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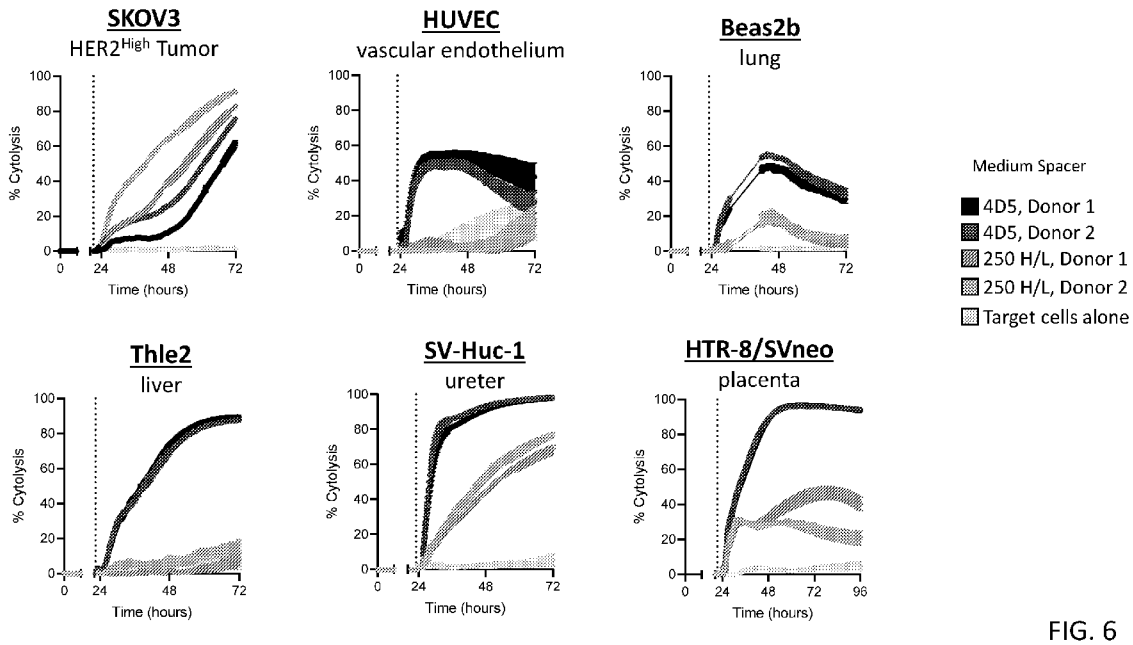

FIG. 6

(57) Abstract: Provided are chimeric antigen receptors (CAR) specific to a selected tumor antigen. Also provided are structure designs and function profiles of provided CAR candidates.

[Continued on next page]

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CHIMERIC ANTIGEN RECEPTOR FOR TUMOR TARGETING

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 63/329,287, filed April 8, 2022, the disclosure of which is hereby incorporated by reference in its entirety.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0002] The Sequence Listing titled 184143-640601_SL.xml, which was created on April 1, 2023 and is 87,714 bytes in size, is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present disclosure is broadly concerned with the field of off-the-shelf immunocellular products. More particularly, the present disclosure is concerned with strategies for developing solid tumor targeting chimeric antigen receptors for use in cancer treatments.

BACKGROUND OF THE INVENTION

[0004] Cancer cells emerge from normal tissues through accumulated genetic and epigenetic aberrations, and differentiate themselves from normal cells by producing proteins that differ in quantity and quality from those of normal cells. However, tumor-specific cell surface expressed membrane proteins that can be exploited as tumor antigens for an effective targeted cancer therapy have very rarely been identified, because as data have shown, these tumor cell surface expressed membrane proteins more likely than not can also be expressed on normal tissues. Therefore, targeting these molecules through chimeric antigen receptors (CARs) in solid tumors comes with an inherent risk of severe toxicities to normal tissues.

SUMMARY OF THE INVENTION

[0005] There is a need for tumor-specific and functionally improved CARs in order to develop targeted cancer treatments, and address issues such as tumor targeting precision, off-target toxicity, and off-tumor effect against solid tumors.

[0006] In one aspect, the invention provides a chimeric antigen receptor (CAR) comprising: (a) an ectodomain comprising an antigen binding domain recognizing a HER2

(human epidermal growth factor receptor 2) antigen, wherein the antigen binding domain comprises: (i) a heavy chain variable (VH) domain comprising a heavy chain complementary determining region 1 (H-CDR1) comprising SEQ ID NO: 1 (NYGMS), a heavy chain complementary determining region 2 (H-CDR2) comprising SEQ ID NO: 2 (TINNNGGGTYYPDSVKG), and a heavy chain complementary determining region 3 (H-CDR3) comprising SEQ ID NO: 3 (PGLLWDA); and (ii) a light chain variable (VL) domain comprising a light chain complementary determining region 1 (L-CDR1) comprising SEQ ID NO: 4 (KSSQSLLDSDGRTYLN), a light chain complementary determining region 2 (L-CDR2) comprising SEQ ID NO: 5 (LVSKLDS), and a light chain complementary determining region 3 (L-CDR3) comprising SEQ ID NO: 6 (WQGTHFPQT); (b) a transmembrane domain; and (c) an endodomain comprising at least one signaling domain, wherein the at least one signaling domain responds specifically to binding of the CAR to a HER2 antigen expressed on a cancer cell, thereby generating a cancer antigen specific response.

[0007] In some embodiments of the CAR, the antigen binding domain: (a) comprises a VH domain with at least 80% sequence identity to SEQ ID NO: 7 (EVQLVESGGGLVQPGGSLKLSCAASGFTFSNYGMSWVRQTPDRRLELVATINNNGGGTYYPDSVKGRFTISRDNAKNTLYLQMSSLKSEDTAMYYCTSPGLLWDAWGAGTTVTVSS); (b) comprises a VL domain with at least 80% sequence identity to SEQ ID NO: 8 (DVVMTQTPLTLSVSIGQPASISCKSSQSLLDSDGRTYLNWLLQRPGQSPKRLIYLVSKLDSGAPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGTKLEIK); (c) comprises a single chain variable fragment (scFV) comprising VH-linker-VL or VL-linker-VH, wherein the linker varies in length and sequence, and optionally wherein the linker has at least 80% sequence identity to SEQ ID NO: 9 (GSTSGGGSGGGSGGGGSS), SEQ ID NO: 10 (GSTSGSGKPGSGEGSTKG), SEQ ID NO: 11 (SSGGGGSGGGGSGGGGS), or SEQ ID NO: 12 (GGGGSGGGGSGGGGS); (d) comprises an scFV represented by an amino acid sequence that is of at least about 99%, about 98%, about 96%, about 95%, about 90%, about 85%, or about 80% identity to SEQ ID NO: 13 (EVQLVESGGGLVQPGGSLKLSCAASGFTFSNYGMSWVRQTPDRRLELVATINNNGGGTYYPDSVKGRFTISRDNAKNTLYLQMSSLKSEDTAMYYCTSPGLLWDAWGAGTTVTVSSGSTSGGGSGGGSGGGGSSDVVMTQTPLTLSVSIGQPASISCKSSQSLLDSDGRTYLNWLLQRPGQSPKRLIYLVSKLDSGAPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGTKLEIK) or SEQ ID NO: 14 (DVVMTQTPLTLSVSIGQPASISCKSSQSLLDSDGRTYLNWLLQRPGQSPKRLIYLVSKLDSGAPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGTKLEIKGSTSGGGSGGGSGGGGSSEVQLVESGGGLVQPGGSLKLSCAASGFTFSNYGMSWVRQTPDRRLELVATI

NNNGGGTYYPDSVKGRFTISRDNAKNTLYLQMSSLKSEDTAMYYCTSPGLLWDAWGAG TTVTVSS), wherein each of SEQ ID NOs: 13 and 14 comprises a linker that varies in length and sequence; and/or (e) is humanized.

[0008] In some embodiments of the CAR, the at least one signaling domain comprises: (a) any one of: 2B4 (Natural killer Cell Receptor 2B4), 4-1BB (Tumor necrosis factor receptor superfamily member 9), CD16 (IgG Fc region Receptor III-A), CD2 (T-cell surface antigen CD2), CD28 (T-cell-specific surface glycoprotein CD28), CD28H (Transmembrane and immunoglobulin domain-containing protein 2), CD3ζ (T-cell surface glycoprotein CD3 zeta chain), DAP10 (Hematopoietic cell signal transducer), DAP12 (TYRO protein tyrosine kinase-binding protein), DNAM1 (CD226 antigen), FcERIγ (High affinity immunoglobulin epsilon receptor subunit gamma), IL21R (Interleukin-21 receptor), IL-2Rβ/IL-15RB (Interleukin-2 receptor subunit beta), IL-2Rγ (Cytokine receptor common subunit gamma), IL-7R (Interleukin-7 receptor subunit alpha), KIR2DS2 (Killer cell immunoglobulin-like receptor 2DS2), NKG2D (NKG2-D type II integral membrane protein), NKp30 (Natural cytotoxicity triggering receptor 3), NKp44 (Natural cytotoxicity triggering receptor 2), NKp46 (Natural cytotoxicity triggering receptor 1), CS1(SLAM family member 7), and CD8 (T-cell surface glycoprotein CD8 alpha chain); (b) an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to the cytoplasmic domain, or a portion thereof, of 2B4, 4-1BB, CD16, CD2, CD28, CD28H, CD3ζ, CD3ζ1XX, DAP10, DAP12, DNAM1, FcERIγ, IL21R, IL2Rβ (IL15Rβ), IL2Rγ, IL7R, KIR2DS2, NKG2D, NKp30, NKp44, NKp46, CS1, or CD8, represented by SEQ ID NOs: 37-59, respectively; and/or (c) an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to the cytoplasmic domain, or a portion thereof, of 2B4, CD28, CD3ζ, DAP10, NKG2D, CD3ζ1XX, DNAM1, CS1, or combinations thereof. In various embodiments of the CAR, the endodomain comprises two different signaling domains, and wherein said endodomain domain comprises fused cytoplasmic domains, or portions thereof, in any one of the forms: 2B4-CD3ζ/1XX, 2B4-DNAM1, 2B4-FcERIγ, 2B4-DAP10, CD16-DNAM1, CD16-DAP10, CD16-DAP12, CD2-CD3ζ/1XX, CD2-DNAM1, CD2-FcERIγ, CD2-DAP10, CD28-DNAM1, CD28-FcERIγ, CD28-DAP10, CD28-DAP12, CD28-CD3ζ/1XX, CD28H-CD3ζ/1XX, DAP10-CD3ζ/1XX, DAP10-DAP12, DAP12-CD3ζ/1XX, DAP12-DAP10, DNAM1-CD3ζ/1XX, KIR2DS2-CD3ζ/1XX, KIR2DS2-DAP10, KIR2DS2-2B4, or NKp46-2B4.

[0009] In various embodiments of the CAR, the transmembrane domain comprises an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to a transmembrane region, or a portion thereof, of: (a) CD2, CD3δ, CD3ε, CD3γ, CD3ζ, CD4, CD8, CD8a, CD8b, CD16, CD27, CD28, CD28H, CD40,

CD84, CD166, 4-1BB, OX40, ICOS, ICAM-1, CTLA4, PD1, LAG3, 2B4, BTLA, DNAM1, DAP10, DAP12, FcERIγ, IL7, IL12, IL15, KIR2DL4, KIR2DS1, KIR2DS2, NKp30, NKp44, NKp46, NKG2C, NKG2D, CS1, or T cell receptor polypeptide; (b) 2B4, CD2, CD16, CD28, CD28H, CD3ζ, DAP10, DAP12, DNAM1, FcERIγ, KIR2DS2, NKG2D, NKp30, NKp44, NKp46, CS1, or CD8; or (c) 2B4, CD28, CD28H, DAP10, DNAM1, KIR2DS2, and NKG2D. In some embodiments of the CAR, the transmembrane domain and its immediately linked signaling domain are from a same protein or from different proteins.

[00010] In various embodiments of the CAR, the ectodomain comprises one or more of: (a) a signal peptide; and/or (b) a spacer/hinge. In some embodiments of the CAR, the spacer/hinge comprises: (a) an IgG4 spacer, a CD28 spacers, a CD8 spacer, a CH3 spacer, a CH2/CH3 spacer, or any combination thereof; (b) a short spacer of about 10 to about 80 amino acids; a medium spacer of more than 80 to about 180 amino acids; or a long spacer of more than 180 amino acids; and/or (c) an amino acid sequence of at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to any of SEQ ID NOs: 81-85. In some embodiments of the CAR, the spacer/hinge comprises a medium spacer, wherein the spacer comprises an amino acid sequence of at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 84.

[00011] In various embodiments of the CAR, the CAR comparises an amino acid sequence of at least about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 88. In various embodiments of the CAR, the cancer cell is a breast cancer cell, an ovary cancer cell, an endometrium cancer cell, a lung cancer cell, an esophageal cancer cell, a salivary gland cancer cell, a bladder cancer cell, a gastric cancer cell, a colorectal cancer cell, or a head and neck cancer cell. In various embodiments of the CAR, the at least one signaling domain does not respond, or has a low level of response, to HER2 expressed on non-cancer cells. In various embodiments of the CAR, the cancer antigen specific responses comprise cytolysis and cytokine production.

[00012] In another aspect, the invention provides a polynucleotide comprising a nucleic acid sequence which encodes a CAR as described herein. In yet another aspect, the invention provides a vector comprising the polynucleotide described herein.

[00013] Various objects and advantages of the compositions and methods as provided herein will become apparent from the following description taken in conjunction with the accompanying drawings wherein are set forth, by way of illustration and example, certain embodiments of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[00014] FIGs. 1A and 1B show cytolytic efficacy and specificity of 4D5-, CasMab214-, or CasMab250- based CARs with short spacers against HER2 expressing tumor cells and non-tumorigenic normal cells that express HER2.

[00015] FIGs. 2A and 2B show production of the inflammatory cytokine IFNγ in donor T cells transduced with 4D5-, CasMab214-, or CasMab250- based CARs having a short spacer in response to $HER2^{High}$ tumor cells and $HER2^{+}$ normal/non-tumorigenic cells.

[00016] FIGs. 3A and 3B show cytolytic efficacy and specificity of 4D5-, CasMab214-, or CasMab250- based CARs with long spacers against HER2 expressing tumor cells and non-tumorigenic normal cells that express HER2.

[00017] FIGs. 4A and 4B show production of the inflammatory cytokine IFNγ in donor T cells transduced with 4D5-, CasMab214-, or CasMab250- based CARs having a long spacer in response to $HER2^{High}$ tumor cells and $HER2^{+}$ normal/non-tumorigenic cells.

[00018] FIGs. 5A and 5B show cytolytic efficacy and tumor specificity of 4D5- or CasMab250- based CARs with short or medium spacers against HER2 expressing tumor cells and non-tumorigenic normal cells that express HER2.

[00019] FIG. 6 shows tumor specificity of CasMab250 (H/L)- based CAR having medium spacers towards additional $HER2^{Low/+}$ normal cell lines.

[00020] FIG. 7 shows HER2-CAR transduced Jurkat cell were activated in response to $HER2^{High}$ tumor cells and $HER2^{+}$ normal/non-tumorigenic cells shown by the NFκB reporter marker expression. The HER2-CAR is either 4D5- or CasMab250 (H/L)- based.

[00021] FIG. 8 shows evaluation of multiple intracellular signaling domain configurations alongside the optimized extracellular domain of a CAR based on CasMab250.

DETAILED DESCRIPTION OF THE INVENTION

[00022] **Definitions**

[00023] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[00024] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[00025] As used herein, the articles "a," "an," and "the" are used herein to refer 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.

[00026] The use of the alternative (e.g., "or") should be understood to mean either one, both, or any combination thereof of the alternatives.

[00027] The term "and/or" should be understood to mean either one, or both of the alternatives.

[00028] As used herein, the term "about" or "approximately" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% compared to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In one embodiment, the term "about" or "approximately" refers a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length ± 15%, ± 10%, ± 9%, ± 8%, ± 7%, ± 6%, ± 5%, ± 4%, ± 3%, ± 2%, or ± 1% about a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

[00029] As used herein, the term "substantially" or "essentially" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that is about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or higher compared to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In one embodiment, the terms "essentially the same" or "substantially the same" refer a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that is about the same as a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

[00030] As used herein, the terms "substantially free of" and "essentially free of" are used interchangeably, and when used to describe a composition, such as a cell population or culture media, refer to a composition that is free of a specified substance or its source thereof, such as, 95% free, 96% free, 97% free, 98% free, 99% free of the specified substance or its source thereof, or is undetectable as measured by conventional means. The term "free of" or "essentially free of" a certain ingredient or substance in a composition also means that no such ingredient or substance is (1) included in the composition at any concentration, or (2) included in the composition at a functionally inert, low concentration. Similar meaning can be applied to the term "absence of," where referring to the absence of a particular substance or its source thereof of a composition.

