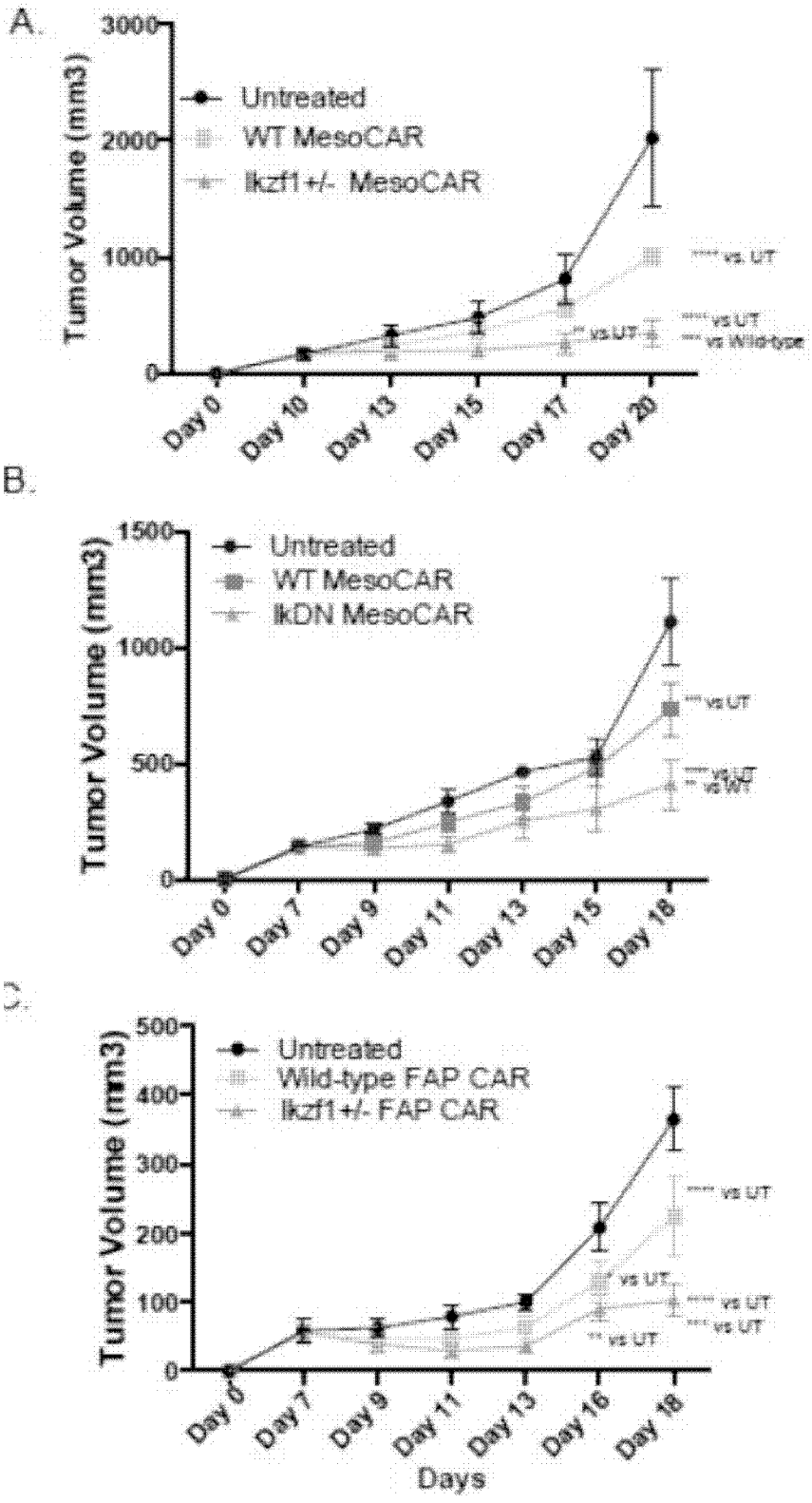

A.
Tumor Volume (mm3)
3000
2000
1000
0
Untreated
WT MesoCAR
Ikzf1+/- MesoCAR
**** vs. UT
**** vs UT
** vs UT
*** vs Wild-type
Day 0
Day 10
Day 13
Day 15
Day 17
Day 20
B.
Tumor Volume (mm3)
1500
1000
500
0
Untreated
WT MesoCAR
IkDN MesoCAR
*** vs UT
**** vs UT
** vs WT
Day 0
Day 7
Day 9
Day 11
Day 13
Day 15
Day 18
C.
Tumor Volume (mm3)
500
400
300
200
100
0
Untreated
Wild-type FAP CAR
Ikzf1+/- FAP CAR
**** vs UT
* vs UT
**** vs UT
**** vs UT
** vs UT
Day 0
Day 7
Day 9
Day 11
Day 13
Day 16
Day 18
Days

FIG. 31

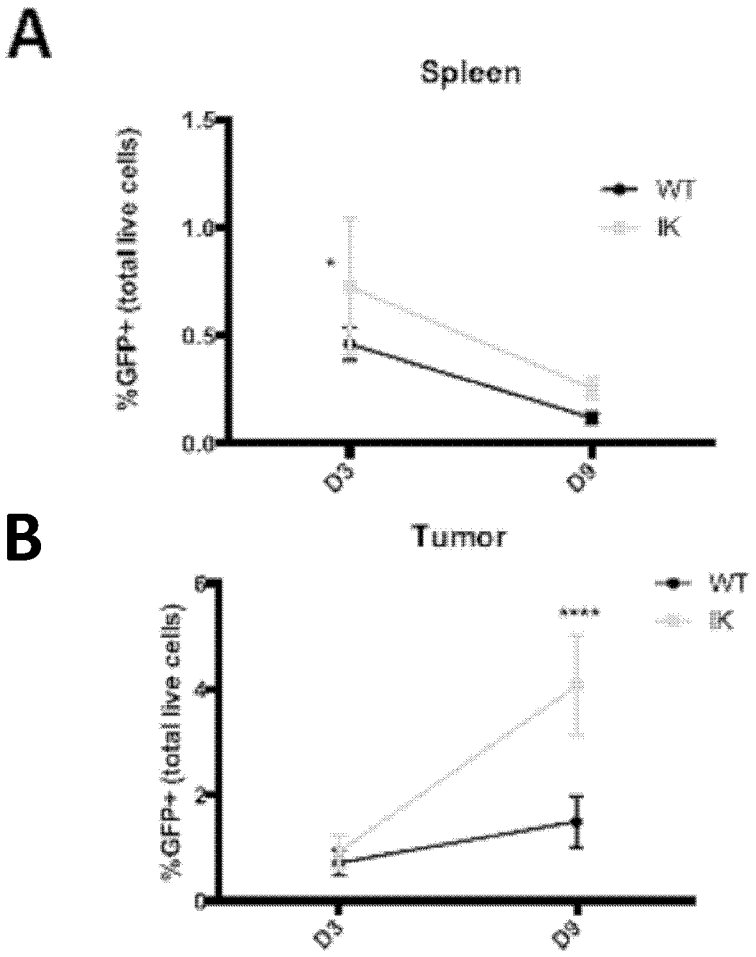

A
Spleen
%GFP+ (total live cells)
1.5
1.0
0.5
0.0
WT
IK
*
D3
D9
B
Tumor
%GFP+ (total live cells)
6
4
2
0
WT
IK
****
D3
D9

FIG. 31

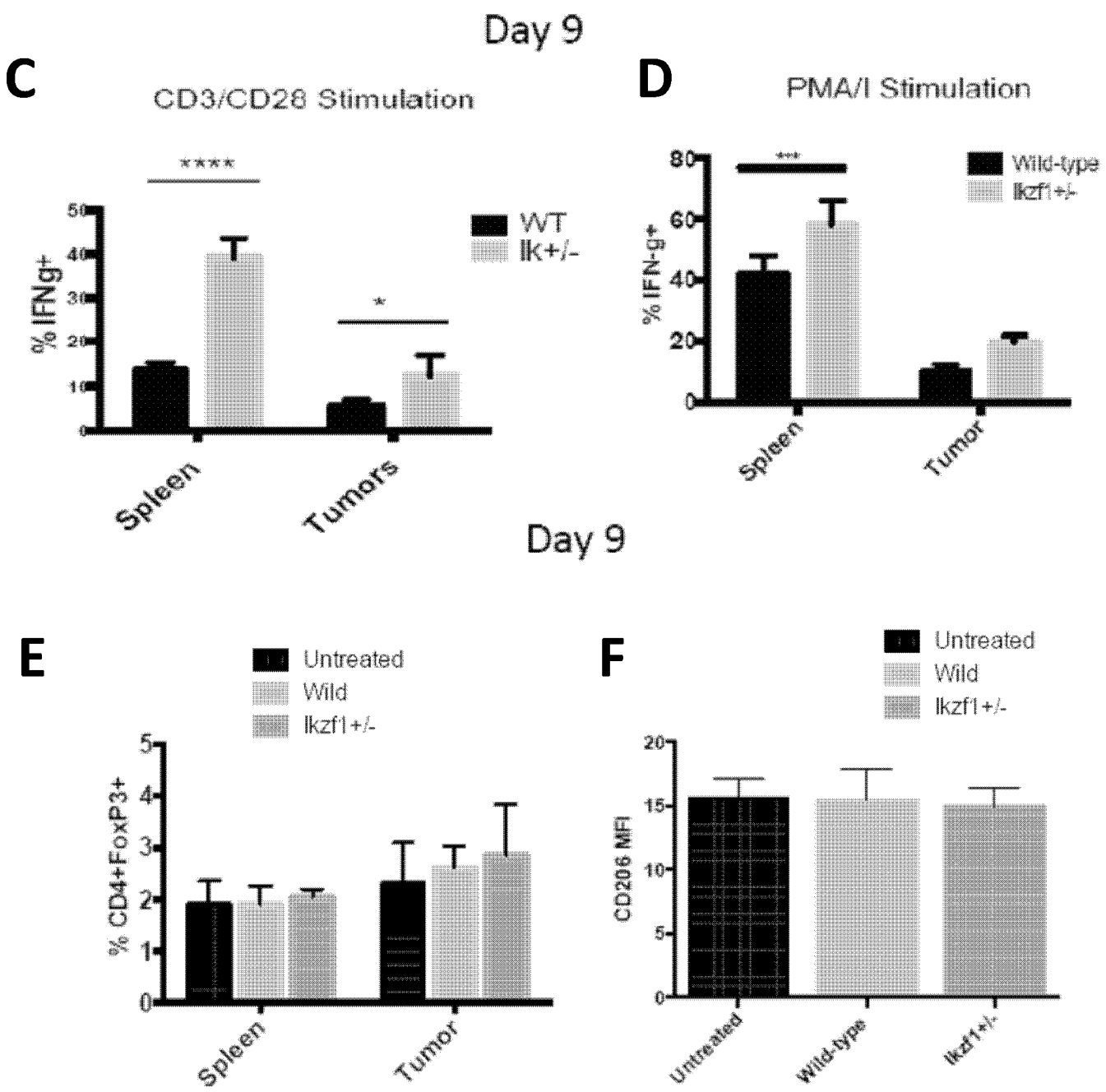

Day 9
C
CD3/CD28 Stimulation
****
*
% IFNg+
WT
Ik+/-
Spleen
Tumors
D
PMA/I Stimulation
***
% IFN-g+
Wild-type
Ikzf1+/-
Spleen
Tumor
Day 9
E
Untreated
Wild
Ikzf1+/-
% CD4+FoxP3+
Spleen
Tumor
F
Untreated
Wild
Ikzf1+/-
CD206 MFI
Untreated
Wild-type
Ikzf1+/-

FIG. 32

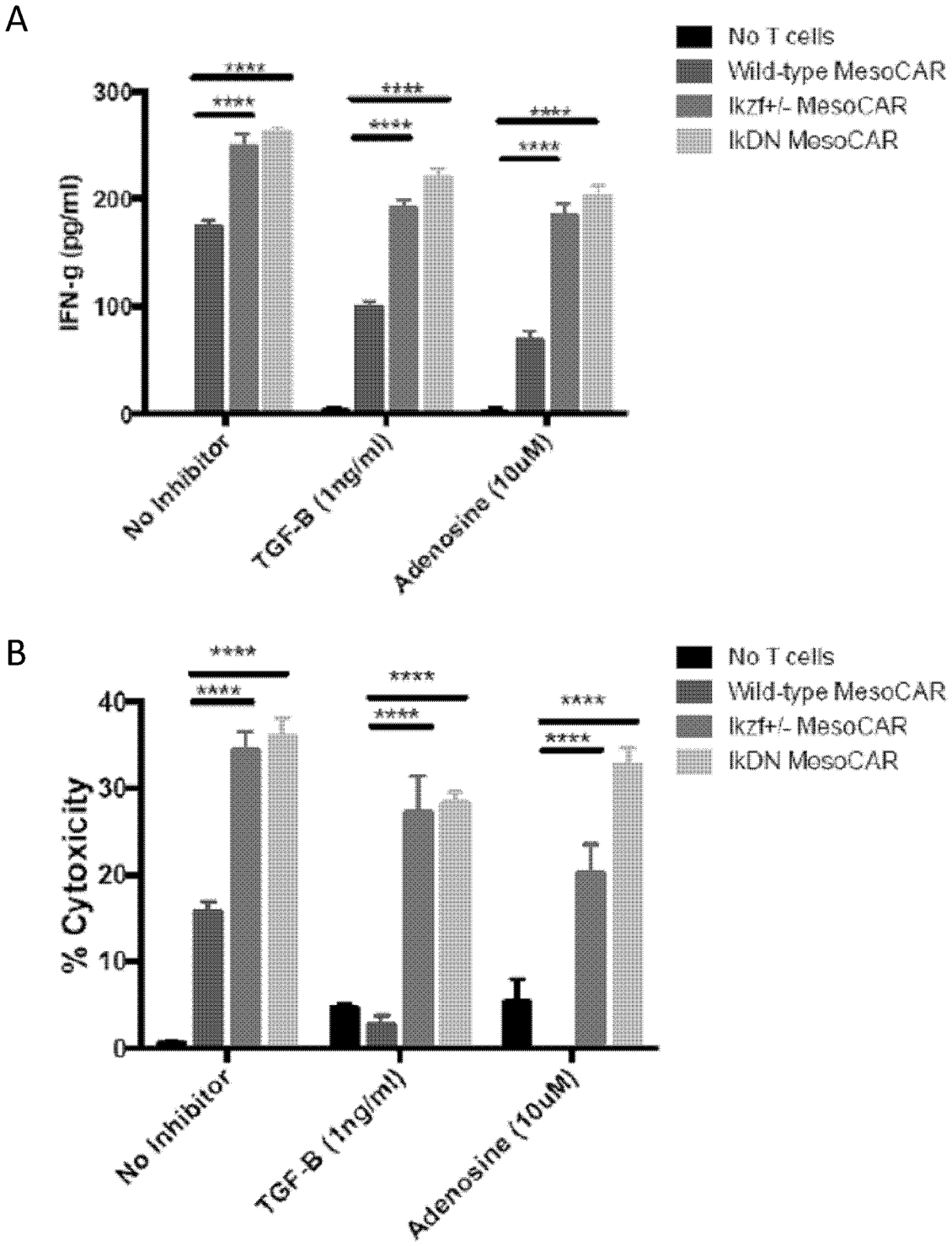
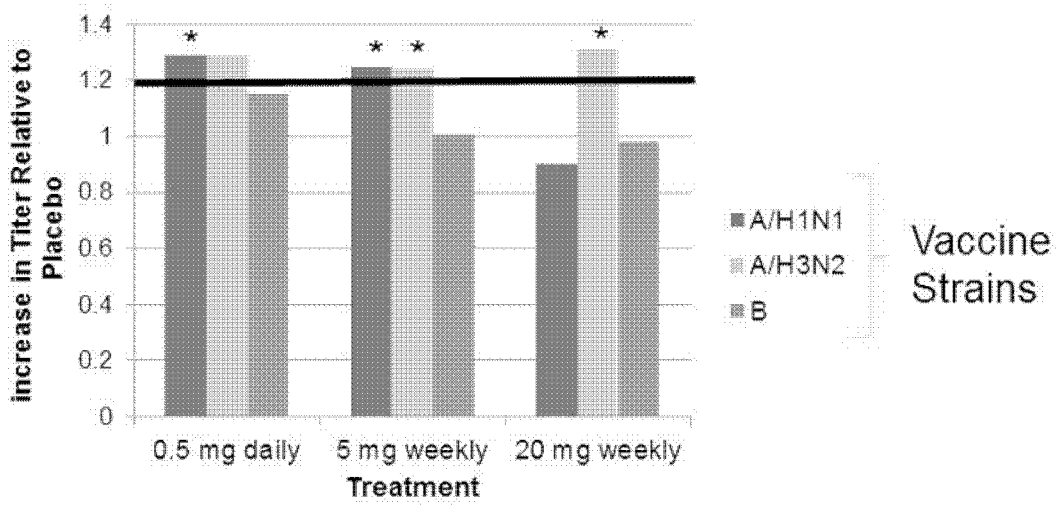

A
No T cells
Wild-type MesoCAR
Ikzf+/- MesoCAR
IkDN MesoCAR
IFN-g (pg/ml)
300
200
100
0
****
No Inhibitor
TGF-B (1ng/ml)
Adenosine (10uM)
B
% Cytoxicity
40
30
20
10
0

FIG. 33

A.


increase in Titer Relative to Placebo
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
* *
*
0.5 mg daily
5 mg weekly
20 mg weekly
Treatment
A/H1N1
A/H3N2
B
Vaccine Strains

B.

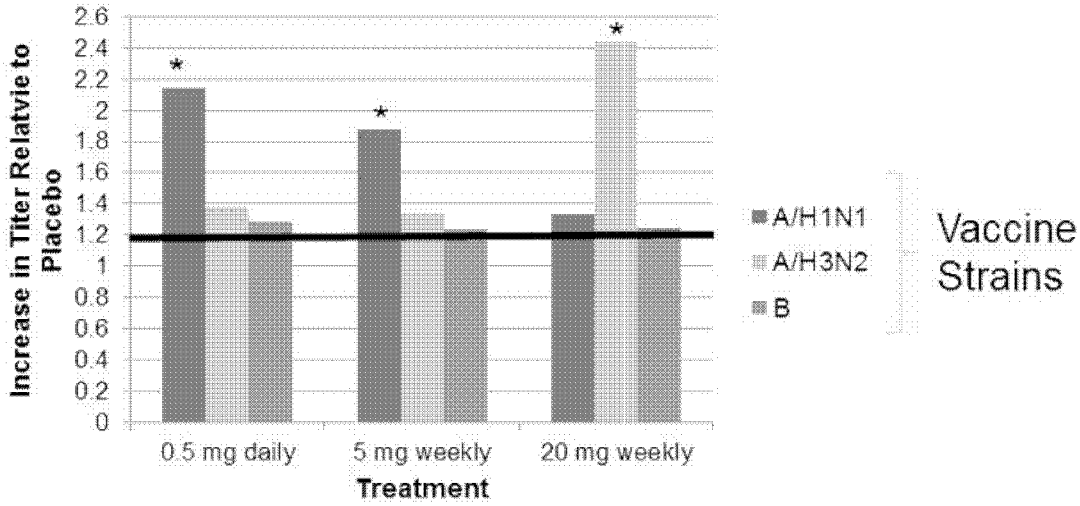

Increase in Titer Relatvie to Placebo
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0.5 mg daily
5 mg weekly
20 mg weekly
Treatment
A/H1N1
A/H3N2
B
Vaccine Strains