[00031] Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises" and "comprising" will be understood to imply the inclusion of a stated

step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. In particular embodiments, the terms "include," "has," "contains," and "comprise" are used synonymously.

[00032] By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

[00033] By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that no other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

[00034] Reference throughout this specification to "one embodiment," "an embodiment," "a particular embodiment," "a related embodiment," "a certain embodiment," "an additional embodiment," or "a further embodiment" or combinations thereof means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

[00035] The term "*ex vivo*" refers generally to activities that take place outside an organism, such as experimentation or measurements done in or on living tissue in an artificial environment outside the organism, preferably with minimum alteration of the natural conditions. In particular embodiments, "*ex vivo*" procedures involve living cells or tissues taken from an organism and cultured in a laboratory apparatus, usually under sterile conditions, and typically for a few hours or up to about 24 hours, but including up to 48 or 72 hours or longer, depending on the circumstances. In certain embodiments, such tissues or cells can be collected and frozen, and later thawed for *ex vivo* treatment. Tissue culture experiments or procedures lasting longer than a few days using living cells or tissue are typically considered to be "*in vitro*," though in certain embodiments, this term can be used interchangeably with *ex vivo*.

[00036] The term "*in vivo*" refers generally to activities that take place inside an organism.

[00037] As used herein, the term "encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or a mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of

amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

[00038] A "construct" refers to a macromolecule or complex of molecules comprising a polynucleotide to be delivered to a host cell, either *in vitro* or *in vivo*. A "vector," as used herein refers to any nucleic acid construct capable of directing the delivery or transfer of a foreign genetic material to target cells, where it can be replicated and/or expressed. The term "vector" as used herein comprises the construct to be delivered. A vector can be a linear or a circular molecule. A vector can be integrating or non-integrating. The major types of vectors include, but are not limited to, plasmids, episomal vectors, viral vectors, cosmids, and artificial chromosomes. Viral vectors include, but are not limited to, adenovirus vectors, adeno-associated virus vectors, retrovirus vectors, lentivirus vectors, Sendai virus vectors, and the like.

[00039] As used herein, the term "exogenous" is intended to mean that the referenced molecule is introduced into, or is non-native to, the host cell. The molecule can be introduced, for example, by introduction of an encoding nucleic acid into the host genetic material such as by integration into a host chromosome or as non-chromosomal genetic material such as a plasmid. Therefore, the term as it is used in reference to expression of an encoding nucleic acid refers to introduction of the encoding nucleic acid in an expressible form into the cell. The term "endogenous" refers to a referenced molecule or activity that is present in the host cell. Similarly, the term when used in reference to expression of an encoding nucleic acid refers to expression of an encoding nucleic acid contained within the cell and not exogenously introduced.

[00040] By "integration" it is meant that one or more nucleotides of a construct is stably inserted into the cellular genome, i.e., covalently linked to the nucleic acid sequence within the cell's chromosomal DNA. By "targeted integration" it is meant that the nucleotide(s) of a construct is inserted into the cell's chromosomal or mitochondrial DNA at a pre-selected site or "integration site". The term "integration" as used herein further refers to a process involving insertion of one or more exogenous sequences or nucleotides of the construct, with or without deletion of an endogenous sequence or nucleotide at the integration site.

[00041] As used herein, a "gene of interest" or "a polynucleotide sequence of interest" is a DNA sequence that is transcribed into RNA and in some instances translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. A gene or polynucleotide of interest can include, but is not limited to, prokaryotic sequences, cDNA from

eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. For example, a gene of interest may encode an miRNA, an shRNA, a native polypeptide (i.e., a polypeptide found in nature) or fragment thereof; a variant polypeptide (i.e., a mutant of the native polypeptide having less than 100% sequence identity with the native polypeptide) or fragment thereof; an engineered polypeptide or peptide fragment, a therapeutic peptide or polypeptide, an imaging marker, a selectable marker, and the like.

[00042] As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. The sequence of a polynucleotide is composed of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for thymine when the polynucleotide is RNA. A polynucleotide can include a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. Polynucleotide also refers to both double- and single-stranded molecules.

[00043] As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably and refer to a molecule having amino acid residues covalently linked by peptide bonds. A polypeptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids of a polypeptide. As used herein, the terms refer to both short chains, which are also commonly 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 polypeptides or proteins. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural polypeptides, recombinant polypeptides, synthetic polypeptides, or a combination thereof.

[00044] As used herein and throughout the application, the percent identity between two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm recognized in the art.

[00045] As used herein, the term "subunit" as used herein refers to each separate polypeptide chain of a protein complex, where each separate polypeptide chain can form a stable folded structure by itself. Many protein molecules are composed of more than one subunit,

where the amino acid sequences can either be identical for each subunit, or similar, or completely different. For example, a CD3 complex is composed of CD3α, CD3ε, CD3δ, CD3γ, and CD3ζ subunits, which form the CD3ε/CD3γ, CD3ε/CD3δ, and CD3ζ/CD3ζ dimers. Within a single subunit, contiguous portions of the polypeptide chain frequently fold into compact, local, semi-independent units that are called "domains". Many protein domains may further comprise independent "structural subunits", also called subdomains, contributing to a common function of the domain. As such, the term "subdomain" as used herein refers to a protein domain inside of a larger domain, for example, a binding domain within an ectodomain of a cell surface receptor; or a stimulatory domain or a signaling domain of an endodomain of a cell surface receptor.

[00046] "Operably-linked" or "operatively linked," interchangeable with "operably connected" or "operatively connected," refers to the association of nucleic acid sequences on a single nucleic acid fragment (or amino acids in a polypeptide with multiple domains) so that the function of one is affected by the other. For example, a promoter is operably-linked with a coding sequence or functional RNA when it is capable of affecting the expression of that coding sequence or functional RNA (i.e., the coding sequence or functional RNA is under the transcriptional control of the promoter). Coding sequences can be operably-linked to regulatory sequences in sense or antisense orientation. As a further example, a receptor-binding domain can be operatively connected to an intracellular signaling domain, such that binding of the receptor to a ligand transduces a signal responsive to said binding.

[00047] "Fusion proteins" or "chimeric proteins", as used herein, are proteins created through genetic engineering to join two or more partial or whole polynucleotide coding sequences encoding separate proteins, and the expression of these joined polynucleotides results in a single peptide or multiple polypeptides with functional properties derived from each of the original proteins or fragments thereof. Between two neighboring polypeptides of different sources in the fusion protein, a linker (or spacer) peptide can be added.

[00048] As used herein, the term "signaling molecule" refers to any molecule that modulates, participates in, inhibits, activates, reduces, or increases, cellular signal transduction. "Signal transduction" refers to the transmission of a molecular signal in the form of chemical modification by recruitment of protein complexes along a pathway that ultimately triggers a biochemical event in the cell. Signal transduction pathways are well known in the art, and include, but are not limited to, G protein coupled receptor signaling, tyrosine kinase receptor signaling, integrin signaling, toll gate signaling, ligand-gated ion channel signaling, ERK/MAPK signaling pathway, Wnt signaling pathway, cAMP-dependent pathway, and IP3/DAG signaling pathway.

[00049] As used herein, the term "specific" or "specificity" can be used to refer to the ability of a molecule, e.g., a receptor or an engager, to selectively bind to a target molecule, in contrast to non-specific or non-selective binding.

[00050] The term "ligand" refers to a substance that forms a complex with a target molecule to produce a signal by binding to a site on the target. The ligand may be a natural or artificial substance capable of specific binding to the target. The ligand may be in the form of a protein, a peptide, an antibody, an antibody complex, a conjugate, a nucleic acid, a lipid, a polysaccharide, a monosaccharide, a small molecule, a nanoparticle, an ion, a neurotransmitter, or any other molecular entity capable of specific binding to a target. The target to which the ligand binds, may be a protein, a nucleic acid, an antigen, a receptor, a protein complex, or a cell. A ligand that binds to and alters the function of the target and triggers a signaling response is called "agonistic" or "an agonist". A ligand that binds to a target and blocks or reduces a signaling response is "antagonistic" or "an antagonist."

[00051] The term "antibody" encompasses antibodies and antibody fragments that contain at least one binding site that specifically binds to a particular target of interest, wherein the target may be an antigen, or a receptor that is capable of interacting with certain antibodies. The term "antibody" includes, but is not limited to, an immunoglobulin molecule or an antigen-binding or receptor-binding portion thereof. A specific piece or portion of an antigen or receptor, or a target in general, to which an antibody binds is known as an epitope or an antigenic determinant. The term antibody also includes, but is not limited to, native antibodies and variants thereof, fragments of native antibodies and variants thereof, peptibodies and variants thereof, and antibody mimetics that mimic the structure and/or function of an antibody or a specified fragment or portion thereof, including single chain antibodies and fragments thereof. An antibody may be a murine antibody, a human antibody, a humanized antibody, a camel IgG, single variable new antigen receptor (VNAR), shark heavy-chain antibody (Ig-NAR), a chimeric antibody, a recombinant antibody, a single-domain antibody (dAb), an anti-idiotype antibody, a bi-specific-, multi-specific- or multimeric- antibody, or antibody fragment thereof. Non-limiting examples of antibody fragments include Fab, Fab', F(ab')2, F(ab')3, Fv, Fabc, pFc, Fd, single chain fragment variable (scFv), tandem scFv (scFv)2, single chain Fab (scFab), disulfide stabilized Fv (dsFv), minibody, diabody, triabody, tetrabody, single-domain antigen binding fragments (sdAb), camelid heavy-chain IgG and Nanobody® fragments, recombinant heavy-chain-only antibody (VHH), and other antibody fragments that maintain the binding specificity of the antibody.

Tumor-Specific Chimeric Antigen Receptor (CAR)

[00052] A CAR is a fusion protein generally including an ectodomain that comprises an antigen recognition region, a transmembrane domain, and an endodomain. In some embodiments, the ectodomain can further include a signal peptide or leader sequence and/or a spacer. In some embodiments, the endodomain comprises a signaling peptide that activates the effector cell expressing the CAR. In some embodiments, the endodomain comprises one or more signaling domains, wherein the signaling domain orginates from a cytoplasmic domain of a signal transducing protein specific to T and/or NK cell activation or functioning. In some embodiments, the antigen recognition domain can specifically bind an antigen. In some embodiments, the antigen recognition domain can specifically bind an antigen associated with a disease or pathogen. In some embodiments, the disease-associated antigen is a tumor antigen, wherein the tumor may be a liquid or a solid tumor.

[00053] In certain embodiments, said antigen recognition region/domain comprises a murine antibody, a human antibody, a humanized antibody, a camel Ig, a single variable new antigen receptor (VNAR), a shark heavy-chain-only antibody (Ig-NAR), a chimeric antibody, a recombinant antibody, or an antibody fragment thereof. Non-limiting examples of antibody fragments include Fab, Fab′, F(ab′)2, F(ab′)3, Fv, single chain antigen binding fragment (scFv), $(scFv)_2$, disulfide stabilized Fv (dsFv), minibody, diabody, triabody, tetrabody, single-domain antigen binding fragments (sdAb, Nanobody), recombinant heavy-chain-only antibody (VHH), and other antibody fragments that maintain the binding specificity of the whole antibody.

[00054] Various aspects of the invention provide a CAR comprising an antigen recognition region that binds to a tumor associated antigen. In various embodiments, the CAR is specific to a tumor cell surface HER2 antigen. In some embodiments, the antigen recognition domain of the ectodomain of the HER2-CAR comprises a heavy chain variable (VH) domain comprising a heavy chain complementary determining region 1 (H-CDR1) comprising SEQ ID NO: 1 (NYGMS), a heavy chain complementary determining region 2 (H-CDR2) comprising SEQ ID NO: 2 (TINNNGGGTYYPDSVKG), and a heavy chain complementary determining region 3 (H-CDR3) comprising SEQ ID NO: 3 (PGLLWDA); and a light chain variable (VL) domain comprising a light chain complementary determining region 1 (L-CDR1) comprising SEQ ID NO: 4 (KSSQSLLDSDGRTYLN), a light chain complementary determining region 2 (L-CDR2) comprising SEQ ID NO: 5 (LVSKLDS), and a light chain complementary determining region 3 (L-CDR3) comprising SEQ ID NO: 6 (WQGTHFPQT).

[00055] In some embodiments, the CAR comprises heavy chain CDRs followed by light chain CDRs (H/L) in an amino to carboxy direction. In some embodiments, the CAR comprises

a heavy chain variable domain followed by a light chain variable domain in an amino to carboxy direction.

[00056] In some embodiments, the antigen binding domain of the CAR comprises a VH domain having a sequence identity of at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 100%, or any percentage in-between, when compared to the exemplary sequence represented by SEQ ID NO: 7. In some embodiments, the VH domain comprises an amino acid sequence of at least about 90% identity to SEQ ID NO: 7. In some embodiments, the VH domain comprises an amino acid sequence of at least about 95% identity to SEQ ID NO: 7. In some embodiments, the VH domain comprises the amino acid sequence of SEQ ID NO: 7. In some other embodiments, the antigen binding domain of the CAR comprises a VL domain having a sequence identity of at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 100%, or any percentage in-between, when compared to the exemplary sequence represented by SEQ ID NO: 8. In some embodiments, the VL domain comprises an amino acid sequence of at least about 90% identity to SEQ ID NO: 8. In some embodiments, the VL domain comprises an amino acid sequence of at least about 95% identity to SEQ ID NO: 8. In some embodiments, the VL domain comprises the amino acid sequence of SEQ ID NO: 8.

SEQ ID NO: 7
EVQLVESGGGLVQPGGSLKLSCAASGFTFSNYGMSWVRQTPDRRLELVATINNNGGGTYYPDSV
KGRFTISRDNAKNTLYLQMSSLKSEDTAMYYCTSPGLLWDAWGAGTTVTVSS

SEQ ID NO: 8
DVVMTQTPLTLSVSIGQPASISCKSSQSLLDSDGRTYLNWLLQRPGQSPKRLIYLVSKLDSGAPDR
FTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGTKLEIK

[00057] In some embodiments the antigen binding domain of the CAR comprises a single chain variable fragment (scFV) having a N to C terminus orientation comprising VH-linker-VL or VL-linker-VH, wherein the linker varies in length and sequence. In some embodiments, the linker has a sequence identity of at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 100%, or any percentage in-between, when compared to the exemplary sequences represented by SEQ ID NOs: 9-12. In some embodiments, the linker comprises an amino acid sequence of at least about 90% identity to any of SEQ ID NOs: 9-12. In some embodiments, the linker comprises an amino acid sequence of at least about 95% identity to any of SEQ ID NOs: 9-12. In some embodiments, the linker comprises the amino acid sequence of any of SEQ ID NOs: 9-12.

SEQ ID NO: 9
GSTSGGGSGGGSGGGGSS

SEQ ID NO: 10
GSTSGSGKPGSGEGSTKG

SEQ ID NO: 11
SSGGGGSGGGGSGGGGS

SEQ ID NO: 12
GGGGSGGGGSGGGGS

[00058] In some embodiments the antigen binding domain of the CAR comprises a single chain variable fragment (scFV) having n sequence identity of at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 100%, or any percentage in-between, when compared to the exemplary sequences represented by SEQ ID NO: 13 or SEQ ID NO: 14, wherein each of SEQ ID NOs: 13 and 14 comprise a linker that can vary in length and/or sequence. In some embodiments, the scFV comprises an amino acid sequence of at least 90% identity to SEQ ID NO: 13 or 14. In some embodiments, the scFV comprises an amino acid sequence of at least 95% identity to SEQ ID NO: 13 or 14. In some embodiments, the scFV comprises the amino acid sequence of SEQ ID NO: 13. In some embodiments, the scFV comprises the amino acid sequence of SEQ ID NO: 14.

SEQ ID NO: 13
EVQLVESGGGLVQPGGSLKLSCAASGFTFSNYGMSWVRQTPDRRLELVATINNNGGGTYYPDSV
KGRFTISRDNAKNTLYLQMSSLKSEDTAMYYCTSPGLLWDAWGAGTTVTVSSGSTSGGGSGGG
SGGGGSSDVVMTQTPLTLSVSIGQPASISCKSSQSLLDSDGRTYLNWLLQRPGQSPKRLIYLVSKL
DSGAPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGTKLEIK

SEQ ID NO: 14
DVVMTQTPLTLSVSIGQPASISCKSSQSLLDSDGRTYLNWLLQRPGQSPKRLIYLVSKLDSGAPDR
FTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGTKLEIKGSTSGGGSGGGSGGGGSSE
VQLVESGGGLVQPGGSLKLSCAASGFTFSNYGMSWVRQTPDRRLELVATINNNGGGTYYPDSV
KGRFTISRDNAKNTLYLQMSSLKSEDTAMYYCTSPGLLWDAWGAGTTVTVSS

[00059] In various embodiments, CARs described herein include at least an ectodomain, a transmembrane domain, and an endodomain. In some embodiments, the endodomain of a CAR

comprises at least one signaling domain that is activated upon antigen binding. In some embodiments of the CAR endodomain, one or more co-stimulation domains (oftentimes referred to as "additional signaling domain(s)") is further included for optimized functionality. Exemplary signal transducing proteins suitable for a CAR design include, but are not limited to, 2B4, 4-1BB (CD137, or "41BB" in illustrative fusion constructs throughout the application), CD16, CD2, CD28, CD28H, CD3ζ/1XX (i.e., CD3ζ or CD3ζ1XX), DAP10, DAP12, DNAM1, FcERIγ, IL21R, IL-2Rβ (IL-15Rβ), IL-2Rγ, IL-7R, KIR2DS2, NKG2D, NKp30, NKp44, NKp46, CS1 and CD8. The description of exemplary signal transducing proteins, including transmembrane and cytoplasmic sequences of the proteins are provided below, and further in Table 1A.