FIG. 34

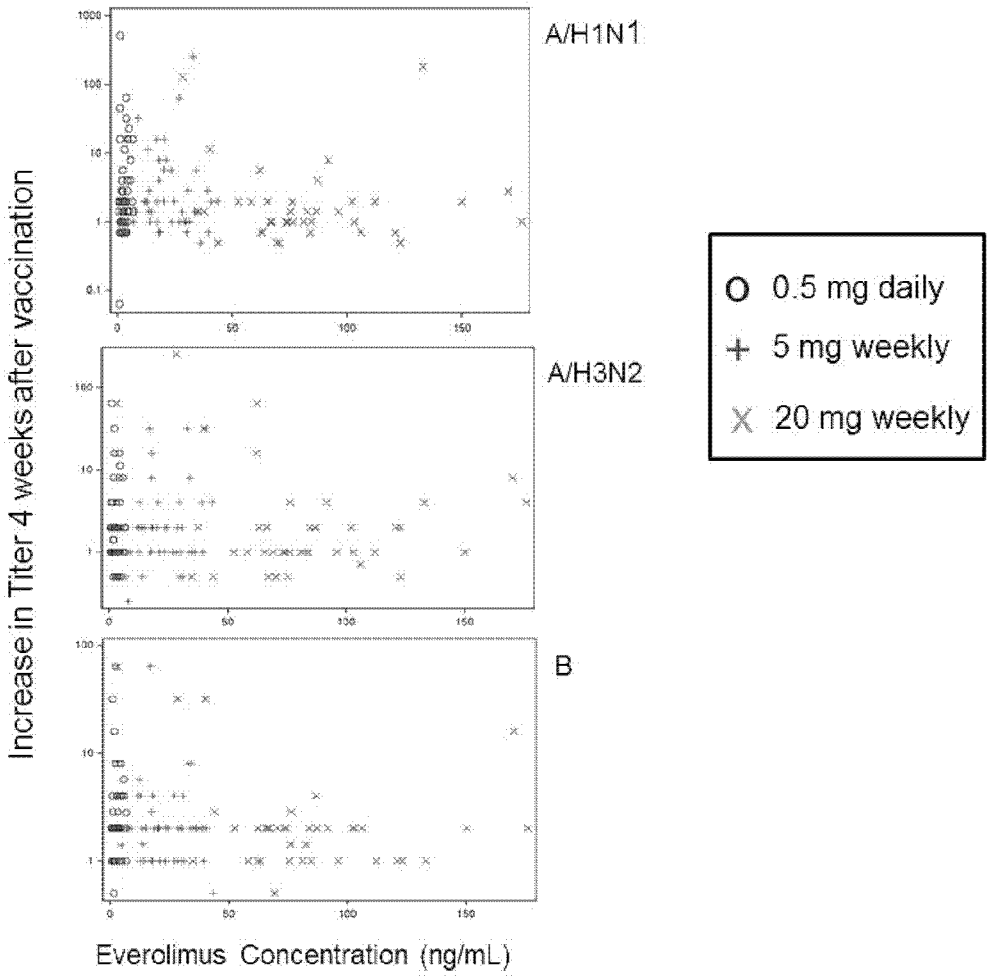

A/H1N1
A/H3N2
B
Increase in Titer 4 weeks after vaccination
Everolimus Concentration (ng/mL)
0.5 mg daily
5 mg weekly
20 mg weekly

FIG. 35

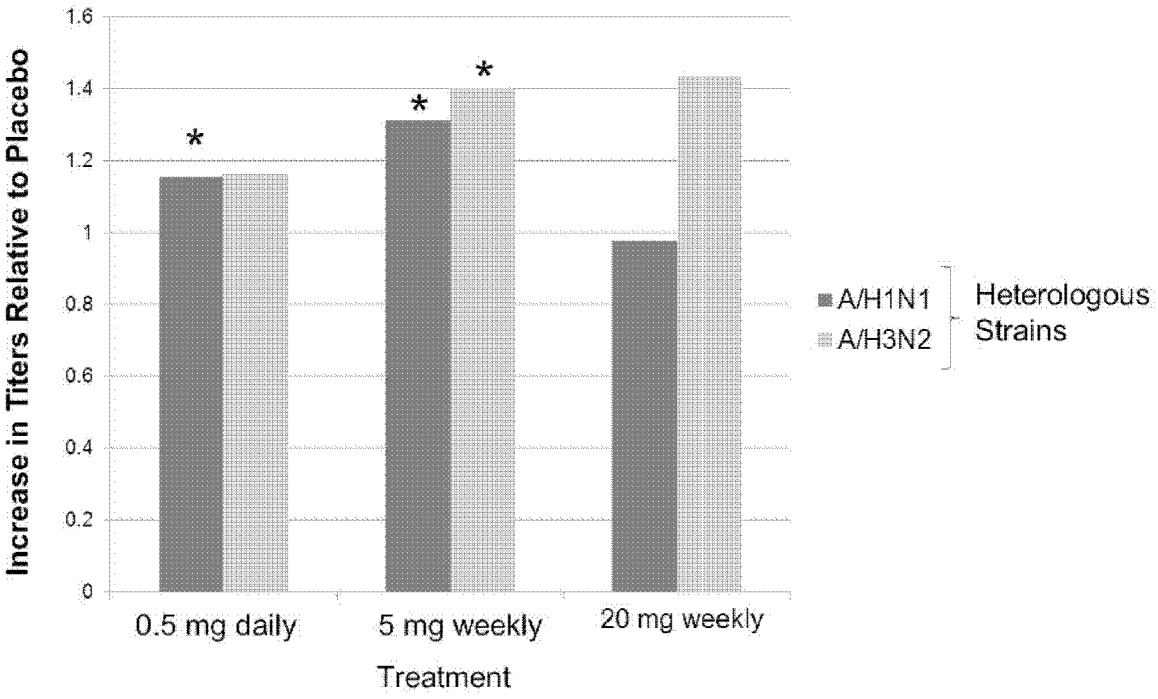
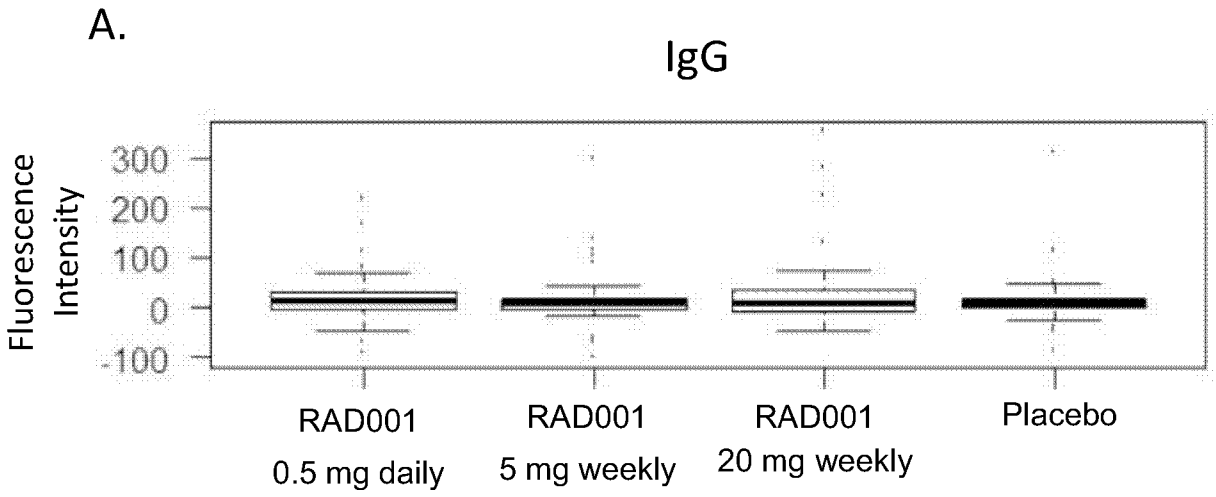

Increase in Titers Relative to Placebo
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0.5 mg daily
5 mg weekly
20 mg weekly
Treatment
A/H1N1
A/H3N2
Heterologous Strains

FIG. 36


A.
IgG
Fluorescence Intensity
300
200
100
0
-100
RAD001 0.5 mg daily
RAD001 5 mg weekly
RAD001 20 mg weekly
Placebo

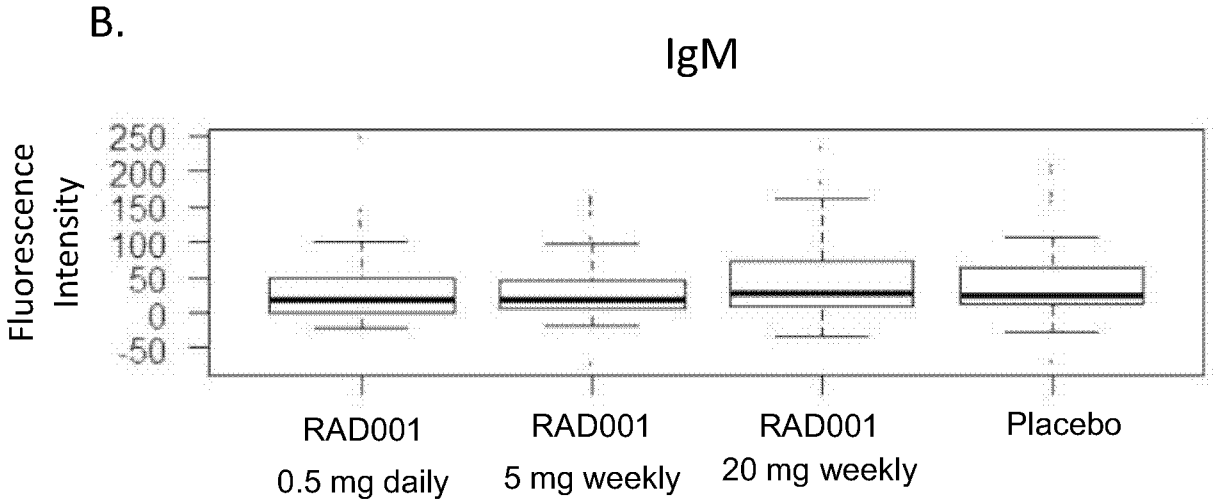
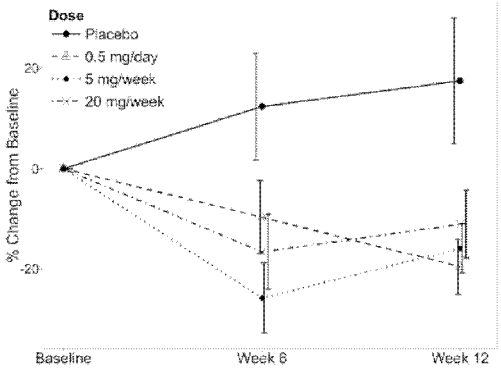
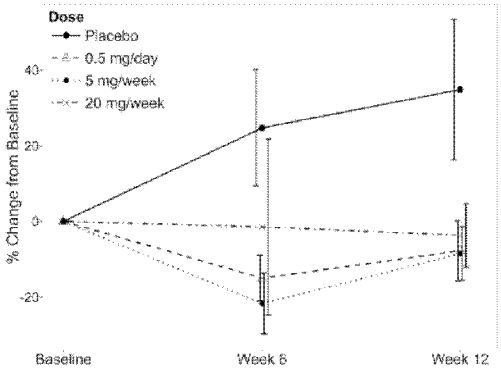

B.
IgM
Fluorescence Intensity
250
200
150
100
50
0
-50
RAD001 0.5 mg daily
RAD001 5 mg weekly
RAD001 20 mg weekly
Placebo

FIG. 37

A.


Dose
Placebo
0.5 mg/day
5 mg/week
20 mg/week
% Change from Baseline
20
0
-20
Baseline
Week 6
Week 12

B.


Dose
Placebo
0.5 mg/day
5 mg/week
20 mg/week
% Change from Baseline
40
20
0
-20
Baseline
Week 6
Week 12

C.

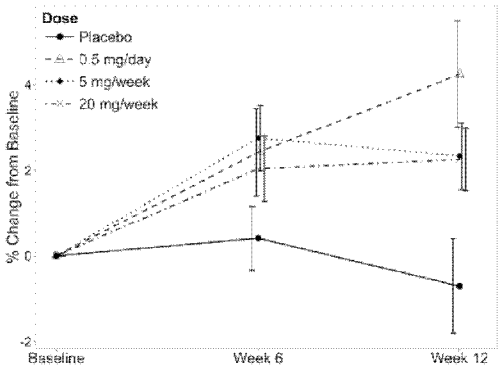
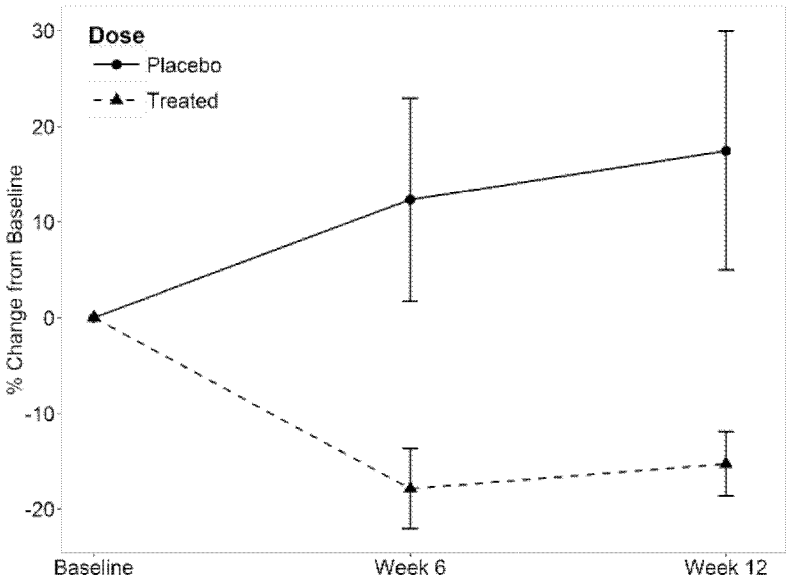

Dose
Placebo
0.5 mg/day
5 mg/week
20 mg/week
% Change from Baseline
4
2
0
-2
Baseline
Week 6
Week 12

FIG. 38

A.


Dose
Placebo
Treated
30
20
10
0
-10
-20
% Change from Baseline
Baseline
Week 6
Week 12

B.

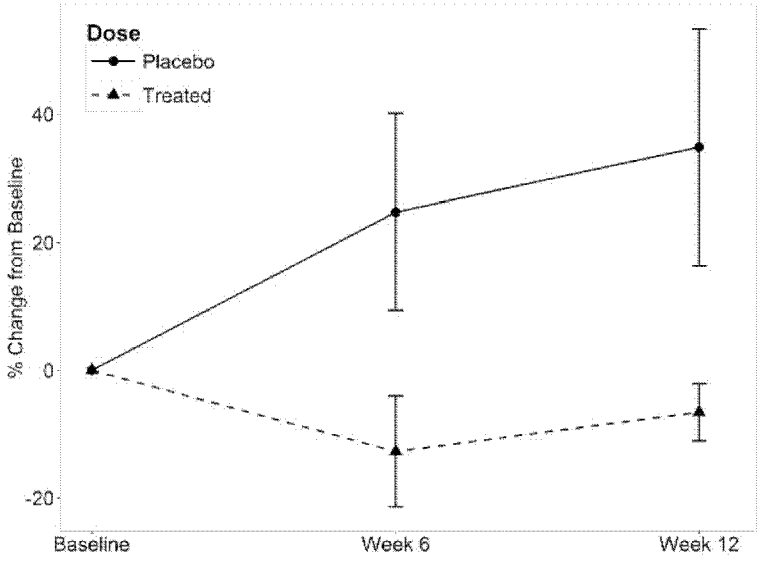

Dose
Placebo
Treated
40
20
0
-20
% Change from Baseline
Baseline
Week 6
Week 12

FIG. 39

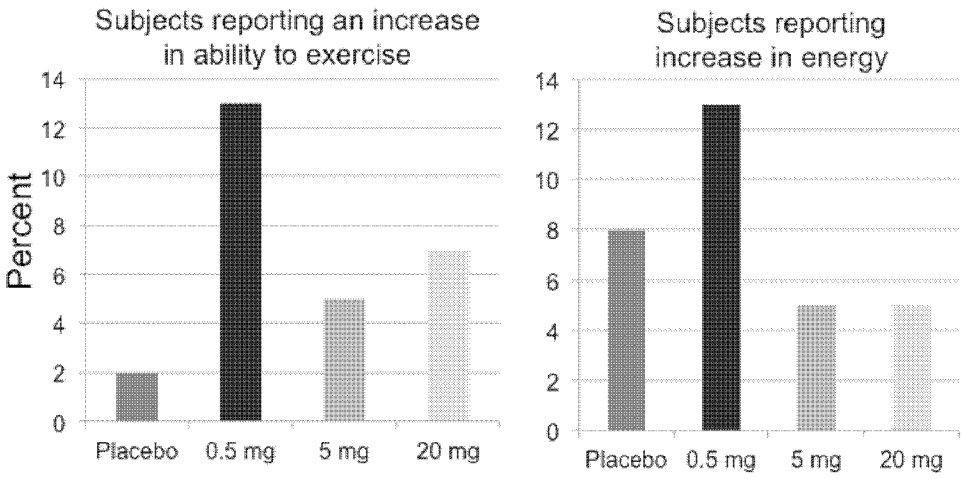
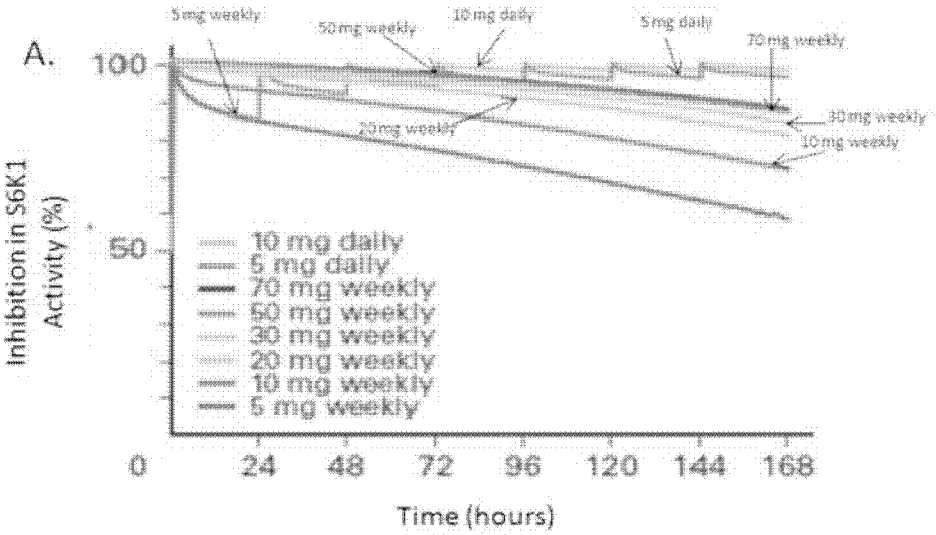

Subjects reporting an increase in ability to exercise
Percent
14
12
10
8
6
4
2
0
Placebo
0.5 mg
5 mg
20 mg
Subjects reporting increase in energy
14
12
10
8
6
4
2
0
Placebo
0.5 mg
5 mg
20 mg