Table 1A:

	Protein name	**UniProtKB Accession No.**	**Transmembrane Sequence**	**Cytoplasmic Sequence**
2B4	Natural killer cell receptor 2B4	Q9BZW8	FLVIIVILSALFLGTL ACFCV (SEQ ID NO: 15)	WRRKRKEKQSETSPKEFLTIYEDVK DLKTRRNHEQEQTFPGGGSTIYSMI QSQSSAPTSQEPAYTLYSLIQPSRKS GSRKRNHSPSFNSTIYEVIGKSQPKA QNPARLSRKELENFDVYS (SEQ ID NO: 37)
4-1BB	Tumor necrosis factor receptor superfamily member 9	Q07011	IISFFLALTSTALLFL LFFLTLRFSVV (SEQ ID NO: 16)	KRGRKKLLYIFKQPFMRPVQTTQEE DGCSCRFPEEEEGGCEL (SEQ ID NO: 38)
CD16	IgG Fc region Receptor III-A	P08637	VSFCLVMVLLFAVD TGLYFSVKTNIRSST RD (SEQ ID NO: 17)	WKDHKFKWRKDPQDK (SEQ ID NO: 39)
CD2	T-cell surface antigen CD2	P06729	IYLIIGICGGGSLLM VFVALLVFYITKRK KQRSRRNDEELETR AHRVATEERGRKPH QIPASTPQNPATSQH PPPPPGHRSQAPSHR PPPPGHRVQ (SEQ ID NO: 18)	HQPQKRPPAPSGTQVHQQKGPPLPR PRVQPKPPHGAAENSLSPSSN (SEQ ID NO: 40)
CD28	T-cell-specific surface glycoprotein CD28	P10747	FWVLVVVGGVLAC YSLLVTVAFIIFWV (SEQ ID NO: 19)	RSKRSRLLHSDYMNMTPRRPGPTR KHYQPYAPPRDFAAYRS (SEQ ID NO: 41)
CD28H	Transmembrane and immunoglobulin domain-	Q96BF3	FLFVLLGVGSMGVA AIVWGAW (SEQ ID NO: 20)	FWGRRSCQQRDSGNSPGNAFYSNV LYRPRGAPKKSEDCSGEGKDQRGQ SIYSTSFPQPAPRQPHLASRPCPSPRP CPSPRPGHPVSMVRVSPRPSPTQQP

	containing protein 2			RPKGFPKVGEE (SEQ ID NO: 42)
CD3ζ/1XX	T-cell surface glycoprotein CD3 zeta chain	P20963	LCYLLDGILFIYGVILTALFL (SEQ ID NO: 21)	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 43; CD3ζ) Or RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLFNELQKDKMAEAFSEIGMKGERRRGKGHDGLFQGLSTATKDTFDALHMQALPPR (SEQ ID NO: 44; containing 2 mutations in ITAM1; CD3ζ1XX)
DAP10	Hematopoietic cell signal transducer	Q9UBK5	LLAGLVAADAVASLLIVGAVF (SEQ ID NO: 22)	LCARPRRSPAQEDGKVYINMPGRG (SEQ ID NO: 45)
DAP12	TYRO protein tyrosine kinase-binding protein	O43914	GVLAGIVMGDLVLTVLIALAV (SEQ ID NO: 23)	YFLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSDVYSDLNTQRPYYK (SEQ ID NO: 46)
DNAM1	CD226 antigen	Q15762	GGTVLLLLFVISITTIIVIFL (SEQ ID NO: 24)	NRRRRRERRDLFTESWDTQKAPNNYRSPISTSQPTNQSMDDTREDIYVNYPTFSRRPKTRV (SEQ ID NO: 47)
FcERIγ	High affinity immunoglobulin epsilon receptor subunit gamma	P30273	CYILDAILFLYGIVLTLLYC (SEQ ID NO: 25)	RLKIQVRKAAITSYEKSDGVYTGLSTRNQETYETLKHEKPPQ (SEQ ID NO: 48)
IL-21R	Interleukin-21 receptor	Q9HBE5	GWNPHLLLLLLLVIVFIPAFW (SEQ ID NO: 26)	SLKTHPLWRLWKKIWAVPSPERFFMPLYKGCSGDFKKWVGAPFTGSSLELGPWSPEVPSTLEVYSCHPPRSPAKRLQLTELQEPAELVESDGVPKPSFWPTAQNSGGSAYSEERDRPYGLVSIDTVTVLDAEGPCTWPCSCEDDGYPALDLDAGLEPSPGLEDPLLDAGTTVLSCGCVSAGSPGLGGPLGSLLDRLKPPLADGEDWAGGLPWGGRSPGGVSESEAGSPLAGLDMDTFDSGFVGSDCSSPVECDFTSPGDEGPPRSYLRQWVVIPPPLSSPGPQAS (SEQ ID NO: 49)

IL-2Rβ (IL-15Rβ)	Interleukin-2 receptor subunit beta	P14784	IPWLGHLLVGLSGAFGFIILVYLLI (SEQ ID NO: 27)	NCRNTGPWLKKVLKCNTPDPSKFFSQLSSEHGGDVQKWLSSPFPSSSFSPGGLAPEISPLEVLERDKVTQLLLQQDKVPEPASLSSNHSLTSCFTNQGYFFFHLPDALEIEACQVYFTYDPYSEEDPDEGVAGAPTGSSPQPLQPLSGEDDAYCTFPSRDDLLLFSPSLLGGPSPPSTAPGGSGAGEERMPPSLQERVPRDWDPQPLGPPTPGVPDLVDFQPPPELVLREAGEEVPDAGPREGVSFPWSRPPGQGEFRALNARLPLNTDAYLSLQELQGQDPTHLV (SEQ ID NO: 50)
IL-2Rγ	Cytokine receptor common subunit gamma	P31785	VVISVGSMGLIISLLCVYFWL (SEQ ID NO: 28)	ERTMPRIPTLKNLEDLVTEYHGNFSAWSGVSKGLAESLQPDYSERLCLVSEIPPKGGALGEGPGASPCNQHSPYWAPPCYTLKPET (SEQ ID NO: 51)
IL-7R	Interleukin-7 receptor subunit alpha	P16871	PILLTISILSFFSVALLVILACVLW (SEQ ID NO: 29)	KKRIKPIVWPSLPDHKKTLEHLCKKPRKNLNVSFNPESFLDCQIHRVDDIQARDEVEGFLQDTFPQQLEESEKQRLGGDVQSPNCPSEDVVITPESFGRDSSLTCLAGNVSACDAPILSSSRSLDCRESGKNGPHVYQDLLLSLGTTNSTLPPPFSLQSGILTLNPVAQGQPILTSLGSNQEEAYVTMSSFYQNQ (SEQ ID NO: 52)
KIR2DS2	Killer cell immunoglobulin-like receptor 2DS2	P43631	VLIGTSVVKIPFTILLFFLL (SEQ ID NO: 30)	HRWCSNKKNAAVMDQEPAGNRTVNSEDSDEQDHQEVSYA (SEQ ID NO: 53)
NKG2D	NKG2-D type II integral membrane protein	P26718	PFFFCCFIAVAMGIRFIIMVA (SEQ ID NO: 31)	IWSAVFLNSLFNQEVQIPLTESYCGPCPKNWICYKNNCYQFFDESKNWYESQASCMSQNASLLKVYSKEDQDLLKLVKSYHWMGLVHIPTNGSWQWEDGSILSPNLLTIIEMQKGDCALYASSFKGYIENCSTPNTYICMQRTV (SEQ ID NO: 54)
NKp30	Natural cytotoxicity triggering receptor 3	O14931	AGTVLLLRAGFYAVSFLSVAV (SEQ ID NO: 32)	GSTVYYQGKCLTWKGPRRQLPAVVPAPLPPPCGSSAHLLPPVPGG (SEQ ID NO: 55)
NKp44	Natural cytotoxicity triggering receptor 2	O95944	LVPVFCGLLVAKSLVLSALLV (SEQ ID NO: 33)	WWGDIWWKTMMELRSLDTQKATCHLQQVTDLPWTSVSSPVEREILYHTVARTKISDDDDEHTL (SEQ ID NO: 56)
NKp46	Natural cytotoxicity triggering receptor 1	O76036	GLAFLVLVALVWFLVEDWLS (SEQ ID NO: 34)	RKRTRERASRASTWEGRRRLNTQTL (SEQ ID NO: 57)
CS1	SLAM family member 7	Q9NQ25	VLLCLLLVPLLLSLFVLGLFL (SEQ ID NO: 35)	WFLKRERQEEYIEEKKRVDICRETPNICPHSGENTEYDTIPHTNRTILKEDPANTVYSTVEIPKKMENPHSLLTMPDTPRLFAYENVI (SEQ ID NO: 58)

CD8	T-cell surface glycoprotein CD8 alpha chain	P01732	IYIWAPLAGTC (SEQ ID NO: 36)	GVLLLSLVITLYCNHRNRRRVCKCP RPVVKSGDKPSLSARYV (SEQ ID NO: 59)

[00060] In some embodiments of the CAR as provided, the endodomain of the CAR comprises at least a first signaling domain having an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to the cytoplasmic domain, or a portion thereof, of 2B4, 4-1BB, CD16, CD2, CD28, CD28H, CD3ζ, CD3ζ1XX, DAP10, DAP12, DNAM1, FcERIγ IL21R, IL-2Rβ (IL-15Rβ), IL-2Rγ, IL-7R, KIR2DS2, NKG2D, NKp30, NKp44, NKp46, CS1, or CD8, represented by SEQ ID NOs: 37-59, respectively. In some embodiments, the first signaling domain comprises an amino acid sequence of at least 90% identity to any of SEQ ID NOs: 37-59. In some embodiments, the first signaling domain comprises an amino acid sequence of at least 95% identity to any of SEQ ID NOs: 37-59. In some embodiments, the first signaling domain comprises the amino acid sequence of any of SEQ ID NOs: 37-59. In some embodiments, the signaling domain of a CAR disclosed herein comprises only a portion of the cytoplasmic domain of 2B4, 4-1BB, CD16, CD2, CD28, CD28H, CD3ζ, CD3ζ1XX, DAP10, DAP12, DNAM1, FcERIγ IL21R, IL-2Rβ (IL-15Rβ), IL-2Rγ, IL-7R, KIR2DS2, NKG2D, NKp30, NKp44, NKp46, CS1, or CD8. In some embodiments, the portion of the cytoplasmic domain selected for the CAR signaling domain comprises an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to an ITAM (immunoreceptor tyrosine-based activation motif), a YxxM motif, a TxYxxV/I motif, FcRγ, hemi-ITAM, and/or an ITT-like motif.

[00061] In some embodiments of the CAR as provided, the endodomain of the CAR comprising a first signaling domain further comprises a second signaling domain comprising an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to the cytoplasmic domain, or a portion thereof, of 2B4, 4-1BB, CD16, CD2, CD28, CD28H, CD3ζ, CD3ζ1XX, DAP10, DAP12, DNAM1, FcERIγ IL21R, IL-2Rβ (IL-15Rβ), IL-2Rγ, IL-7R, KIR2DS2, NKG2D, NKp30, NKp44, NKp46, CS1 or CD8, represented by SEQ ID NOs: 37-59, respectively, wherein the second signaling domain is different from the first signaling domain. In some embodiments, the second signaling domain comprises an amino acid sequence of at least 90% identity to any of SEQ ID NOs: 37-59. In some embodiments, the second signaling domain comprises an amino acid sequence of at least 95% identity to any of SEQ ID NOs: 37-59. In some embodiments, the second signaling domain comprises the amino acid sequence of any of SEQ ID NOs: 37-59.

[00062] In some embodiments of the CAR as provided, the endodomain of the CAR comprising a first and a second signaling domain further comprises a third signaling domain comprising an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to the cytoplasmic domain, or a portion thereof, of 2B4, 4-1BB, CD16, CD2, CD28, CD28H, CD3ζ, CD3ζ1XX, DAP10, DAP12, DNAM1, FcERIγ, IL21R, IL-2Rβ (IL-15Rβ), IL-2Rγ, IL-7R, KIR2DS2, NKG2D, NKp30, NKp44, NKp46, CS1, or CD8, represented by SEQ ID NOs: 37-59, respectively, wherein the third signaling domain is different from the first and the second signaling domains. In some embodiments, the third signaling domain comprises an amino acid sequence of at least 90% identity to any of SEQ ID NOs: 37-59. In some embodiments, the third signaling domain comprises an amino acid sequence of at least 95% identity to any of SEQ ID NOs: 37-59. In some embodiments, the third signaling domain comprises the amino acid sequence of any of SEQ ID NOs: 37-59. In some embodiments, signal transducing proteins suitable for designing a signaling domain of a CAR endodomain further comprise CD27, OX40, ICOS, PD-1, LAG-3, BTLA, or CTLA-4.

[00063] In some exemplary embodiments of a CAR having an endodomain comprised of only one signaling domain, said endodomain comprises an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to the cytoplasmic domain or a portion thereof, of a protein including, but not limited to, DNAM1, CD28H, KIR2DS2, DAP12 or DAP10.

[00064] In some exemplary embodiments of a CAR having an endodomain comprised of two different signaling domains, said endodomain comprises fused cytoplasmic domains, or portions thereof, in a form including, but not limited to, 2B4-CD3ζ/1XX (i.e., 2B4-CD3ζ or 2B4-CD3ζ1XX; same below), 2B4-DNAM1, 2B4-FcERIγ, 2B4-DAP10, CD16-DNAM1, CD16-DAP10, CD16-DAP12, CD2-CD3ζ/1XX, CD2-DNAM1, CD2-FcERIγ, CD2-DAP10, CD28-DNAM1, CD28-FcERIγ, CD28-DAP10, CD28-DAP12, CD28-CD3ζ/1XX, CD28H-CD3ζ/1XX, DAP10-CD3ζ/1XX, DAP10-DAP12, DAP12-CD3ζ/1XX, DAP12-DAP10, DNAM1-CD3ζ/1XX, KIR2DS2-CD3ζ/1XX, KIR2DS2-DAP10, KIR2DS2-2B4, or NKp46-2B4.

[00065] In some exemplary embodiments of a CAR having an endodomain comprised of three different signaling domains, said endodomain comprises fused cytoplasmic domains, or portions thereof, in a form including, but not limited to, 2B4-DAP10-CD3ζ/1XX, 2B4-IL21R-DAP10, 2B4-IL2RB-DAP10, 2B4-IL2RB-CD3ζ/1XX, 2B4-41BB-DAP10, CD16-2B4-DAP10, or KIR2DS2-2B4-CD3ζ/1XX.

[00066] In some embodiments, the transmembrane domain of a CAR comprises an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about

98%, or about 99% identity to a full length or a portion of the transmembrane region of CD2, CD3δ, CD3ε, CD3γ, CD3ζ, CD4, CD8, CD8a, CD8b, CD16, CD27, CD28, CD28H, CD40, CD84, CD166, 4-1BB, OX40, ICOS, ICAM-1, CTLA4, PD1, LAG3, 2B4, BTLA, DNAM1, DAP10, DAP12, FcERIγ, IL7, IL12, IL15, KIR2DL4, KIR2DS1, KIR2DS2, NKp30, NKp44, NKp46, NKG2C, NKG2D, CS1, or T cell receptor polypeptide. In some other embodiments, the transmembrane domain of a CAR comprises an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to a full length or a portion of the transmembrane region of (a) 2B4, CD16, CD2, CD28, CD28H, CD3ζ, DAP10, DAP12, DNAM1, FcERIγ, KIR2DS2, NKG2D, NKp30, NKp44, NKp46, CS1, or CD8, represented by SEQ ID NOs: 15, 17-25, 30-36, respectively; or of (b) 2B4, CD28, CD28H, DAP10, DNAM1, KIR2DS2, and NKG2D. In some embodiments, the transmembrane domain comprises an amino acid sequence of at least about 90% identity to any of SEQ ID NOs: 15, 17-25, 30-36. In some embodiments, the transmembrane domain comprises an amino acid sequence of at least about 95% identity to any of SEQ ID NOs: 15, 17-25, 30-36. In some embodiments, the transmembrane domain comprises the amino acid sequence of any of SEQ ID NOs: 15, 17-25, 30-36. In some embodiments of the CAR, the transmembrane domain and its immediately linked signaling domain are from the same protein. In some other embodiments of the CAR, the transmembrane domain and the signaling domain that is immediately linked are from different proteins.