FIG. 40


A.
5 mg weekly
50 mg weekly
10 mg daily
5 mg daily
70 mg weekly
30 mg weekly
20 mg weekly
10 mg weekly
Inhibition in S6K1 Activity (%)
100
50
10 mg daily
5 mg daily
70 mg weekly
50 mg weekly
30 mg weekly
20 mg weekly
10 mg weekly
5 mg weekly
0
24
48
72
96
120
144
168
Time (hours)

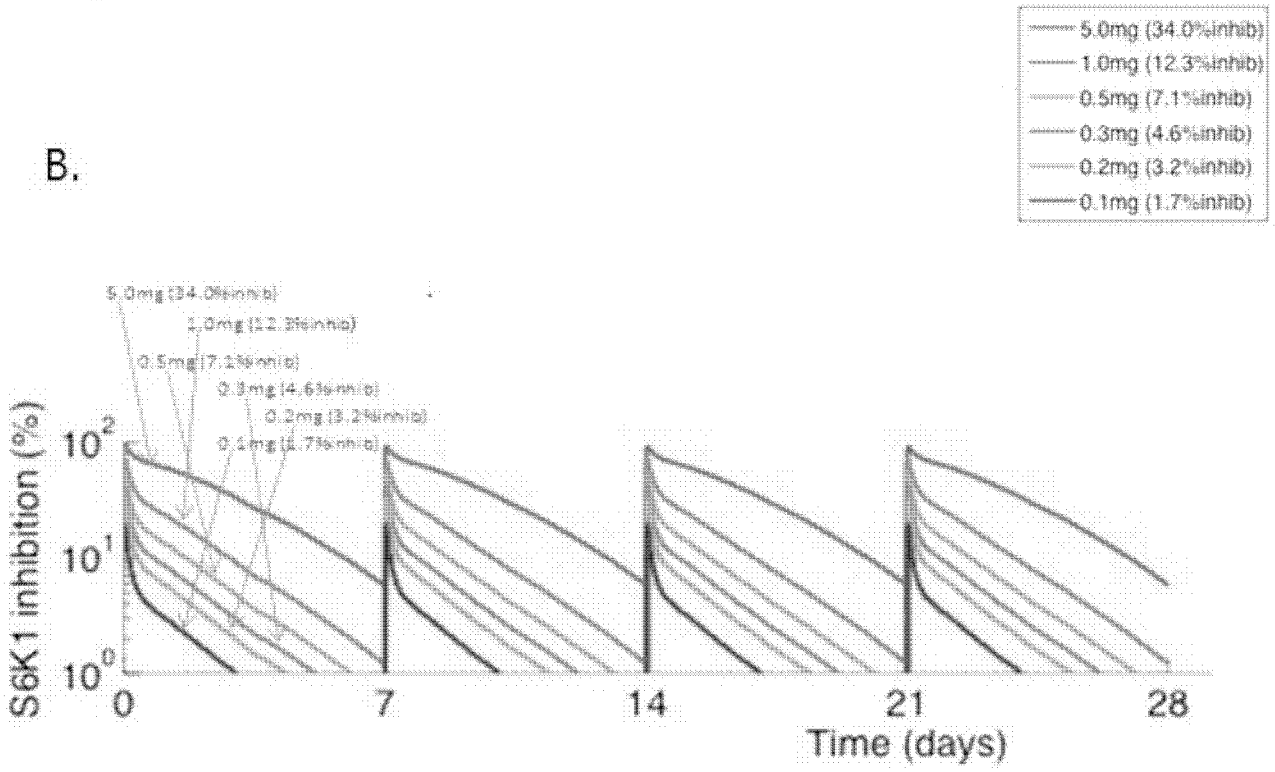

B.
5.0mg (34.0%inhib)
1.0mg (12.3%inhib)
0.5mg (7.1%inhib)
0.3mg (4.6%inhib)
0.2mg (3.2%inhib)
0.1mg (1.7%inhib)
S6K1 inhibition (%)
10^2
10^1
10^0
0
7
14
21
28
Time (days)