[00067] Table 1B of the application provides non-limiting examples of CAR constructs comprising a transmembrane domain (TM) and an endodomain (labelled as: TM-(endodomain)). In general, the illustrated CAR constructs each comprise a transmembrane domain, and an endodomain comprising one or more signaling domains derived from the cytoplasmic region of one or more signal transducing proteins. In general, a transmembrane domain is a three-dimensional protein structure which is thermodynamically stable in a membrane such as the phospholipid bilayer of a biological membrane (e.g., a membrane of a cell or cell vesicle). Thus, in some embodiments, the transmembrane domain of a CAR of the present invention comprises a single alpha helix, a stable complex of several transmembrane alpha helices, a transmembrane beta barrel, a beta-helix of gramicidin A, or any combination thereof. In various embodiments, the transmembrane domain of the CAR comprises all or a portion of a "transmembrane protein" or "membrane protein" that is within the membrane. As used herein, a "transmembrane protein" or "membrane protein" is a protein located at and/or within a membrane. Examples of transmembrane proteins that are suitable for providing a transmembrane domain comprised in a CAR of embodiments of the invention include, but are not limited to, a receptor, a ligand, an immunoglobulin, a glycophorin, or any combination thereof. In some embodiments, the

transmembrane domain comprised in the CAR comprises all or a portion of a transmembrane domain of 2B4, 4-1BB, BTLA, CD2, CD3δ, CD3ε, CD3γ, CD3ζ, CD4, CD8, CD8a, CD8b, CD16, CD27, CD28, CD28H, CD40, CD84, CD166, CS1, CTLA-4, DNAM1, DAP10, DAP12, FcERIγ, ICOS, ICAM-1, IL7, IL12, IL15, KIR2DL4, KIR2DS1, KIR2DS2, LAG3, PD1, NKp30, NKp44, NKp46, NKG2C, NKG2D, OX40, T cell receptor polypeptide (such as TCRα and/or TCRβ), a nicotinic acetylcholine receptor, a GABA receptor, or any combination thereof.

[00068] In some embodiments, one or more signaling domains comprised in the CAR endodomain are derived from the same or a different protein from which the TM is derived. As shown in Table 1B, the portion representing the transmembrane domain of the CAR is underlined, the domains comprised in the endodomain appear in parenthesis, "()", with each of the TM and signaling domains designated by the name of the signal transducing protein from which the domain sequence is derived. In embodiments, the amino acid sequence of each TM or signaling domains may be of about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to a full length or a portion of the corresponding transmembrane or cytoplasmic regions of the designated signal transducing protein. Exemplary CAR constructs comprising a transmembrane domain and an endodomain as provided herein include, but are not limited to: NKG2D-(2B4-IL2RB-CD3ζ), CD8-(41BB-CD3ζ1XX), CD28-(CD28-2B4-CD3ζ), CD28-(CD28-CD3 ζ 1XX), CD28H-(CD28H-CD3ζ), DNAM1-(DNAM1-CD3ζ), DAP10-(DAP10-CD3ζ), KIR2DS2-(KIR2DS2-CD3ζ), KIR2DS2-(KIR2DS2-DAP10), KIR2DS2-(KIR2DS2-2B4), CD16-(CD16-2B4-DAP10), CD16-(CD16-DNAM1), NKp46-(NKp46-2B4), NKp46-(NKp46-2B4-CD3ζ), NKp46-(NKp46-CD2-DAP10), CD2-(CD2-CD3ζ), 2B4-(2B4-CD3ζ), 2B4-(2B4-FcERIγ), and CS1-(CS1-CD3ζ). In some embodiments, each of the above exemplary CAR constructs comprising a transmembrane domain and an endodomain comprises an amino acid sequence of at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% identity to a sequence represented by each of SEQ ID NOs: 60-78 in Table 1B. In some embodiments, the CAR comprises an amino acid sequence of at least about 90% identity to any of SEQ ID NOs: 60-78. In some embodiments, the CAR comprises an amino acid sequence of at least about 95% identity to any of SEQ ID NOs: 60-78. In some embodiments, the CAR comprises the amino acid sequence of any of SEQ ID NOs: 60-78. The illustrative sequence for each construct provided in Table 1B has text formatted to match the formatting of the corresponding region in the illustration at left of the sequence (i.e., underlined, normal, or bolded text). For most of the illustrative constructs of Table 1B, the TM is the first sequence region; however, constructs may include an extracellular domain preceeding the TM (see, e.g., Construct 7 in Table 1B), and may be from the same or a different protein as the TM.

In some embodiments, two or more signaling domains comprised in the CAR endodomain may be separated by one or more additional sequences, such as a spacer or a linker.

Table 1B:

Construct	Sequence Domains TM-(endodomain)	Illustrative Sequence	SEQ ID NO
1	NKG2D-(**2B4**-IL2Rβ-**CD3ζ**)	SNLFVASWIAVMIIFRIGMAVAIFCCFFFPS**WRRKRKEK QSETSPKEFLTIYEDVKDLKTRRNHEQEQTFPGGGSTI YSMIQSQSSAPTSQEPAYTLYSLIQPSRKSGSRKRNHSP SFNSTIYEVIGKSQPKAQNPARLSRKELENFDVYS**NCR NTGPWLKKVLKCNTPDPSKFFSQLSSEHGGDVQKWLSSP FPSSSFSPGGLAPEISPLEVLERDKVTQLLLQQDKVPEPAS LSSNHSLTSCFTNQGYFFFHLPDALEIEACQVYFTYDPYS EEDPDEGVAGAPTGSSPQPLQPLSGEDDAYCTFPSRDDLL LFSPSLLGGPSPPSTAPGGSGAGEERMPPSLQERVPRDWD PQPLGPPTPGVPDLVDFQPPPELVLREAGEEVPDAGPREG VSFPWSRPPGQGEFRALNARLPLNTDAYLSLQELQGQDP THLV**RVKFSRSADAPAYQQGQNQLYNELNLGRREEY DVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKM AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA LHMQALPPR**	60
2	CD8-(**41BB**-CD3ζ1XX)	IYIWAPLAGTCGVLLLSLVITLYC**KRGRKKLLYIFKQPF MRPVQTTQEEDGCSCRFPEEEEGGCEL**RVKFSRSADA PAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLFNELQKDKMAEAFSEIGMKGERRRGKG HDGLFQGLSTATKDTFDALHMQALPPR	61
3	CD28-(CD28-**2B4**-CD3ζ)	FWVLVVVGGVLACYSLLVTVAFIIFWV**RSKRSRLLHSDY** MNMTPRRPGPTRKHYQPYAPPRDFAAYRS**WRRKRKEK QSETSPKEFLTIYEDVKDLKTRRNHEQEQTFPGGGSTI YSMIQSQSSAPTSQEPAYTLYSLIQPSRKSGSRKRNHSP SFNSTIYEVIGKSQPKAQNPARLSRKELENFDVYS**RVK FSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE RRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	62
4	CD28-(CD28-**CD3ζ1XX**)	FWVLVVVGGVLACYSLLVTVAFIIFWV**RSKRSRLLHSDY** MNMTPRRPGPTRKHYQPYAPPRDFAAYRS**RVKFSRSAD APAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPE MGGKPRRKNPQEGLFNELQKDKMAEAFSEIGMKGE RRRGKGHDGLFQGLSTATKDTFDALHMQALPPR**	63
5	CD28H-(CD28H-**CD3ζ**)	FLFVLLGVGSMGVAAIVWGAWFWGRRSCQQRDSGNSP GNAFYSNVLYRPRGAPKKSEDCSGEGKDQRGQSIYSTSF PQPAPRQPHLASRPCPSPRPCPSPRPGHPVSMVRVSPRPSP TQQPRPKGFPKVGEE**RVKFSRSADAPAYQQGQNQLYN ELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGL YNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGL STATKDTYDALHMQALPPR**	64
6	DNAM1-(DNAM1-**CD3ζ**)	GGTVLLLLFVISITTIIVIFLNRRRRRERRDLFTESWDTQK APNNYRSPISTSQPTNQSMDDTREDIYVNYPTFSRRPKTR V**RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVL DKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEA YSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH MQALPPR**	65
7	DAP10-(DAP10-**CD3ζ**)	TTPGERSSLPAFYPGTSGSCSGCGSLSLPLLAGLVAADAV ASLLIVGAVFLCARPRRSPAQEDGKVYINMPGRG**RVKFS RSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLSTATKDTYDALHMQALPP R**	66

8	KIR2DS2-(KIR2DS2-**CD3ζ)**	VLIGTSVVKIPFTILLFFLLHRWCSNKKNAAVMDQEPAG NRTVNSEDSDEQDHQEVSYA**RVKFSRSADAPAYQQGQ NQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRK NPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHD GLYQGLSTATKDTYDALHMQALPPR**	67
9	KIR2DS2-(KIR2DS2-**DAP10)**	VLIGTSVVKIPFTILLFFLLHRWCSNKKNAAVMDQEPAG NRTVNSEDSDEQDHQEVSYA**LCARPRRSPAQEDGKVYI NMPGRG**	68
10	KIR2DS2-(KIR2DS2-**2B4)**	VLIGTSVVKIPFTILLFFLLHRWCSNKKNAAVMDQEPAG NRTVNSEDSDEQDHQEVSYA**WRRKRKEKQSETSPKEF LTIYEDVKDLKTRRNHEQEQTFPGGGSTIYSMIQSQSS APTSQEPAYTLYSLIQPSRKSGSRKRNHSPSFNSTIYEV IGKSQPKAQNPARLSRKELENFDVYS**	69
11	CD16-(CD16-**2B4**-DAP10)	VSFCLVMVLLFAVDTGLYFSVKTNIRSSTRDWKDHKFK WRKDPQDK**WRRKRKEKQSETSPKEFLTIYEDVKDLK TRRNHEQEQTFPGGGSTIYSMIQSQSSAPTSQEPAYTL YSLIQPSRKSGSRKRNHSPSFNSTIYEVIGKSQPKAQNP ARLSRKELENFDVYS**LCARPRRSPAQEDGKVYINMPGR G	70
12	CD16-(CD16-**DNAM1)**	VSFCLVMVLLFAVDTGLYFSVKTNIRSSTRDWKDHKFK WRKDPQDK**NRRRRRERRDLFTESWDTQKAPNNYRSPI STSQPTNQSMDDTREDIYVNYPTFSRRPKTRV**	71
13	NKp46-(NKp46-**2B4)**	MGLAFLVLVALVWFLVEDWLSRKRTRERASRASTWEGR RRLNTQTL**WRRKRKEKQSETSPKEFLTIYEDVKDLKT RRNHEQEQTFPGGGSTIYSMIQSQSSAPTSQEPAYTLY SLIQPSRKSGSRKRNHSPSFNSTIYEVIGKSQPKAQNPA RLSRKELENFDVYS**	72
14	NKp46-(NKp46-**2B4**-CD3ζ)	MGLAFLVLVALVWFLVEDWLSRKRTRERASRASTWEGR RRLNTQTL**WRRKRKEKQSETSPKEFLTIYEDVKDLKT RRNHEQEQTFPGGGSTIYSMIQSQSSAPTSQEPAYTLY SLIQPSRKSGSRKRNHSPSFNSTIYEVIGKSQPKAQNPA RLSRKELENFDVYS**RVKFSRSADAPAYQQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPR	73
15	NKp46-(NKp46-**CD2**-DAP10)	MGLAFLVLVALVWFLVEDWLSRKRTRERASRASTWEGR RRLNTQTL**KRKKQRSRRNDEELETRAHRVATEERGR KPHQIPASTPQNPATSQHPPPPPGHRSQAPSHRPPPPG HRVQHQPQKRPPAPSGTQVHQQKGPPLPRPRVQPKP PHGAAENSLSPSSN**LCARPRRSPAQEDGKVYINMPGRG	74
16	CD2-(CD2-**CD3ζ)**	IYLIIGICGGGSLLMVFVALLVFYITKRKKQRSRRNDEELE TRAHRVATEERGRKPHQIPASTPQNPATSQHPPPPPGHRS QAPSHRPPPPGHRVQHQPQKRPPAPSGTQVHQQKGPPLP RPRVQPKPPHGAAENSLSPSSN**RVKFSRSADAPAYQQG QNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGH DGLYQGLSTATKDTYDALHMQALPPR**	75
17	2B4-(2B4-**CD3ζ)**	FLVIIVILSALFLGTLACFCVWRRKRKEKQSETSPKEFLTI YEDVKDLKTRRNHEQEQTFPGGGSTIYSMIQSQSSAPTSQ EPAYTLYSLIQPSRKSGSRKRNHSPSFNSTIYEVIGKSQPK AQNPARLSRKELENFDVYS**RVKFSRSADAPAYQQGQN QLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNP QEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGL YQGLSTATKDTYDALHMQALPPR**	76
18	2B4-(2B4-**FcERIγ)**	FLVIIVILSALFLGTLACFCVWRRKRKEKQSETSPKEFLTI YEDVKDLKTRRNHEQEQTFPGGGSTIYSMIQSQSSAPTSQ EPAYTLYSLIQPSRKSGSRKRNHSPSFNSTIYEVIGKSQPK AQNPARLSRKELENFDVYS**RLKIQVRKAAITSYEKSDG VYTGLSTRNQETYETLKHEKPPQ**	77
19	CS1-(CS1-**CD3ζ)**	VLLCLLLVPLLLSLFVLGLFLWFLKRERQEEYIEEKKRVD ICRETPNICPHSGENTEYDTIPHTNRTILKEDPANTVYSTV EIPKKMENPHSLLTMPDTPRLFAYENVI**RVKFSRSADAP**	78

		AYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMG GKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERR RGKGHDGLYQGLSTATKDTYDALHMQALPPR	

[00069] In some embodiments, the ectodomain can further include a signal peptide or leader sequence and/or a spacer/hinge. In some embodiments, there is a spacer/hinge between the antigen recognition region and the transmembrane domain of the CAR, although in some other embodiments such spacer/hinge is not required. Exemplary N-terminal signal peptides include MALPVTALLLPLALLLHA (SEQ ID NO: 79; CD8asp) or MDFQVQIFSFLLISASVIMSR (SEQ ID NO: 80; IgKsp), or any signal peptide sequence or functional variants thereof known in the art. Exemplary spacers that may be included in a CAR are commonly known in the art, including, but not limited to, IgG4 spacers, CD28 spacers, CD8 spacers, or combinations of more than one spacer. The length of the spacers may also vary, from about 15 amino acids (a.a.) to about 300 a.a. or more. In this application, for ease of description, a spacer of less than around 80 a.a., for example 10-80 a.a., is considered short; a spacer of about 80-180 a.a. is considered medium; and a spacer of more than 180 a.a. is considered long. Non-limiting exemplary spacer peptides include those represented by an amino acid sequence of at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to any of SEQ ID NOs: 81-85. In some embodiments, the spacer peptide comprises an amino acid sequence of at least about 90% identity to any of SEQ ID NOs: 81-85. In some embodiments, the spacer peptide comprises an amino acid sequence of at least about 95% identity to any of SEQ ID NOs: 81-85. In some embodiments, the spacer peptide comprises the amino acid sequence of any of SEQ ID NOs: 81-85.

SEQ ID NO: 81
IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKP
(39 a.a.)

SEQ ID NO: 82
ESKYGPPCPPCPGGGSSGGGSGGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFL
(88 a.a.)

SEQ ID NO: 83
ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVE
VHNAKTKPREEQFQSTYRVVSVLT
(89 a.a.)

SEQ ID NO: 84
ESKYGPPCPPCPGGGSSGGGSGGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
(129 a.a.)

SEQ ID NO: 85
ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVE
VHNAKTKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQ
VYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTV
DKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
(229 a.a.)

[00070] In one embodiment, the CAR provided herein comprises a co-stimulatory domain derived from CD28, and a signaling domain comprising the native or modified ITAM1 of CD3ζ, represented by an amino acid sequence having at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 63. In some embodiments, the signaling domain comprises an amino acid sequence with at least about 90% identity to SEQ ID NO: 63. In some embodiments, the signaling domain comprises an amino acid sequence with at least about 95% identity to SEQ ID NO: 63. In some embodiments, the signaling domain comprises the amino acid sequence of SEQ ID NO: 63. In a further embodiment, the CAR comprising a co-stimulatory domain derived from CD28, and a native or modified ITAM1 of CD3ζ also comprises a hinge domain (or "spacer") and trans-membrane domain derived from CD28, wherein an scFv may be connected to the transmembrane domain through the hinge, and the CAR comprises an amino acid sequence of at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 86, wherein the spacer may vary in length and sequence. In some embodiments, the CAR comprises an amino acid sequence of at least 80% to SEQ ID NO: 86, wherein the spacer may vary in length and sequence. In some embodiments, the CAR comprises an amino acid sequence of at least 90% to SEQ ID NO: 86, wherein the spacer may vary in length and sequence. In some embodiments, the CAR comprises an amino acid sequence of at least 95% to SEQ ID NO: 86, wherein the spacer may vary in length and sequence. In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 86.

SEQ ID NO: 86

ESKYGPPCPPCPGGGSSGGGSGGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNG
*QPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK*MFWVL
VVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLFNELQKDK
MAEAFSEIGMKGERRRGKGHDGLFQGLSTATKDTFDALHMQALPPR

(*spacer*-CD28 TM-CD28 Costim-CD3ζ1XX *activation*)

[00071] In another embodiment, the CAR provided herein comprises a transmembrane domain derived from NKG2D, a co-stimulatory domain derived from 2B4, and a signaling domain comprising the native or modified CD3ζ, represented by an amino acid sequence of at

least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 87. In some embodiments, the CAR comprises an amino acid sequence of at least about 90% identity to SEQ ID NO: 87. In some embodiments, the CAR comprises an amino acid sequence of at least about 95% identity to SEQ ID NO: 87. In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 87. Said CAR comprising a transmembrane domain derived from NKG2D, a co-stimulatory domain derived from 2B4, and a signaling domain comprising the native or modified CD3ζ may further comprise a hinge.

SEQ ID NO: 87

SNLFVASWIAVMIIFRIGMAVAIFCCFFFPSWRRKRKEKQSETSPKEFLTIYEDVKDLKTRRNHEQEQTFP
GGGSTIYSMIQSQSSAPTSQEPAYTLYSLIQPSRKSGSRKRNHSPSFNSTIYEVIGKSQPKAQNPARLSRK
ELENFDVYS*RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL*
QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR
(NKG2D TM-2B4-*CD3ζ*)

[00072] In one embodiment, the CAR provided herein comprises an amino acid sequence of at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 88, wherein the linker in the ectodomain and the spacer between the ectodomain and transmembrane domain may vary in length and sequence. In some embodiments, the CAR comprises an amino acid sequence of at least about 90% identity to SEQ ID NO: 88, wherein the linker in the ectodomain and the spacer between the ectodomain and transmembrane domain may vary in length and sequence. In some embodiments, the CAR comprises an amino acid sequence of at least about 95% identity to SEQ ID NO: 88, wherein the linker in the ectodomain and the spacer between the ectodomain and transmembrane domain may vary in length and sequence. In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 88. In some embodiments, the CAR provided herein recognizes a HER2 antigen specific to cells of solid tumors. In some embodiments, the CAR provided herein recognizes a HER2 antigen of a tumor comprising breast cancer, ovary cancer, endometrium cancer, lung cancer, esophageal cancer, salivary gland cancer, bladder cancer, gastric cancer, colorectal cancer, or head and neck cancer. In yet some other embodiments, the CAR provided herein recognizes a HER2 antigen of a tumor and does not respond, or has a low level of response, to HER2 expressed on non-cancer or normal cells.

SEQ ID NO: 88

EVQLVESGGGLVQPGGSLKLSCAASGFTFSNYGMSWVRQTPDRRLELVATINNNGGGTYYPDSVKGRFTIS
RDNAKNTLYLQMSSLKSEDTAMYYCTSPGLLWDAWGAGTTVTVSSGSTSGGGSGGGSGGGGSSDVVMTQTP
LTLSVSIGQPASISCKSSQSLLDSDGRTYLNWLLQRPGQSPKRLIYLVSKLDSGAPDRFTGSGSGTDFTLK

ISRVEAEDLGVYYCWQGTHFPQTFGGGTKLEIK*ESKYGPPCPPCPGGGSSGGGSGGQPREPQVYTLPPSQE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSV MHEALHNHYTQKSLSLSLGK*MFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPT RKHYQPYAPPRDFAAYRS*RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLFNELQKDKMAEAFSEIGMKGERRRGKGHDGLFQGLSTATKDTFDALHMQALPPR*

(**anti-HER2 scFV[linker]**- *spacer*- CD28 TM- CD28 Costim-CD3ζ1XX *activation*)

[00073] Non-limiting CAR strategies further include a heterodimeric, conditionally activated CAR through dimerization of a pair of intracellular domains (see for example, U.S. Pat. No. 9,587,020); a split CAR, where homologous recombination of antigen binding, hinge, and endodomains to generate a CAR (see for example, U.S. Pub. No. 2017/0183407); a multi-chain CAR that allows non-covalent linking between two transmembrane domains connected to an antigen binding domain and a signaling domain, respectively (see for example, U.S. Pub. No. 2014/0134142); CARs having bi-specific antigen binding domains (see for example, U.S. Pat. No. 9,447,194), or having a pair of antigen binding domains recognizing the same or different antigens or epitopes (see for example, U.S. Pat No. 8,409,577), or a tandem CAR (see for example, Hegde et al., *J Clin Invest*. 2016;126(8):3036-3052); an inducible CAR (see for example, U.S. Pub. Nos. 2016/0046700, 2016/0058857, and 2017/0166877); a switchable CAR (see for example, U.S. Pub. No. 2014/0219975); and any other designs known in the art.

[00074] In some embodiments, the polynucleotide encoding a CAR as disclosed is operatively linked to an exogenous promoter. The promoters may be inducible, or constitutive, and may be temporal-, tissue- or cell type- specific. Suitable constitutive promoters for methods disclosed herein include, but are not limited to, cytomegalovirus (CMV), elongation factor 1α (EF1α), phosphoglycerate kinase (PGK), hybrid CMV enhancer/chicken β-actin (CAG) and ubiquitin C (UBC) promoters. In one embodiment, the exogenous promoter is CAG. The CAR construct may be introduced into a cell, such as a primary T cell, for expression using plasmid vectors (e.g., pAl- 11, pXTl, pRc/CMV, pRc/RSV, pcDNAI/Neo) or viral vectors (e.g. adenovirus vectors, adeno-associated virus vectors, retrovirus vectors, lentivirus vectors, or Sendai virus vectors).. Available endonucleases capable of introducing targeted insertion to a cell include, but are not limited to, zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), RNA-guided CRISPR (Clustered Regular Interspaced Short Palindromic Repeats) systems.

EXAMPLES

[00075] The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1 – Design and Functional Profiling of CAR Candidates

[00076] Various chimeric antigen receptor (CAR) constructs containing CDRs of a HER2 cancer-specific monoclonal antibody (CasMab) specific for HER2 were designed, transduced, and expressed in a cell model, including primary T cells, suitable for CAR characterization and function determination. The variables in the CAR designs include, but are not limited to, variable region orientation, linker sequence and/or length, spacer sequence and/or length, compatibility with endodomain signaling and co-stimulatory domains, which all directly or indirectly impact the CAR functionality profile including, but not limited to, expression level, specificity and efficacy, which need to be properly defined and adjusted through CAR design.

[00077] A group of candidate CARs incorporating spacers of differing length within the ectodomain (e.g., short spacer of about 10 to about 80 amino acids; a medium spacer of more than 80 to about 180 amino acids; or a long spacer of more than 180 amino acids) and/or VH and VL in different orientations were constructed and expressed for cancer antigen specific response analysis. By comparing the cytolytic activity of the distinct CAR constructs targeting the same cancer specific antigen, the following assays were performed to determine which constructs, and more specifically which configurations of ectodomain (i.e., spacer length) and endodomain components confer signaling domain responses specific to binding of the CAR to a HER2 antigen expressed on a cancer cell.

[00078] 4D5-CAR with a HER2 binding domain derived from a known HER2 antibody, Herceptin, with short spacers (for example, a CD28 hinge that is less than 80 a.a.) and CD28-CD3ζ1XX intracellular signaling domains was used as control for comparison with the candidate HER2-CARs based on novel CasMab antibodies. CasMab214-CAR (abbreviated as 214-CAR or 214 in the figures) with the same short spacer and intracellular signaling domain was designed and constructed based on the CasMab214 antibody VH domain (SEQ ID NO: 89--QVTLKESGPGILQPSQTLSLTCSFSGFSLSTSGMGVSWIRQPSGKGLEWLAHIFWDDDKRYNPSLKSRLTISKDTSRNKVFLKITSVDTADTATYYCARRVVATDWYFDVWGAGTTVTVSS) and VL domain (SEQ ID NO: 90--DIVLTQSPASLAVSLGQRATISCRASESVEYYGTTLMQWYQQKPGQPPKLLIYAASKVESGVPARFSGSGSGTDFSLNIHPVEEDDVAMYFCQQSRKVPLTFGAGTKLEL); whereas a CasMab250-CAR (abbreviated as 250-CAR or 250 in the figures) of the same configuration was designed based on CasMab250 antibody CDRs and VH/VL as disclosed in this application.

[00079] The cytolytic efficacy and specificity of the above CARs was evaluated via xCELLigence™ real time cell analysis. 24 hours after 10^4 HER2 expressing target cells (SKOV3, SKBR3, MCF10a, or Met5a) were plated, the CAR expressing effector cells were added at a 1:1 effector:target (E:T) ratio for tumor cells SKOV3 and SKBR3 with high HER2 expressing level ($HER2^{High}$) (FIG. 1A), or at a 5:1 E:T ratio for normal/non-tumorigenic HER2 expressing cells MCF10a or Met5a ($HER2^{+}$) (FIG. 1B). The levels of HER2 expression in the target cells were determined by immunohistochemistry (IHC) staining. The higher the IHC score the higher the expression of the HER2 cell surface antigen. An IHC score of 3+ is labled as $HER2^{High}$; an IHC score of 2+ is labled as $HER2^{+}$, representing a medium HER2 expressor; while an IHC score of 1+ is labled as $HER2^{Low}$, which represents a low HER2 expressor. Cell indices were monitored, normalized to effector addition, and percent cytolysis was calculated with RTCA Software Pro™.

[00080] As shown in FIG. 1A, 4D5-, CasMab214-, and CasMab250- based CAR expressing effector cells all recognized and eliminated the $HER2^{High}$ ovarian (SKOV3) and breast (SKBR3) tumor cell line cells, reaching ~100% percent cytolysis within 48 hours of adding the CAR expressing effector cells. Across all primary donors evaluated, the anti-tumor efficacy through the CasMab214- and CasMab250- based CARs was at least as rapid as, or in most cases preceded the targeting and elimination of tumor lines by up to 12 hours than, donor matched 4D5-based CAR, highlighting the robust anti-tumor potential of CasMab214- and CasMab250- CARs.

[00081] For the non-tumorigenic/normal $HER2^{+}$ cell lines MCF10a (breast) and Met5a (mesothelium), 4D5-CAR expressing donor T cells demonstrated robust cytolytic efficacy, indiscrimitively and rapidly eliminating 100% of normal breast cell line cells (MCF10a) and about 30-50% of normal mesothelium cell line cells (Met5a), indicating a lack of cancer cell targeting specificity of 4D5-CAR based on the HER2 antibody, Herceptin. As such, the 4D5-based HER2-CAR presented an "on-target off-tumor" problem, resulting from a direct attack on normal tissues that have shared expression of the targeted antigen. CasMab214- and CasMab250- based CARs, on the other hand, displayed no cytolytic efficacy against the normal breast MCF10a line, even at an E:T ratio of 5:1, while having efficacy against the HER2 expressing tumorous cell line cells. While a limited level of donor-dependent recognition was observed with normal mesothelial Met5a cells by CasMab214- and CasMab250- based CARs, their cytolytic efficacy was significantly reduced when reacting to non-tumorigenic normal cells compared to that of 4D5-based CAR. Also noted is that although anti-tumor efficacy was largely similar between the cancer cell specific CasMab HER2 antibody based 214- and 250- CARs, CasMab250-CAR demonstrated the least recognition of normal mesothelial Met5a cell lines. Overall, these data demonstrate that CasMab-derived CARs possess robust anti-tumor HER2-

targeted efficacy with limited recognition of HER2 on normal/non-tumorigenic cell lines, as compared to the 4D5-CAR based on the widely known and used HER2 antibody, Herceptin.

[00082] Cytokine production in the donor T cells transduced with 4D5-, CasMab214-, or CasMab250- based CARs having a short spacer in response to $HER2^{High}$ tumor cells (SKOV3 and SKBR3; FIG. 2A), and $HER2^{+}$ normal/non-tumorigenic cells (MCF10a, Met5a and keratinocyte HaCat cell lines; FIG. 2B) were then evaluated. About 24 hours after $5x10^4$ target cells (SKOV3, SKBR3, MCF10A, HaCat, or Met5a) were plated, the CAR transduced cells were added at a 1:1 E:T ratio. After about 48 hours of co-culturing, supernatants were collected for IFNγ production assessment. For all donors evaluated, the relative production of IFNγ by cells expressing 4D5-CAR targeting either the tumor or non-tumorigenic HER2 expressing cells was substantially greater than that of CasMab214- or CasMab250- based CARs (FIGs. 2A and 2B). Most notably, 4D5-CAR transduced cells also produced about 300 pg/ml to about 4500 pg/ml of IFNγ in response to normal cell co-culture, demonstrating significant reactivity to HER2 expressed on normal cells. CasMab214- and CasMab250- based CAR-T cells, on the other hand, produced substantially less IFNγ across all donors and evaluated normal cell lines, showing much less "on-target off-tumor" effect. CAR-T cells expressing CasMab250-based CAR, in particular, produced no detectable IFNγ (lower than the lower limit of detection of about 5 pg/ml) when exposed to normal HER2-expressing cells, further underscoring the highly specific tumor antigen selectivity of CasMab250-based CAR.

[00083] 4D5-, CasMab214-, or CasMab250- based CARs with long spacers (for example, IgG4 hinge & CH2/3 domains having more than 180 a.a.) were constructed and assessed for cytolytic efficacy and tumor cell specificity using the same methods described above. When against the $HER2^{High}$ ovarian (SKOV3) and breast (SKBR3) tumor cell lines, similar observations were made with the HER2-CARs having long spacers as compared to the CARs having a short spacer. As shown in FIG. 3A, all evaluated long spacer CARs reached maximal efficacy between 72 and 96 hours, with the CasMab214- and CasMab250- based CARs conveying cytolytic efficacy faster than 4D5-CAR both in initiation and in maximization.

[00084] When against the non-tumorigenic/normal $HER2^{+}$ cell lines MCF10a (breast) and Met5a (mesothelium), the 4D5-CAR with a long spacer demonstrated robust cytolytic efficacy, clearing up to 100% of the normal breast MCF10a and up to 75% of the normal mesothelial Met5a cell lines. For 214- and 250- CARs with a long spacer, although differentials in efficacy and selectivity were observed across donors compared to 4D5, the lengthening of the spacer to a long spacer restored substantial levels of reactivity to HER2 expressing normal/non-tumor cells, see FIG. 3B as compared to FIG. 1B. It is noted, however, even with increased reactivity to normal cells, CasMab250-based CAR still conveys the least amount of cytolytic efficacy across

the tested donors. Further, the fact that 4D5-, CasMab214-, and CasMab250- based CARs conveyed no differentials in selectivity on Met5a demonstrate: (i) spacer length is important for CasMab-CAR selectivity of tumor rather than normal $HER2^{+}$ cells; and (ii) CasMab250-based CAR-T cells demonstrate the least amount of normal cell reactivity across spacer configurations.

[00085] The assay for inflammatory cytokine IFNγ production conveyed by the long spacer CARs in response to HER2-expressing tumor (FIG. 4A) and normal/non-tumorigenic cell lines (FIG. 4B) further confirmed the notion above in terms of the differential effect of spacer length of the HER2-CAR candidates; and more particularly the differential targeting specificity toward non-tumor cells reflected at the level of a CAR, but not at the level of antibody, considering that CasMab214 and CasMab250 were verified antibodies specific for tumor HER2 antigens which were obtained using the same screening method specifically designed for tumor antigens.

[00086] Overall, these data demonstrate that (i) CasMab250-based CAR has a tumor selective advantage over similarly configured 4D5- and CasMab214- based CARs; and (ii) spacer length is important for functional properties of CasMab HER2 antibody based CARs, where shorter spacers result in substantially more tumor selectivity than long spacers.

EXAMPLE 2 – Efficacy and Specificity of CasMab250-CAR with Medium Spacers

[00087] Since the CasMab250-based CAR has a tumor selective advantage over similarly configured 4D5- and CasMab214- based CARs, this experiment focuses on a comparison of the 250-CAR having a short (less than 80 a.a) or a medium spacer (80-180 a.a.) for any differential functional properties.