FIG. 41A

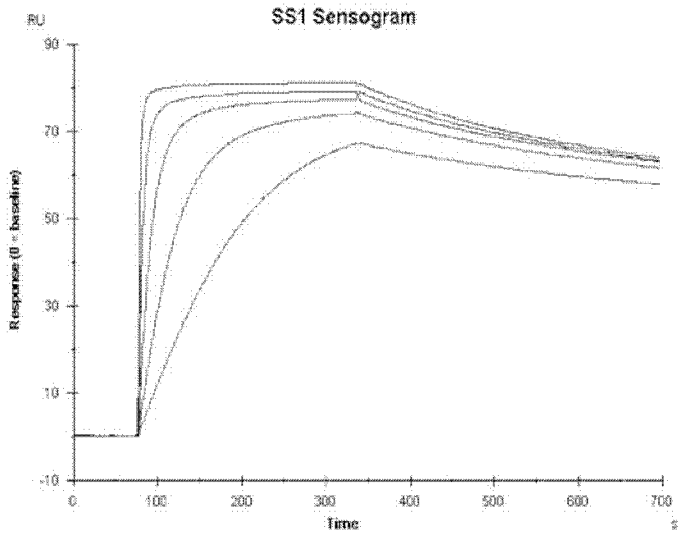


FIG. 41B

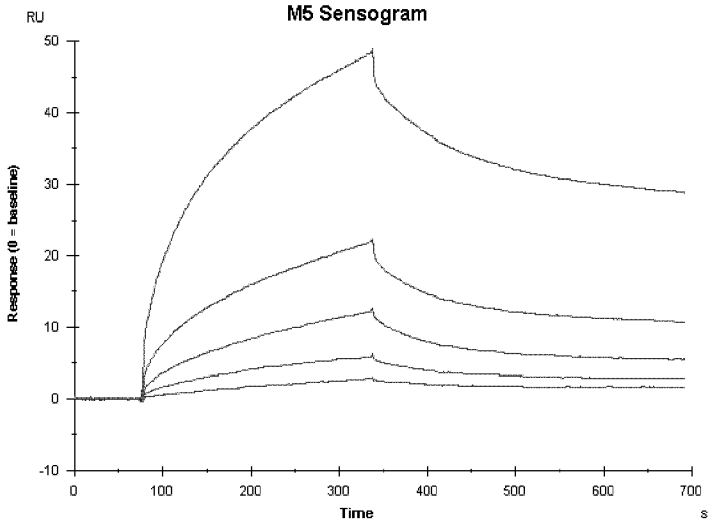


FIG. 41C

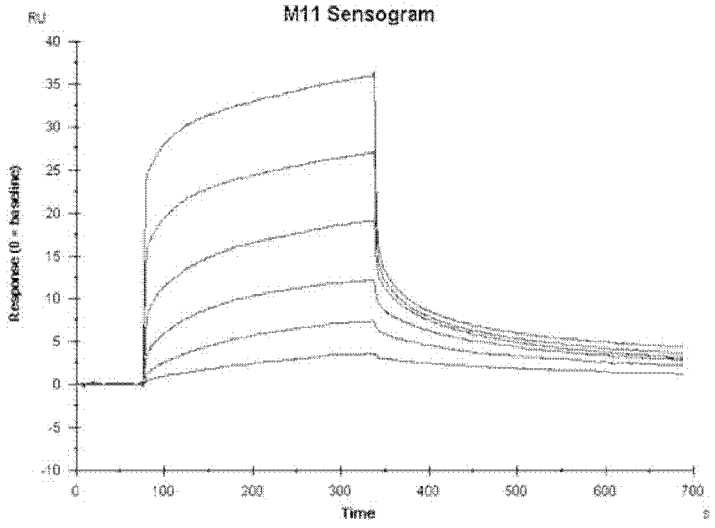


FIG. 42A

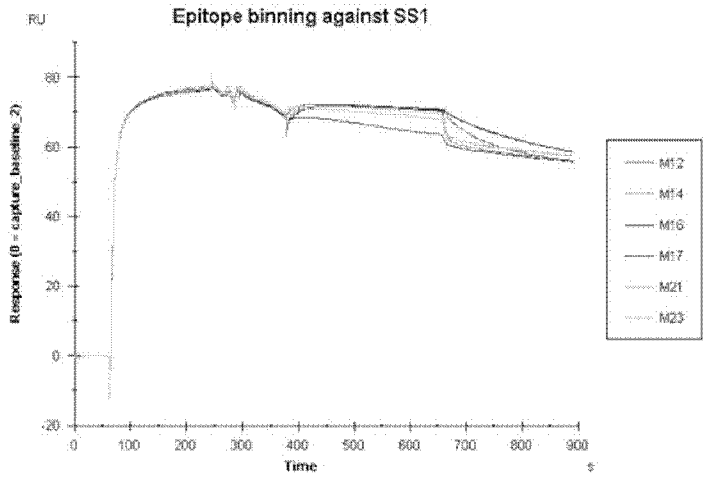


FIG. 42B

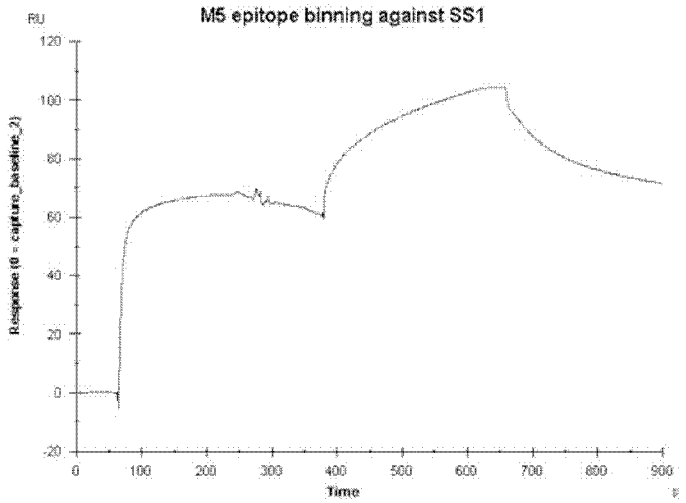


FIG. 42C

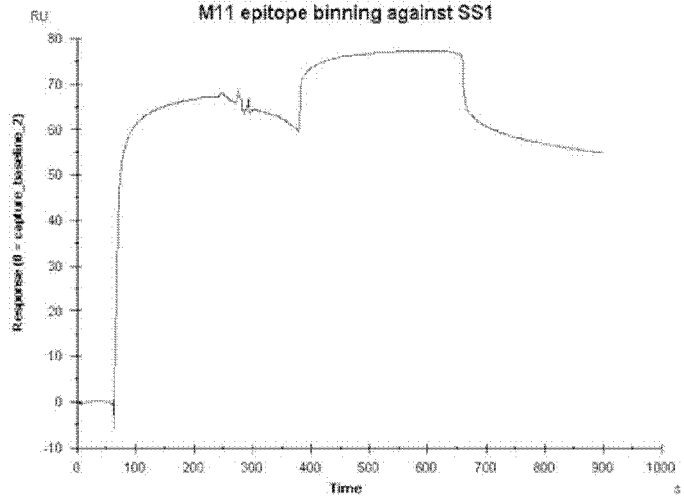


FIG. 43

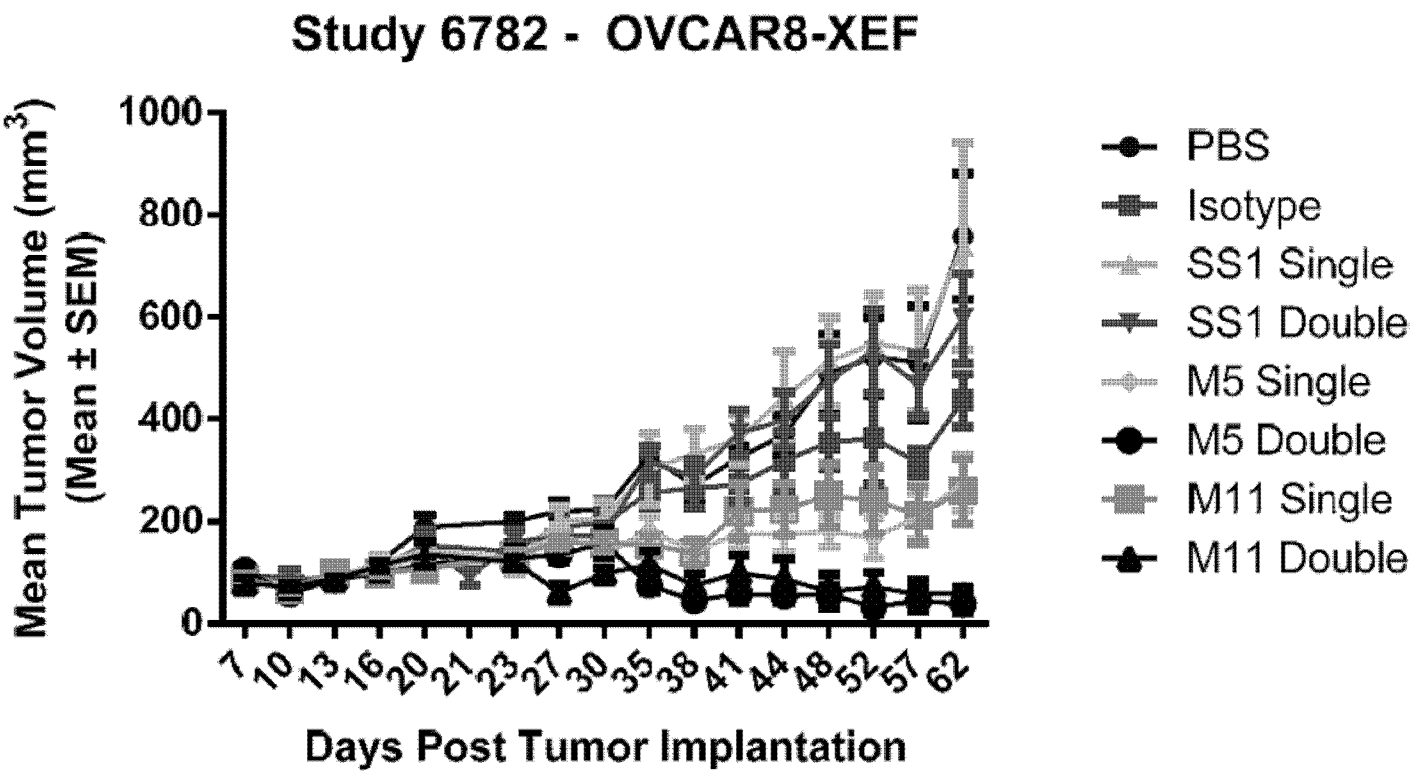
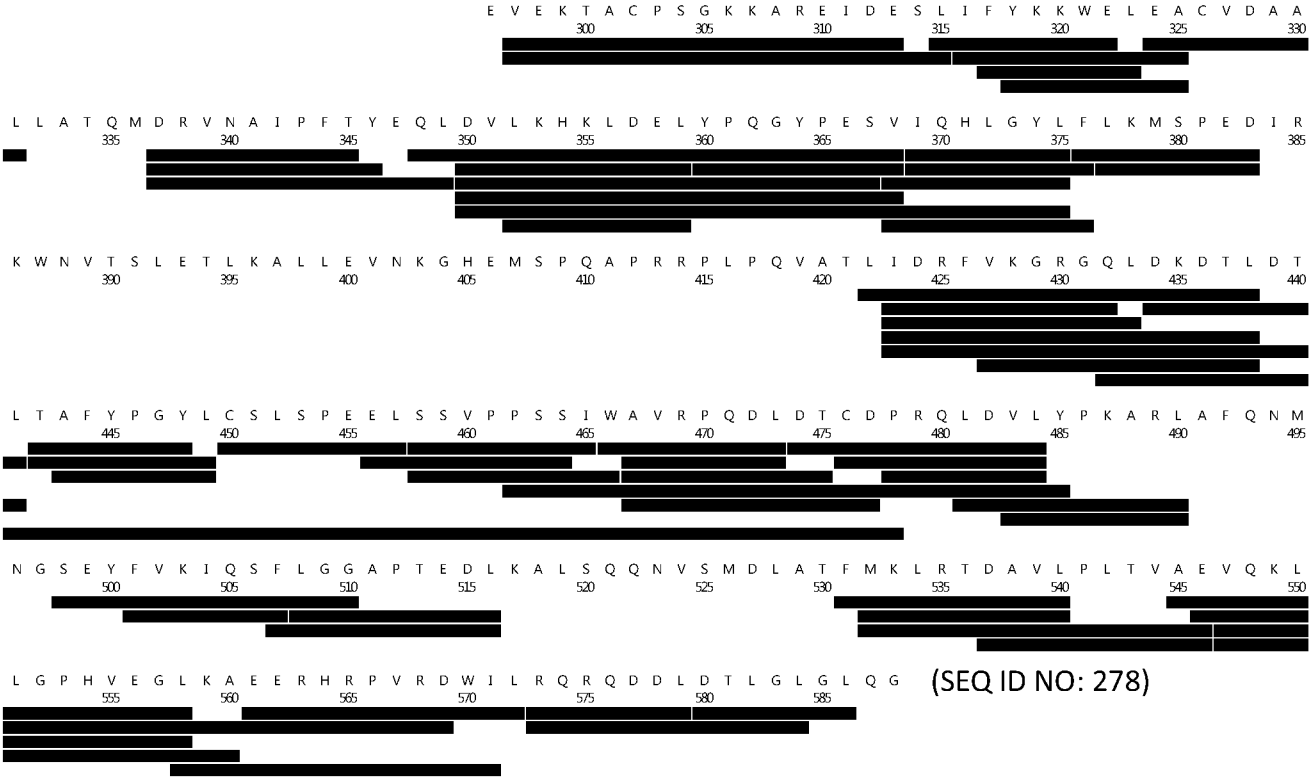

Study 6782 - OVCAR8-XEF
Mean Tumor Volume (mm3)
(Mean ± SEM)
1000
800
600
400
200
0
7
10
13
16
20
21
23
27
30
35
38
41
44
48
52
57
62
Days Post Tumor Implantation
PBS
Isotype
SS1 Single
SS1 Double
M5 Single
M5 Double
M11 Single
M11 Double

FIG. 44



(SEQ ID NO: 278)