[00088] 4D5- or CasMab250- based CARs with short or medium spacers were evaluated for cytolytic efficacy and tumor specificity using the same methods described herein. As in previous Examples, 10^4 target cells (SKOV3, SKBR3, BT474 Clone 5, OE19, MCF10a, or Met5a) were plated and allowed to adhere. Approximately 24 hours later, the CAR expressing primary T cells were added at a 1:1 E:T ratio.

[00089] In line with the previous observations, as shown in FIG. 5A, 4D5- and CasMab250-based CARs with short (panel i.) or medium (panel ii.) spacers induced the targeted killing of $HER2^{High}$ SKOV3, SKBR3, BT474 clone 5 (breast), and OE19 (esophageal) tumor cell line cells, reaching maximal cytolytic efficacy within 48 to 96 hours. Moreover, CasMab250-CARs generally induce faster targeted killing than their equivalently configured 4D5-based CAR. Additionally, CasMab250-CARs with medium spacers outperformed those with short spacers across all evaluated $HER2^{High}$ tumor cell lines (FIG. 5A).

[00090] As shown in FIG. 5B, 4D5-based CARs with either short or medium spacers indiscriminately induced high cytotoxicity towards non-tumor/normal HER2 expressing cells.

CasMab250-based CARs with either the short or medium spacer configuration, on the other hand, induced no or minimal reactivity to these normal HER2 expressing cells, demonstrating highly selective efficacy against HER2 expressing tumor cells.

[00091] In a separate experiment, the tumor specific efficacy of CasMab250-CAR having a medium spacer and an H/L variable region orientation was further evaluated with additional $HER2^{Low/+}$ normal cell lines (HUVEC, Beas2b, Thle2, SV-Huc-1, and HTR-8/SVneo). A similarly constructed 4D5-CAR was included as control. Target cells (including a HER2 tumor cell line, SKOV3, as a control) were plated. Approximately 24 hours later, the CAR transduced primary T cells were added at a 1:1 E:T ratio. As shown in FIG. 6, CasMab250-CAR (H/L; medium spacer) induced low to no detectable cytoxicity (about 5-40% maximal cytolytic efficacy) across the evaluated normal HER2 expressing cell line cells. These data demonstrate that the CasMab250-based CAR is highly selective for tumor-expressed HER2, rather than non-tumor HER2 (thus presenting the least "on-target off-tumor" problem), and this selectivity extends to a diverse number of HER2 expressing normal/non-tumorigenic cell lines from multiple sources.

EXAMPLE 3—CAR Intracellular Signaling Domains and CasMab250-CAR Cell Activation

[00092] To show CAR cell activation, NFκB reporter Jurkat cells were transduced to express either 4D5- or CasMab250 (H/L)- based CARs with medium spacers (IgG4 hinge & CH3 domain), and CD28-CD3ζ1XX intracellular signaling domains, as described above. Jurkats stably expressing either 4D5- or CasMab250 (H/L)- CARs were co-cultured overnight with $HER2^{High}$ tumor (SKOV3, SKBR3) or $HER2^{+}$ normal/non-tumorigenic (MCF10a) cell lines. The expressions of GFP (a readout of NFκB and therefore CAR activation) and CD69 (a general activation marker) were assessed via flow cytometry. PMA/Ionomycin and parallel unstimulated Jurkat cell lines served as positive and negative controls, respectively.

[00093] As shown in FIG. 7, PMA/Ionomycin (PMA/I) treatment resulted in nearly 100% $GFP^{+}/CD69^{+}$ co-expressing cells. Similarly, no CAR-specific cell activation was detected in unstimulated Jurkat reporter lines expressing 4D5- or CasMab250 (H/L)- based CAR (<~1% $GFP^{+}/CD69^{+}$). Both 4D5- and CasMab250 (H/L)- CAR expressing Jurkat cells demonstrated specific cell activation following co-culture with SKOV3 or SKBR3 tumor lines ($GFP^{+}/CD69^{+}$ about 48-52% for SKOV3 and about 32-38% for SKBR3). Importantly, co-culture of CasMab250 (H/L)-CAR expressing Jurkats with $HER2^{+}$ normal/non-tumorigenic MCF10a cells induced minimal cell activation (about 5% $GFP^{+}/CD69^{+}$), and co-culture of 4D5-CAR expressing Jurkats with MCF10a cells resulted in robust cell activation (about 37% $GFP^{+}/CD69^{+}$), which

was similar to the cell activation observed under the $HER2^{High}$ SKBR3 tumor cell line. These data further demonstrate that CARs constructed from CasMab250 (H/L) with medium spacers are highly selective and efficacious for tumor-expressed HER2.

[00094] In a separate experiment, multiple intracellular signaling domain configurations (from wildtype CD28 or CD28 YMFM mutant, in combination with CD3ζ1XX or CD3ζXX3) were evaluated alongside the optimized extracellular CAR domain of CasMab250 (H/L) ScFv and spacer. CD3ζXX3 is another mutant form of CD3ζ, with two substitutions in ITAM1 (sequence not dislosed). 10^4 target cells (SKOV3) were plated and allowed to adhere. Approximately 24 hours later, the CAR expressing donor T cells were added at a 1:1 E:T ratio. Cell indices were monitored, normalized to effector addition, and percent cytolysis was calculated with RTCA Software Pro™. As shown in FIG. 8, although all CARs having different intracellular signaling domain configurations exhibited rapid and strong tumor killing, CasMab250-CAR with the CD28-CD3ζ1XX intracellular signaling domain outperformed all other counterparts with ~90% maximal cytolysis, and had better persistence/duration in conveying the cytotoxicity. These data demonstrate that regardless of intracellular signaling domain used, CARs constructed from CasMab250 (H/L) with medium spacers are highly selective and efficacious for tumor-expressed HER2.

[00095] One skilled in the art would readily appreciate that the methods, compositions, and products described herein are representative of exemplary embodiments, and not intended as limitations on the scope of the invention. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the present disclosure disclosed herein without departing from the scope and spirit of the invention.

[00096] All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the present disclosure pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated as incorporated by reference.

[00097] The present disclosure illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising," "consisting essentially of," and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the present disclosure

claimed. Thus, it should be understood that although the present disclosure has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A chimeric antigen receptor (CAR) comprising:
   (a) an ectodomain comprising an antigen binding domain recognizing a HER2 (human epidermal growth factor receptor 2) antigen, wherein the antigen binding domain comprises:
      (i) a heavy chain variable (VH) domain comprising a heavy chain complementary determining region 1 (H-CDR1) comprising SEQ ID NO: 1 (NYGMS), a heavy chain complementary determining region 2 (H-CDR2) comprising SEQ ID NO: 2 (TINNNGGGTYYPDSVKG), and a heavy chain complementary determining region 3 (H-CDR3) comprising SEQ ID NO: 3 (PGLLWDA); and
      (ii) a light chain variable (VL) domain comprising a light chain complementary determining region 1 (L-CDR1) comprising SEQ ID NO: 4 (KSSQSLLDSDGRTYLN), a light chain complementary determining region 2 (L-CDR2) comprising SEQ ID NO: 5 (LVSKLDS), and a light chain complementary determining region 3 (L-CDR3) comprising SEQ ID NO: 6 (WQGTHFPQT);
   (b) a transmembrane domain; and
   (c) an endodomain comprising at least one signaling domain;
   wherein the at least one signaling domain responds specifically to binding of the CAR to a HER2 antigen expressed on a cancer cell, thereby generating a cancer antigen specific response.

2. The CAR of claim 1, wherein the antigen binding domain:
   (a) comprises a VH domain with at least 80% sequence identity to SEQ ID NO: 7;
   (b) comprises a VL domain with at least 80% sequence identity to SEQ ID NO: 8;
   (c) comprises a single chain variable fragment (scFV) comprising VH-linker-VL or VL-linker-VH, wherein the linker varies in length and sequence, and optionally wherein the linker has at least 80% sequence identity to SEQ ID NOs: 9-12;
   (d) comprises an scFV represented by an amino acid sequence that is of at least about 99%, about 98%, about 96%, about 95%, about 90%, about 85%, or about 80%

identity to SEQ ID NO: 13 or SEQ ID NO: 14, wherein each of SEQ ID NOs: 13 and 14 comprises a linker that varies in length and sequence; and/or

(e) is humanized.

3. The CAR of claim 1, wherein the at least one signaling domain comprises:

(a) any one of: 2B4 (Natural killer Cell Receptor 2B4), 4-1BB (Tumor necrosis factor receptor superfamily member 9), CD16 (IgG Fc region Receptor III-A), CD2 (T-cell surface antigen CD2), CD28 (T-cell-specific surface glycoprotein CD28), CD28H (Transmembrane and immunoglobulin domain-containing protein 2), CD3ζ (T-cell surface glycoprotein CD3 zeta chain), DAP10 (Hematopoietic cell signal transducer), DAP12 (TYRO protein tyrosine kinase-binding protein), DNAM1 (CD226 antigen), FcERIγ (High affinity immunoglobulin epsilon receptor subunit gamma), IL21R (Interleukin-21 receptor), IL-2Rβ/IL-15RB (Interleukin-2 receptor subunit beta), IL-2Rγ (Cytokine receptor common subunit gamma), IL-7R (Interleukin-7 receptor subunit alpha), KIR2DS2 (Killer cell immunoglobulin-like receptor 2DS2), NKG2D (NKG2-D type II integral membrane protein), NKp30 (Natural cytotoxicity triggering receptor 3), NKp44 (Natural cytotoxicity triggering receptor 2), NKp46 (Natural cytotoxicity triggering receptor 1), CS1(SLAM family member 7), and CD8 (T-cell surface glycoprotein CD8 alpha chain);

(b) an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to the cytoplasmic domain, or a portion thereof, of 2B4, 4-1BB, CD16, CD2, CD28, CD28H, CD3ζ, CD3ζ1XX, DAP10, DAP12, DNAM1, FcERIγ, IL21R, IL2Rβ (IL15Rβ), IL2Rγ, IL7R, KIR2DS2, NKG2D, NKp30, NKp44, NKp46, CS1, or CD8, represented by SEQ ID NOs: 37-59, respectively; and/or

(c) an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to the cytoplasmic domain, or a portion thereof, of 2B4, CD28, CD3ζ, DAP10, NKG2D, CD3ζ1XX, DNAM1, CS1, or combinations thereof.

4. The CAR of claim 3, wherein the endodomain comprises two different signaling domains, and wherein said endodomain domain comprises fused cytoplasmic domains, or portions thereof, in any one of the forms: 2B4-CD3ζ/1XX, 2B4-DNAM1, 2B4-FcERIγ, 2B4-DAP10, CD16-DNAM1, CD16-DAP10, CD16-DAP12, CD2-CD3ζ/1XX, CD2-DNAM1, CD2-FcERIγ, CD2-DAP10, CD28-DNAM1, CD28-FcERIγ, CD28-DAP10, CD28-DAP12, CD28-CD3ζ/1XX, CD28H-CD3ζ/1XX, DAP10-CD3ζ/1XX, DAP10-DAP12, DAP12-CD3ζ/1XX, DAP12-DAP10, DNAM1-CD3ζ/1XX, KIR2DS2-CD3ζ/1XX, KIR2DS2-DAP10, KIR2DS2-2B4, or NKp46-2B4.

5. The CAR of claim 1, wherein the transmembrane domain comprises an amino acid sequence that has at least about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to a transmembrane region, or a portion thereof, of:

(a) CD2, CD3δ, CD3ε, CD3γ, CD3ζ, CD4, CD8, CD8a, CD8b, CD16, CD27, CD28, CD28H, CD40, CD84, CD166, 4-1BB, OX40, ICOS, ICAM-1, CTLA4, PD1, LAG3, 2B4, BTLA, DNAM1, DAP10, DAP12, FcERIγ, IL7, IL12, IL15, KIR2DL4, KIR2DS1, KIR2DS2, NKp30, NKp44, NKp46, NKG2C, NKG2D, CS1, or T cell receptor polypeptide;

(b) 2B4, CD2, CD16, CD28, CD28H, CD3ζ, DAP10, DAP12, DNAM1, FcERIγ, KIR2DS2, NKG2D, NKp30, NKp44, NKp46, CS1, or CD8; or

(c) 2B4, CD28, CD28H, DAP10, DNAM1, KIR2DS2, and NKG2D.

6. The CAR of claim 1, wherein the transmembrane domain and its immediately linked signaling domain are from a same protein or from different proteins.

7. The CAR of claim 1, wherein the ectodomain comprises one or more of:

(a) a signal peptide; and/or

(b) a spacer/hinge.

8. The CAR of claim 7, wherein the spacer/hinge comprises

(a) an IgG4 spacer, a CD28 spacers, a CD8 spacer, a CH3 spacer, a CH2/CH3 spacer, or any combination thereof;

(b) a short spacer of about 10 to about 80 amino acids; a medium spacer of more than 80 to about 180 amino acids; or a long spacer of more than 180 amino acids; and/or

(c) an amino acid sequence of at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to any of SEQ ID NOs: 81-85.

9. The CAR of claim 8, wherein the spacer/hinge comprises a medium spacer, wherein the spacer comprises an amino acid sequence of at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 84.

10. The CAR of claim 1, wherein the CAR comprises an amino acid sequence of at least about 90%, about 95%, about 96%, about 97%, about 98%, or about 99% identity to SEQ ID NO: 88.

11. The CAR of claim 1, wherein the cancer cell is a breast cancer cell, an ovary cancer cell, an endometrium cancer cell, a lung cancer cell, an esophageal cancer cell, a salivary gland cancer cell, a bladder cancer cell, a gastric cancer cell, a colorectal cancer cell, or a head and neck cancer cell.

12. The CAR of claim 1, wherein the at least one signaling domain does not respond, or has a low level of response, to HER2 expressed on non-cancer cells.

13. The CAR of claim 1, wherein the cancer antigen specific responses comprise cytolysis and cytokine production.

14. The CAR of any one of claims 1-13, wherein the CAR comprises the VH domain followed by the VL domain in an amino to carboxy direction.

15. A polynucleotide comprising a nucleic acid sequence which encodes a CAR according to any one of the claims 1-14.

16. A vector comprising the polynucleotide according to claim 15.

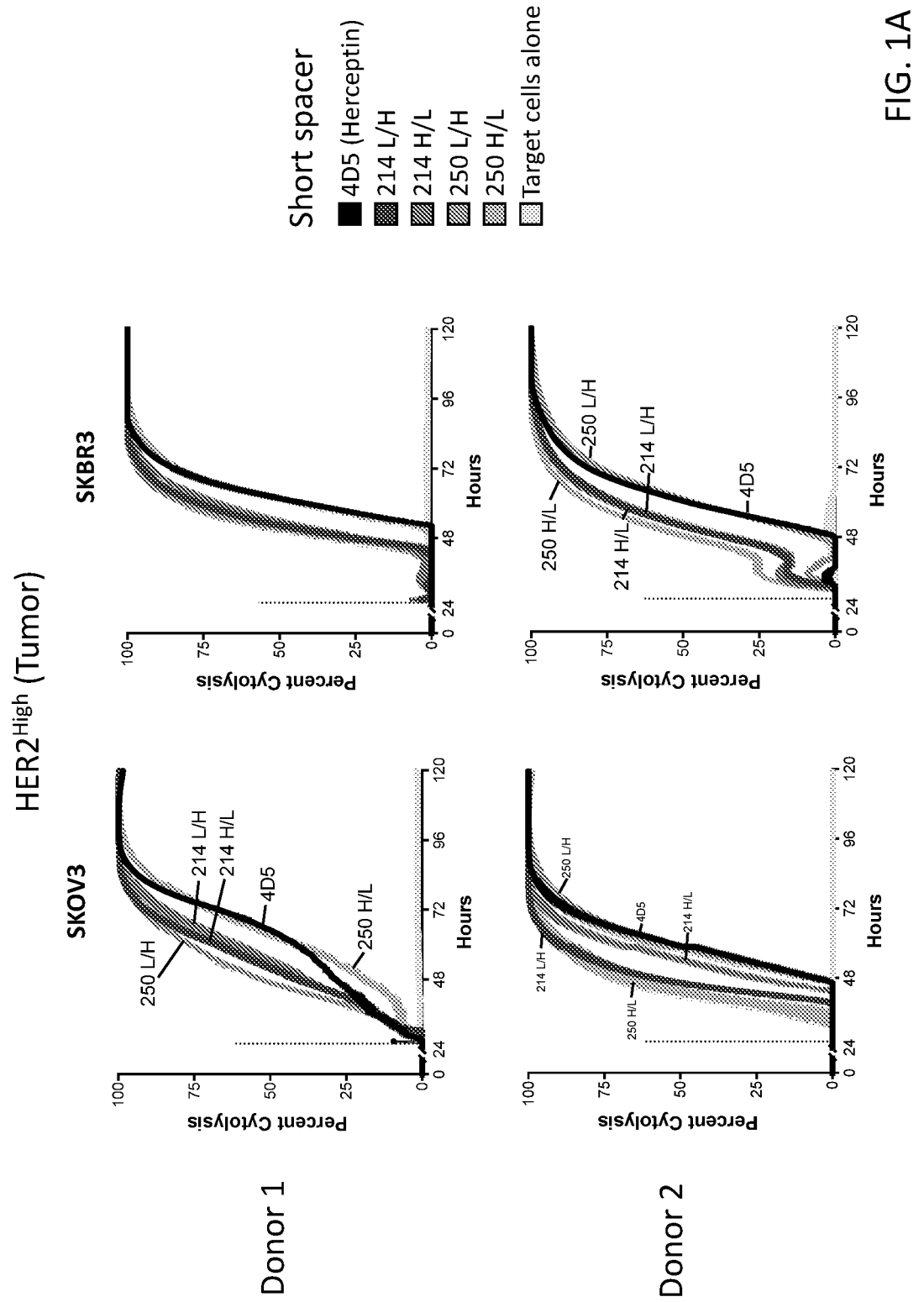

HER2High (Tumor)
SKOV3
SKBR3
Donor 1
Donor 2
Percent Cytolysis
Hours
Short spacer
4D5 (Herceptin)
214 L/H
214 H/L
250 L/H
250 H/L
Target cells alone

FIG. 1A

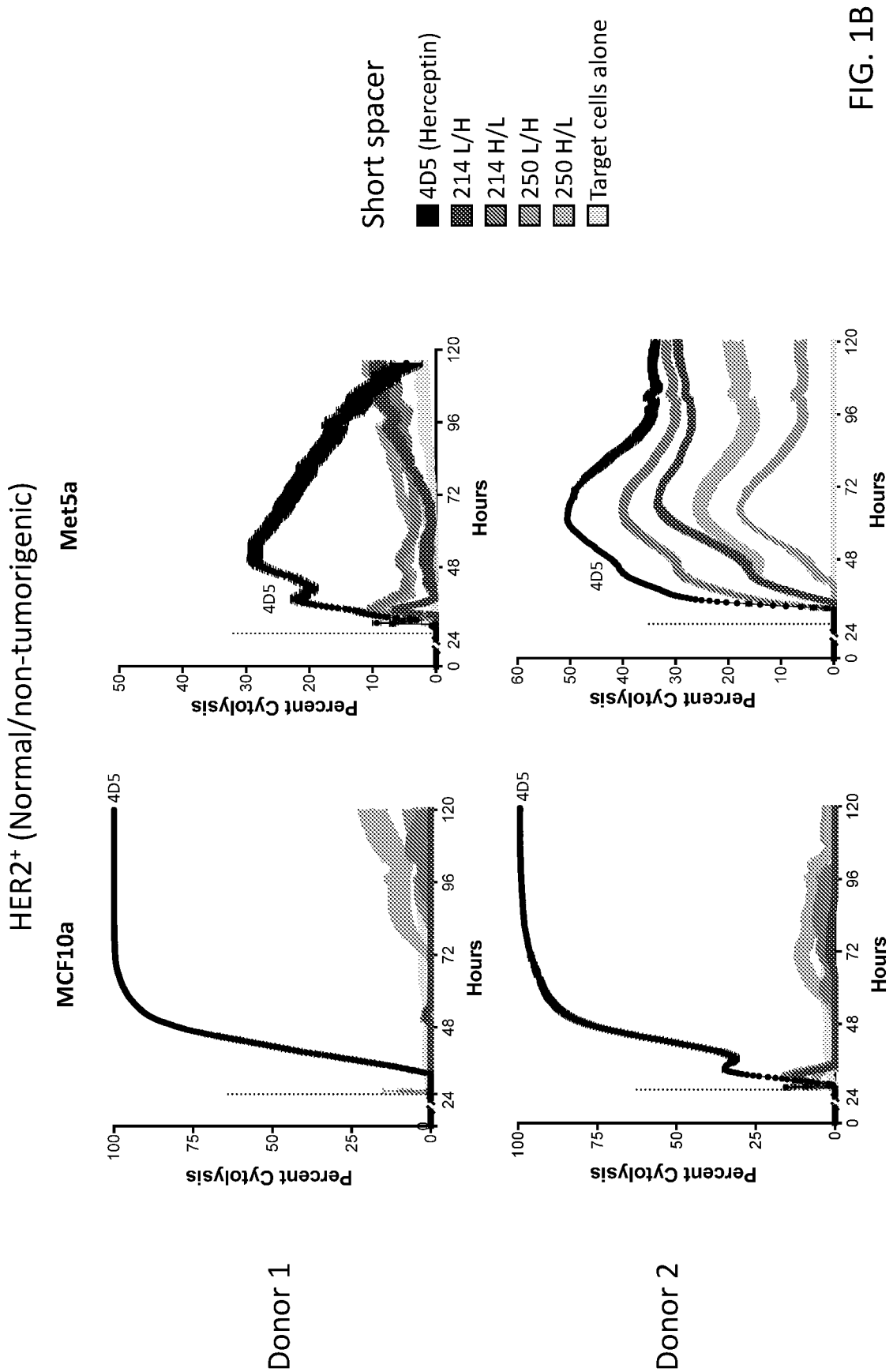

HER2+ (Normal/non-tumorigenic)
MCF10a
Met5a
Donor 1
Donor 2
Percent Cytolysis
Hours
4D5
Short spacer
4D5 (Herceptin)
214 L/H
214 H/L
250 L/H
250 H/L
Target cells alone

FIG. 1B

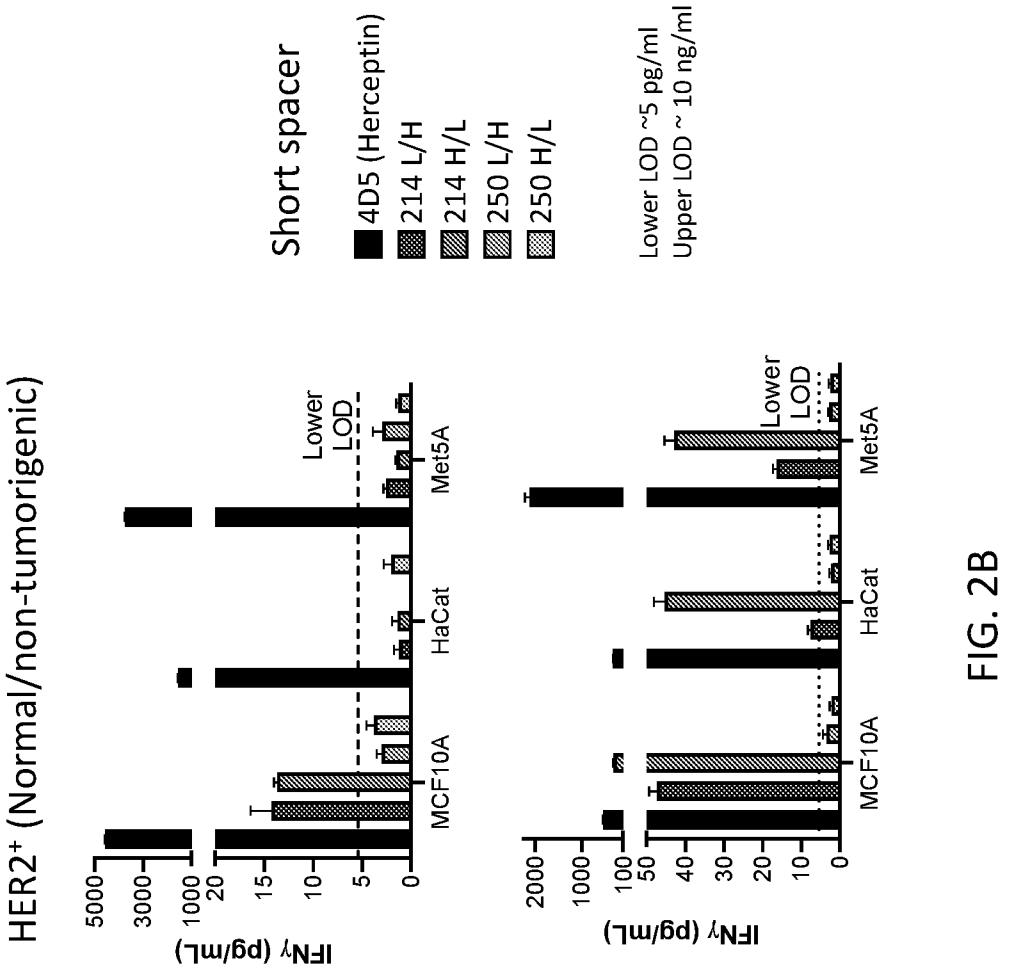

HER2+ (Normal/non-tumorigenic)
Short spacer
4D5 (Herceptin)
214 L/H
214 H/L
250 L/H
250 H/L
Lower LOD ~5 pg/ml
Upper LOD ~ 10 ng/ml
IFNγ (pg/mL)
Lower LOD
MCF10A
HaCat
Met5A
HER2High (Tumor)
Upper LOD
SKOV
SKBR
Donor 1
Donor 2

FIG. 2A

FIG. 2B

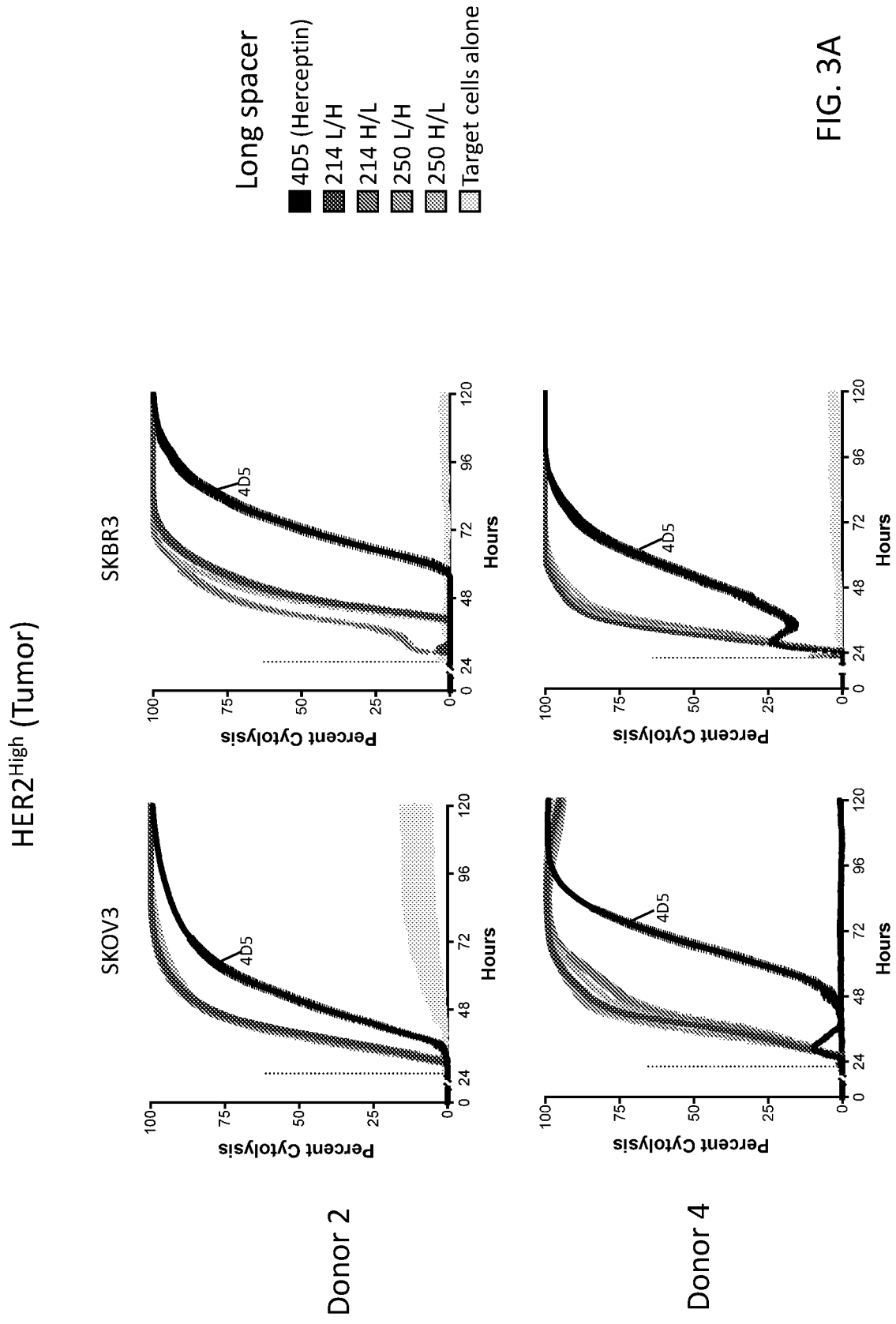

HER2High (Tumor)
SKOV3
SKBR3
Donor 2
Donor 4
Percent Cytolysis
Hours
4D5
Long spacer
4D5 (Herceptin)
214 L/H
214 H/L
250 L/H
250 H/L
Target cells alone

FIG. 3A

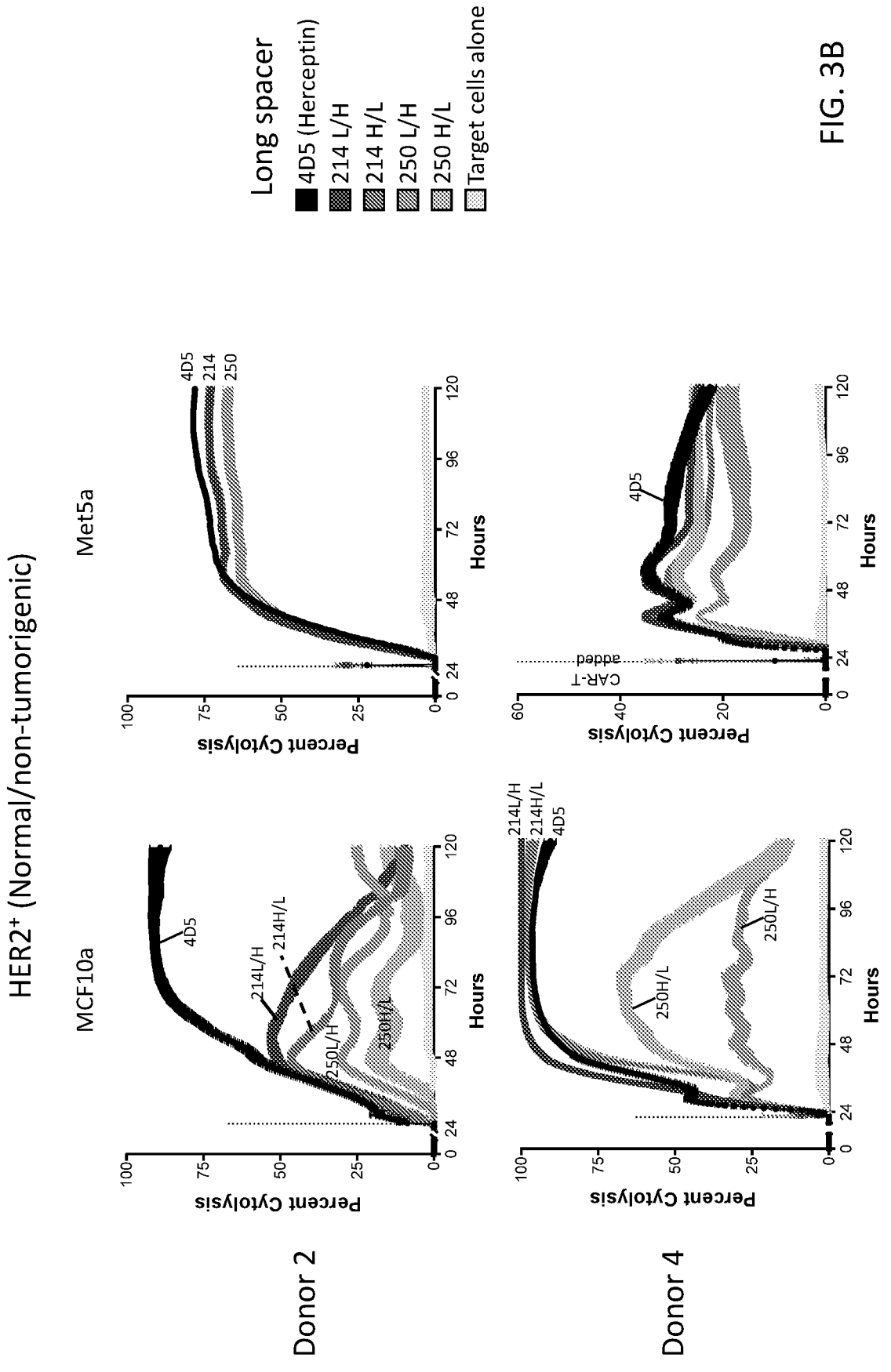
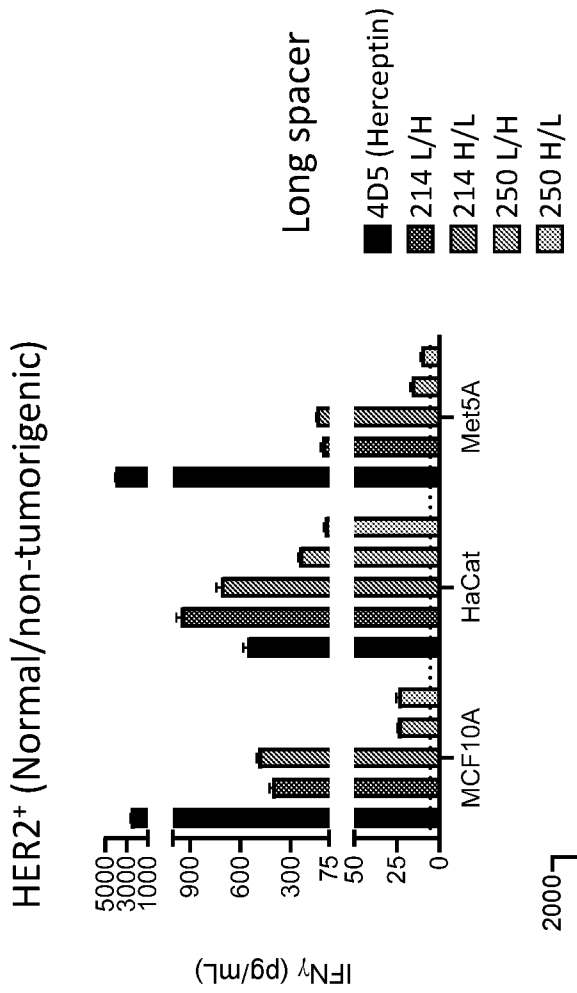