FIG. 45A

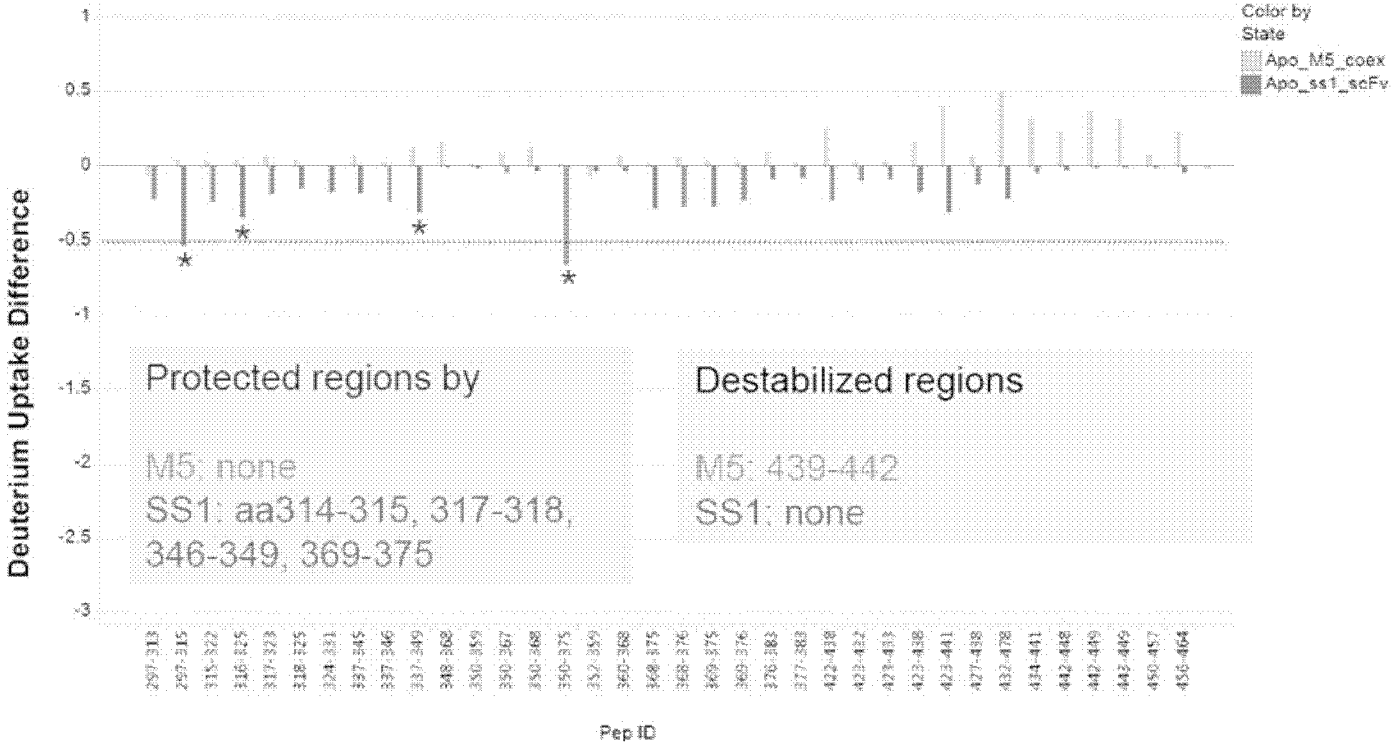


FIG. 45B

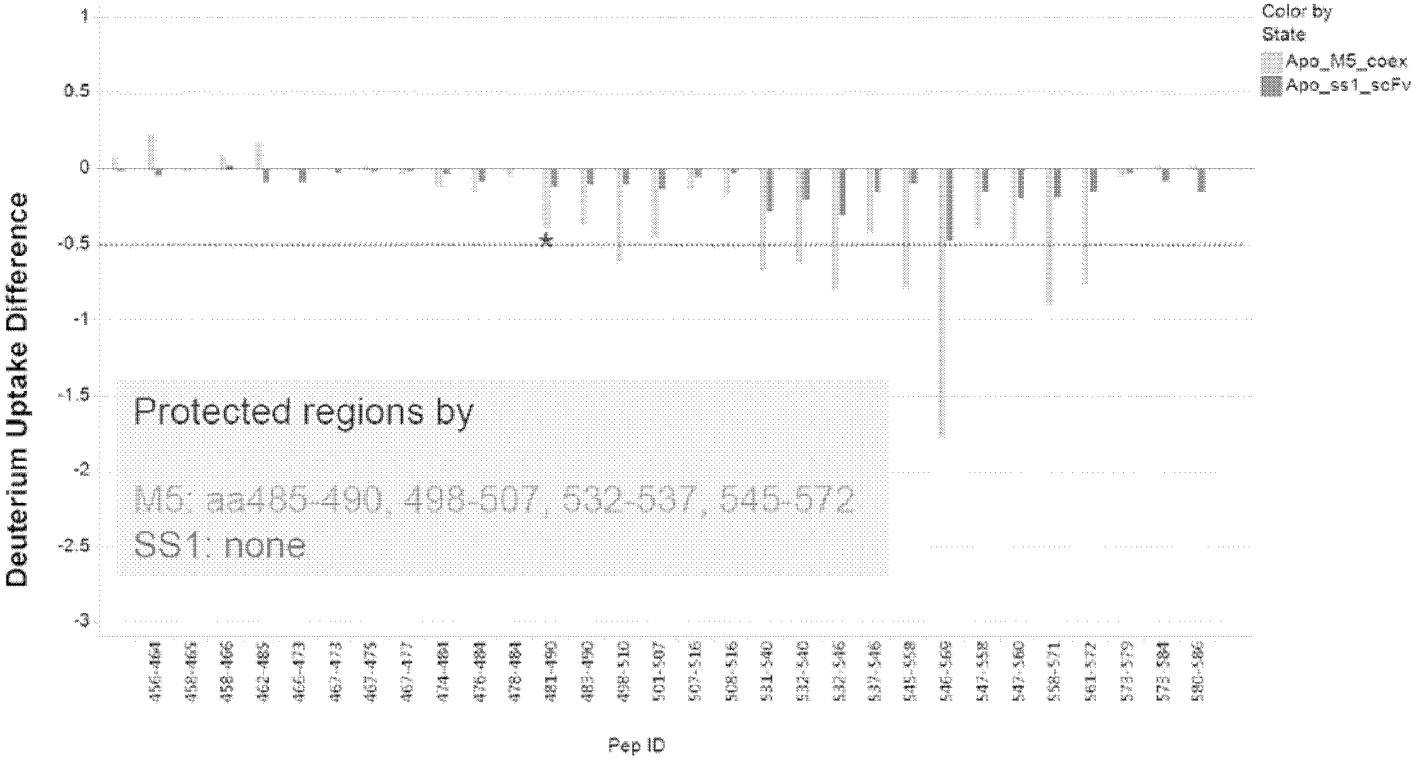


FIG. 46

$E_{296}VEKT_{300}$

$A_{301}CPSGKKAREIDESLIFYKKWELEACVDAALLATQMDRVNAIPFTYEQLD_{350}$

$V_{351}LKHKLDELYPQGYPESVIQHLGYLFLKMSPEDIRKWNVTSLETLKALLE_{400}$

$V_{401}NKGHEMSPQAPRRPLPQVATLIDRFVKGRGQLDKDTLDTLTAFYPGYLC_{450}$

$S_{451}LSPEELSSVPPSSIWAVRPQDLDTCDPRQLDVLYPKARLAFQNMNGSEY_{500}$

$F_{501}VKIQSFLGGAPTEDLKALSQQNVSMDLATFMKLRTDAVLPLTVAEVQKL_{550}$

$L_{551}GPHVEGLKAEERHRPVRDWILRQRQDDLDTLGLGLQG_{588}$ (SEQ ID NO: 278)

Protected by M5

Protected by SS1

FIG. 47

Vector constructs

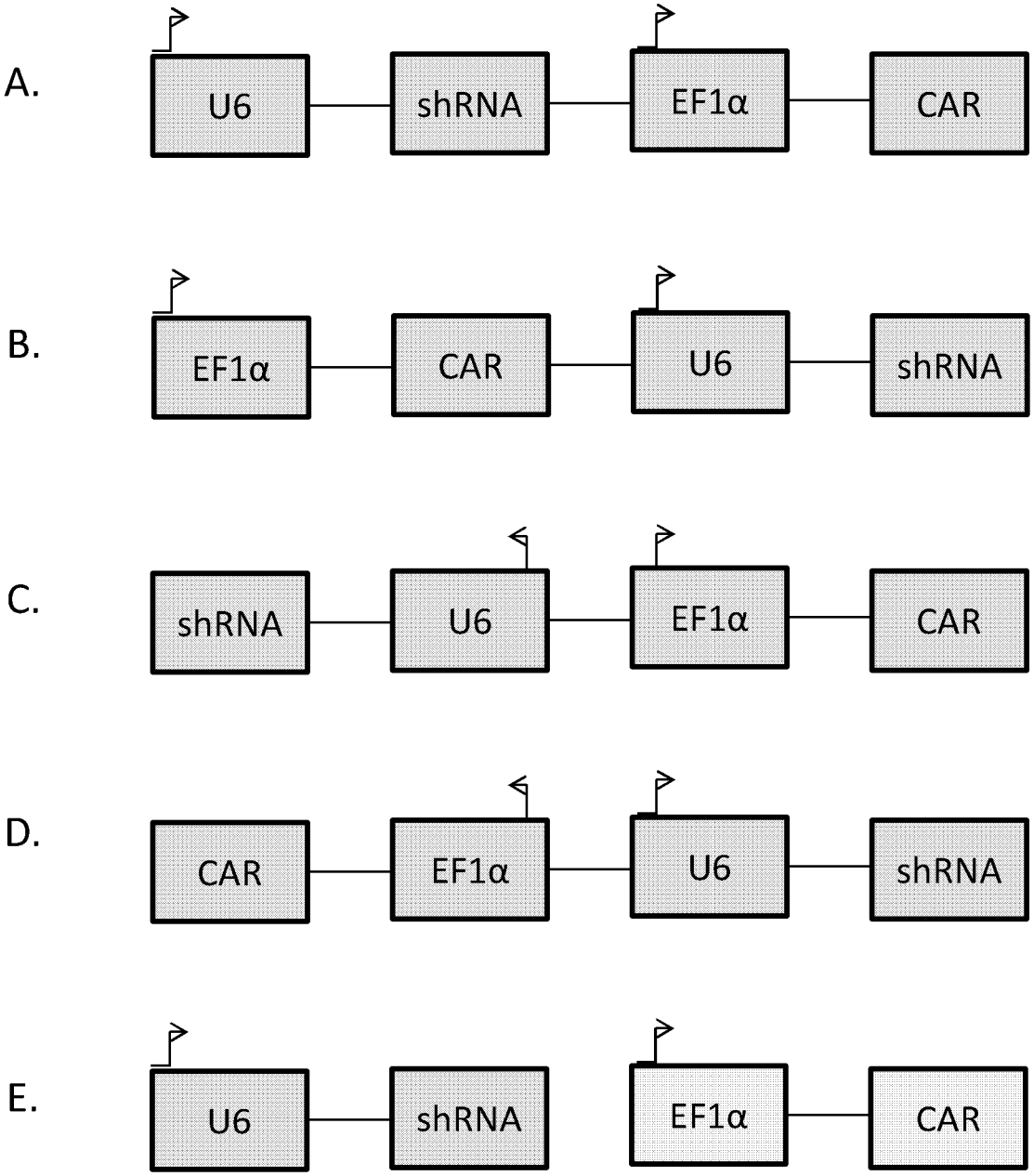
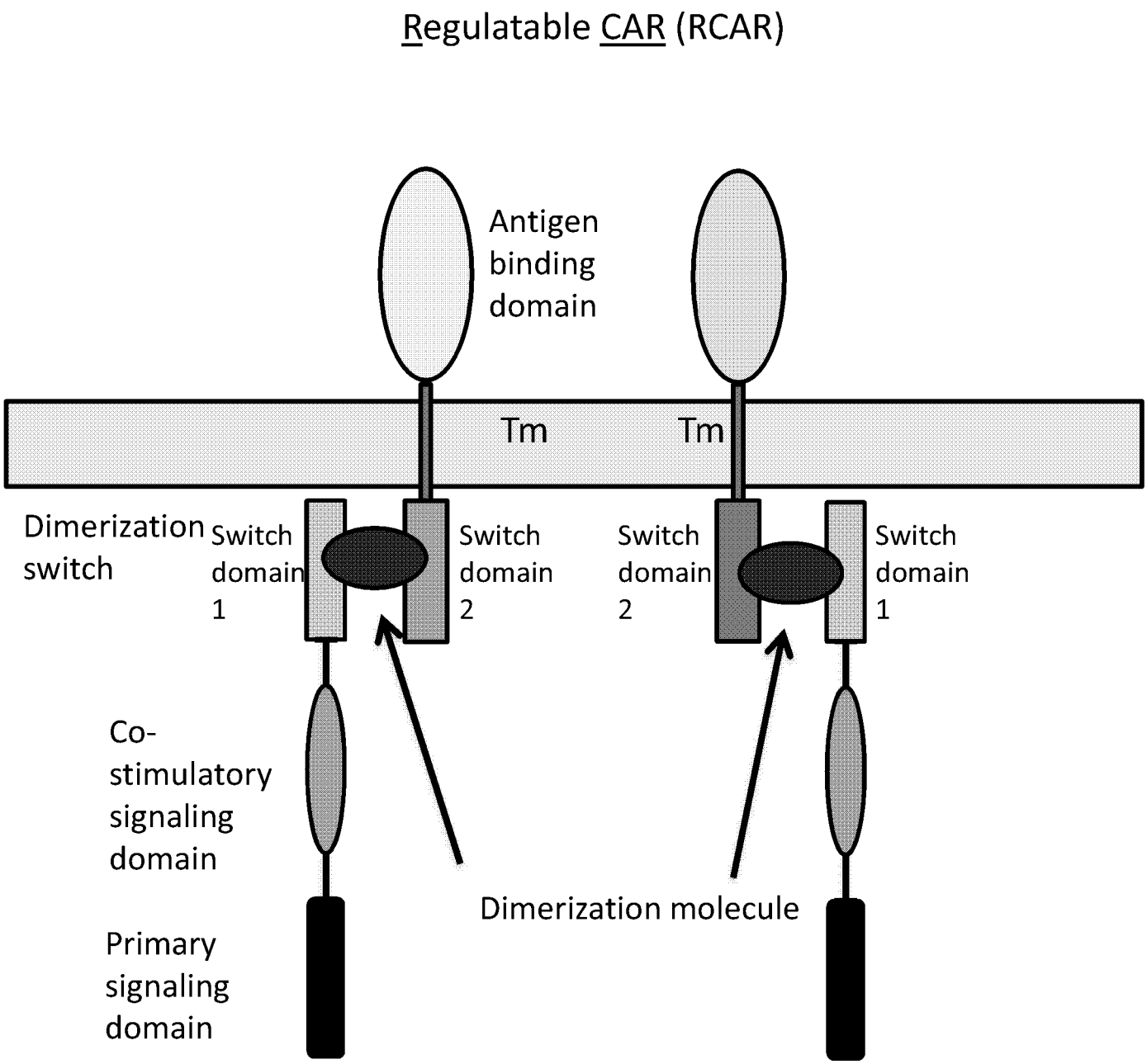


FIG. 48


Regulatable CAR (RCAR)
Antigen binding domain
Tm
Tm
Dimerization switch
Switch domain 1
Switch domain 2
Switch domain 2
Switch domain 1
Co-stimulatory signaling domain
Primary signaling domain
Dimerization molecule

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2014/094393

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/62(2006.01)i; C12N 15/13(2006.01)i; C12N 15/63(2006.01)i; C07K 16/46(2006.01)i; C07K 14/725(2006.01)i; A61K 48/00(2006.01)i; A61P 35/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N; C07K; A61K; A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CPRSABS;CNABS;DWPI;SIPOABS;CNTXT,USTXT;WOTXT;EPTXT;JPTXT;CATXT;CNKI;ISI;PUBMED,ELSEVIER: chimeric antigen receptor,CAR,mesothelin,MSLN,anti-mesothelin domain,transmembrane domainjntracellular signaling domain, stimulatory domain,leader sequence,hige region,antibody,antibody fragmentjight chainjight chain complementary determining region,LC CDR,heavy chain complementary determing region HC CDR,T-cell receptor,CD28,promoter, vector, virus,retrovirus vector,T-cell, tumor, anti-tumor,cancer,treatment; GenBank;DDBJ;EMBL: sequences search on SEQ IDs: 1,2,6,7, 9,10,1 1,39,43,49,63,67,73,87,1 11,278 and 279.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013063419 A2 (UNIV PENNSYLVANIAET AL.) 02 May 2013 (2013-05-02) see claims 1-20, description, pages 32-42, figure 1A, and SEQ ID NOs: 9, 13-14, 17-20, 23-24	1-35, 37, 42-69, 76-88, 99-102(all partially)
Y	WO 2013063419 A2 (UNIV PENNSYLVANIAET AL.) 02 May 2013 (2013-05-02) see claims 1-20, description, pages 32-42, figure 1A, and SEQ ID NOs: 9, 13-14, 17-20, 23-24	36, 38-41, 70-75(all partially)
X	WO 2013074916 A1 (UNIV TEXAS) 23 May 2013 (2013-05-23) see claims 1-37, the abstract	34-35, 46(all partially)
Y	CN 103347897 A (HOFFMANN LA ROCHE & CO AG F) 09 October 2013 (2013-10-09) see the abstract, claims 1-70, SEQ ID NO:45	36, 38-41, 70-75(all partially)

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 March 2015	**30 March 2015**
Name and mailing address of the ISA/CN **STATE INTELLECTUAL PROPERTY OFFICE OF THE P.R.CHINA(ISA/CN)** **6,Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088, China** Facsimile No. **(86-10)62019451**	Authorized officer **LI,Xiaoqu** Telephone No. **(86-10)62089432**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2014/094393

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CA 2863799 A1 (UNIV PENNSYLVANIA) 29 August 2013 (2013-08-29) see the whole document	1-88, 99-102 (all partially)
A	Marcela V. Maus et al. "T Cells Expressing Chimeric Antigen Receptors Can Cause Anaphylaxis in Humans" *Cancer Immunology Research*, Vol. vol.1, No. no.l, 31 July 2013 (2013-07-31), ISSN: ISSN:2326-6066, pages 26-31, see the whole document	1-88, 99-102 (all partially)
A	Evripidis Lanitis et al. "Redirected Antitumor Activity of Primary Human Lymphocytes Transduced With a Fully Human Anti-mesothelin Chimeric Receptor" *Molecular Therapy*, Vol. no.3, No. vol.20, 31 March 2012 (2012-03-31), ISSN: ISSN: 1525-0016, pages 633-643, see the whole document	1-88, 99-102 (all partially)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2014/094393

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. 0 Claims Nos.: **89-98,103-110**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] The subject matter of claims 89-98, 103-110 relates to a method of treatment for the human or animal body and therefore does not warrant an international search according to the criteria set out in Rule 39.1(iv).

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. Ill Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

[1] Invention 1: claims 1-88,99-102 (all partially), relate to a chimeric antigen receptor (CAR) comprising a human anti-mesothelin binding domain M1 comprising a sequence of SEQ ID NO: 39 or 63, and subject-matter relating thereto;

[2] Invention 2: claims 1-88, 99-102 (all partially), relate to a chimeric antigen receptor (CAR) comprising a human anti-mesothelin binding domain M2 comprising a sequence of SEQ ID NO: 40 or 64, and subject-matter relating thereto;

[3] ……

[4] Invention 24: claims 1-88, 99-102 (all partially), relate to a chimeric antigen receptor (CAR) comprising a human anti-mesothelin binding domain M24 comprising a sequence of SEQ ID NO: 62 or 86, and subject-matter relating thereto.

[5] Dl: WO 2013063419 A2(THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA), 2 May 2013(02.05.2013), see claims 1-11, figure 1.

[6] The common or corresponding technical features among inventions 1-24 are a CAR molecule comprising a human anti-mesothelin binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain; however, said common or corresponding technical features have been disclosed by Dl, which discloses a human mesothelin binding domain and a CAR molecule comprising a human mesothelin binding domain such as anti-Meso P4 scFv, a transmembrane domain such as CD8a TM or CD28 TM, an intracellular signaling domain such as CD3 zeta signaling domain, and a co-stimulatory domain such as CD28 or 4-lBB signaling domain (see claims 1-11 and figure 1). Therefore, said technical features can not be construed as special technical features, resulting in the 24 inventions lack common or corresponding special technical features, and do not linked by a single general inventive concept, hence do not meet the requirements of unity of invention as defined in PCT Rule 13.1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2014/094393

Box No. Ill Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☑ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.: **claims 1-88,99-102 (all partially), related to a chimeric antigen receptor (CAR) comprising a human anti-mesothelin binding domain M1,M5 and Mll,and subject-matter relating thereto.**

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☑ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2014/094393

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