HER2+ (Normal/non-tumorigenic)
MCF10a
Met5a
Donor 2
Donor 4
Percent Cytolysis
Hours
4D5
214L/H
214H/L
250L/H
250H/L
214
250
CAR-T added
Long spacer
4D5 (Herceptin)
214 L/H
214 H/L
250 L/H
250 H/L
Target cells alone

FIG. 3B


Long spacer
4D5 (Herceptin)
214 L/H
214 H/L
250 L/H
250 H/L
HER2+ (Normal/non-tumorigenic)
IFNγ (pg/mL)
5000
3000
1000
900
600
300
75
50
25
0
MCF10A
HaCat
Met5A

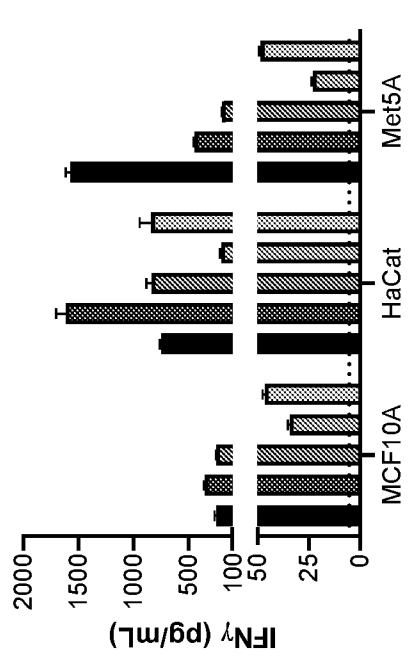
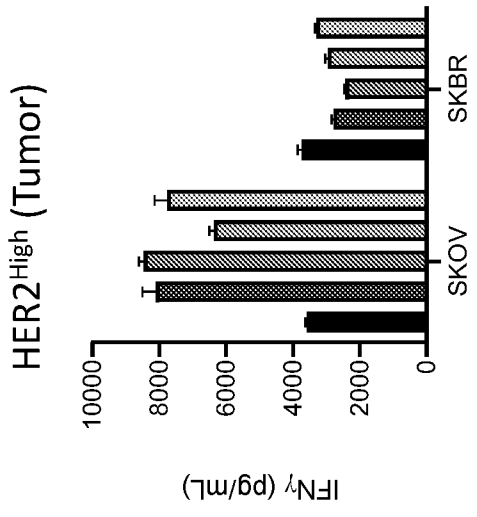

IFNγ (pg/mL)
2000
1500
1000
500
100
50
25
0
MCF10A
HaCat
Met5A

FIG. 4B


Donor 2
HER2High (Tumor)
IFNγ (pg/mL)
10000
8000
6000
4000
2000
0
SKOV
SKBR

Donor 4
IFNγ (pg/mL)
40000
30000
20000
10000
0
Upper LOD
SKOV
SKBR

FIG. 4A

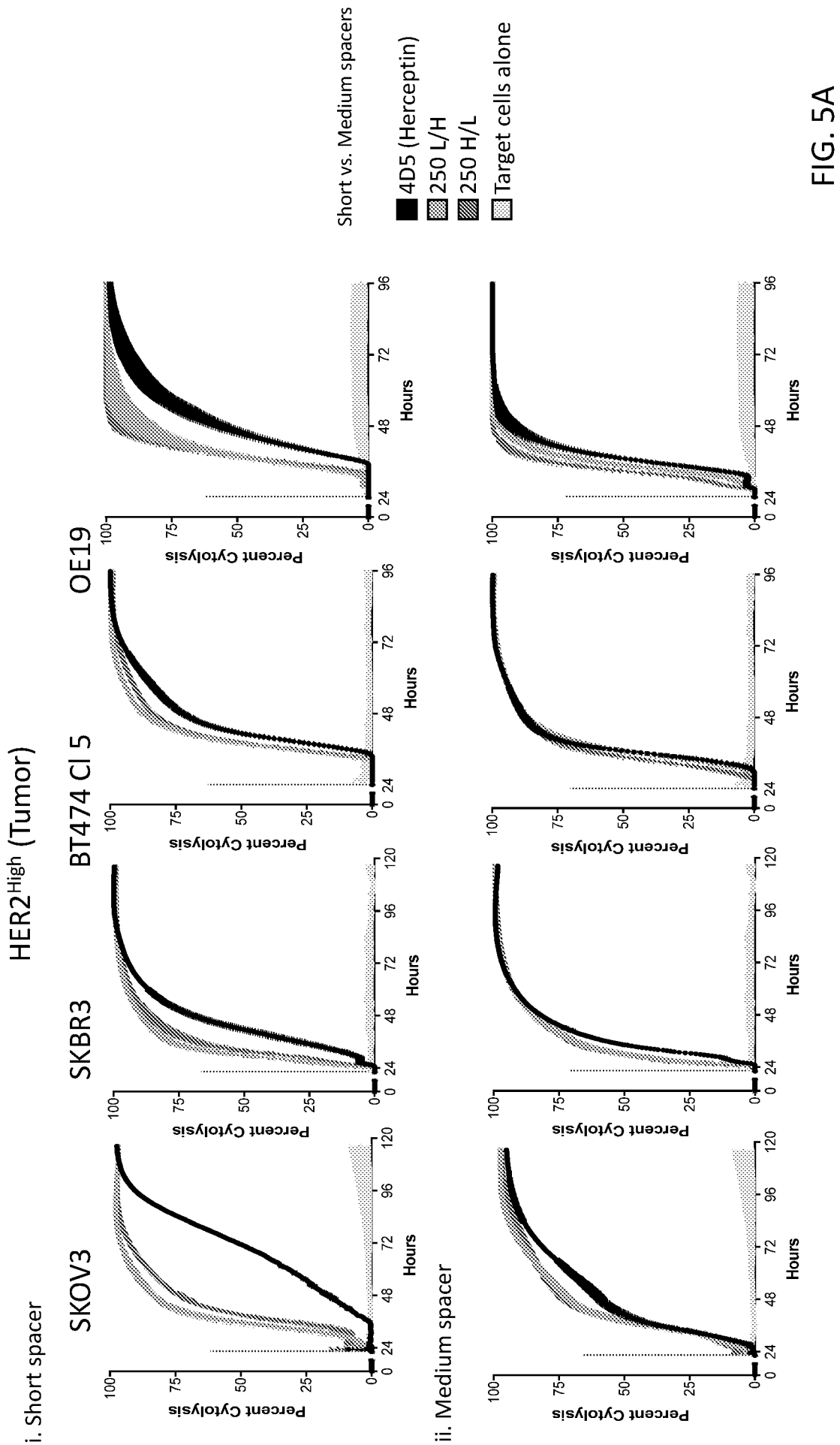

HER2High (Tumor)
i. Short spacer
ii. Medium spacer
SKOV3
SKBR3
BT474 Cl 5
OE19
Percent Cytolysis
Hours
Short vs. Medium spacers
4D5 (Herceptin)
250 L/H
250 H/L
Target cells alone

FIG. 5A

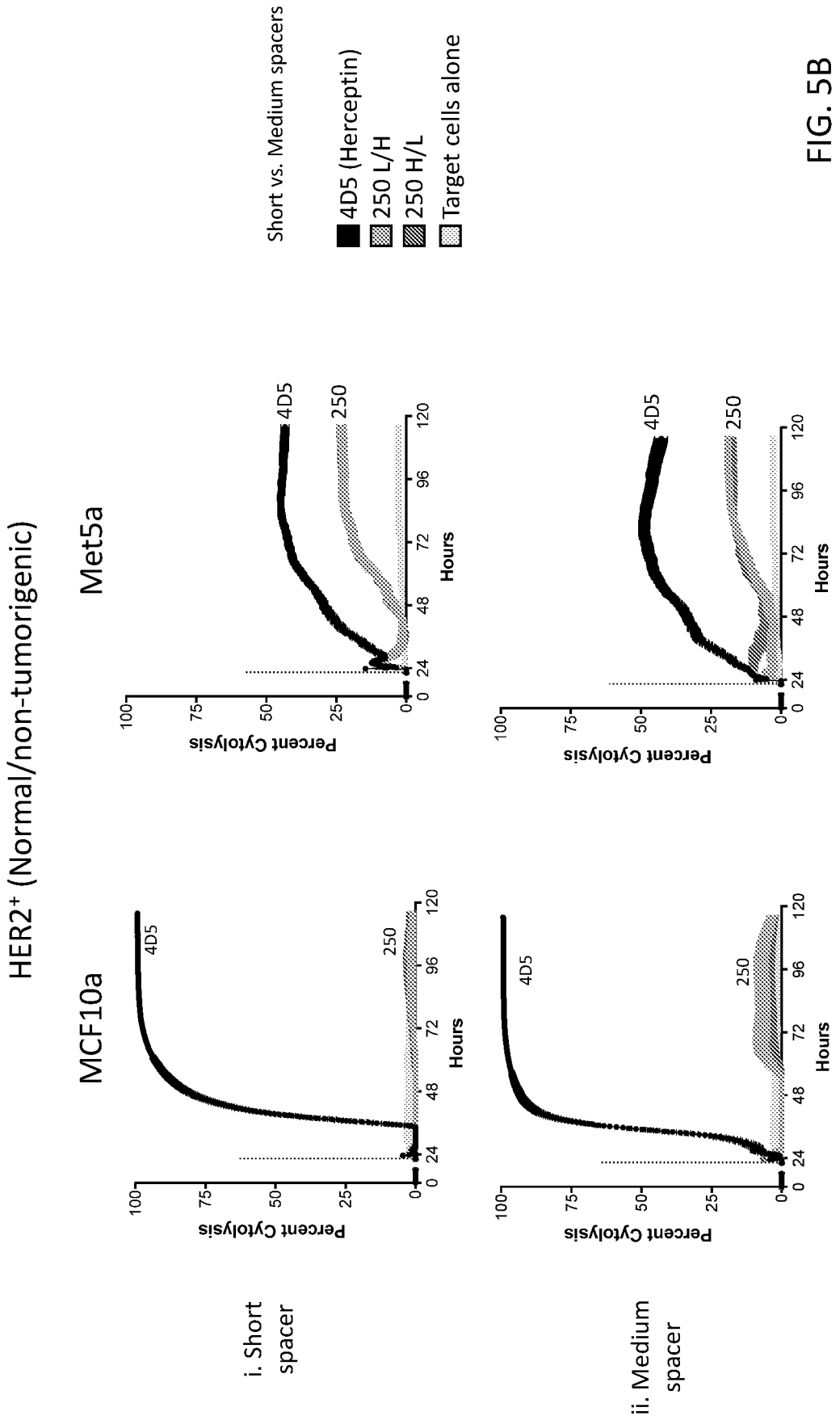

HER2+ (Normal/non-tumorigenic)
MCF10a
Met5a
Short vs. Medium spacers
4D5 (Herceptin)
250 L/H
250 H/L
Target cells alone
Percent Cytolysis
Hours
4D5
250
i. Short spacer
ii. Medium spacer

FIG. 5B

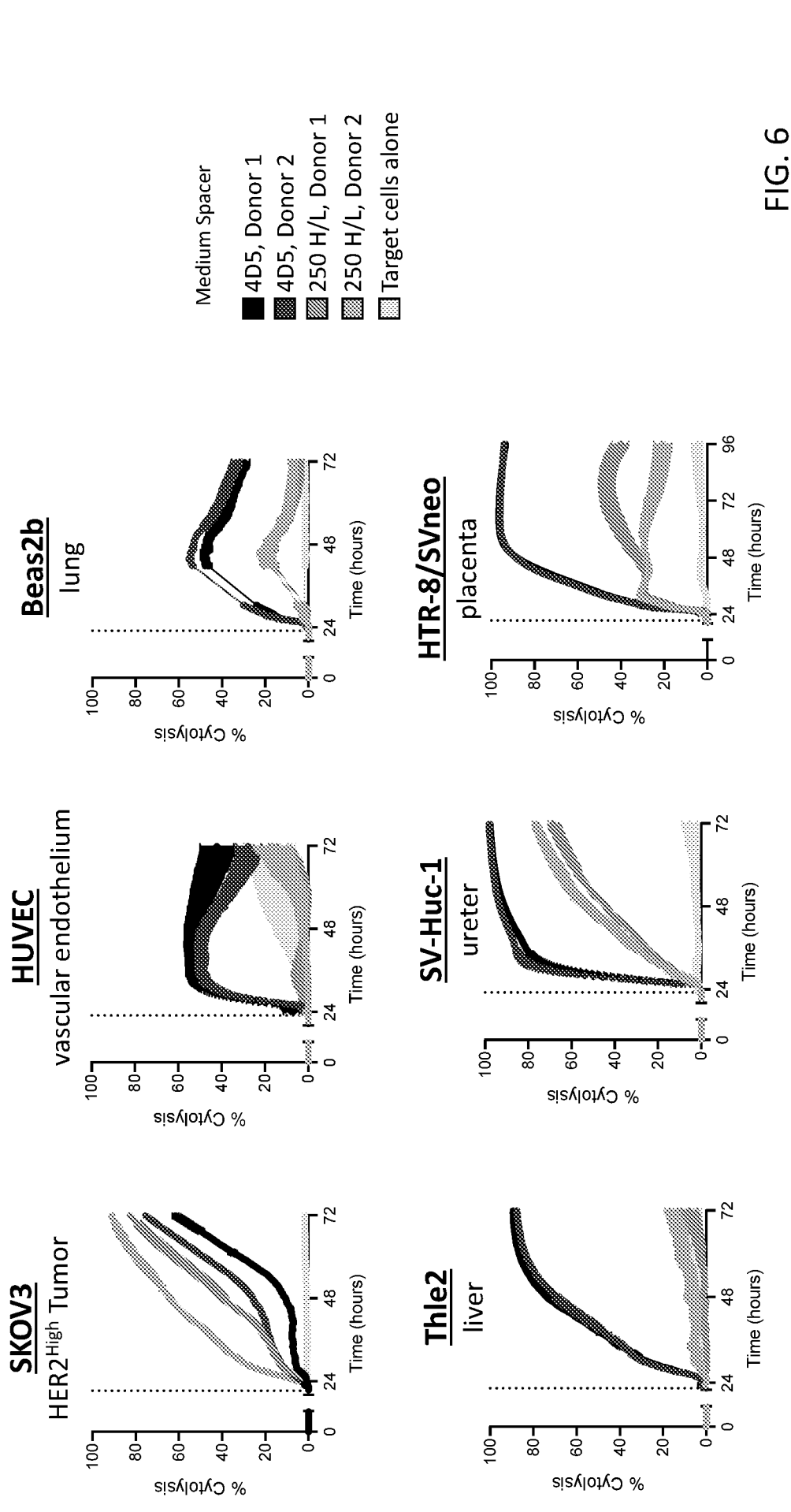

Medium Spacer
4D5, Donor 1
4D5, Donor 2
250 H/L, Donor 1
250 H/L, Donor 2
Target cells alone
Beas2b
lung
HUVEC
vascular endothelium
HTR-8/SVneo
placenta
SKOV3
HER2High Tumor
SV-Huc-1
ureter
Thle2
liver
% Cytolysis
Time (hours)

FIG. 6

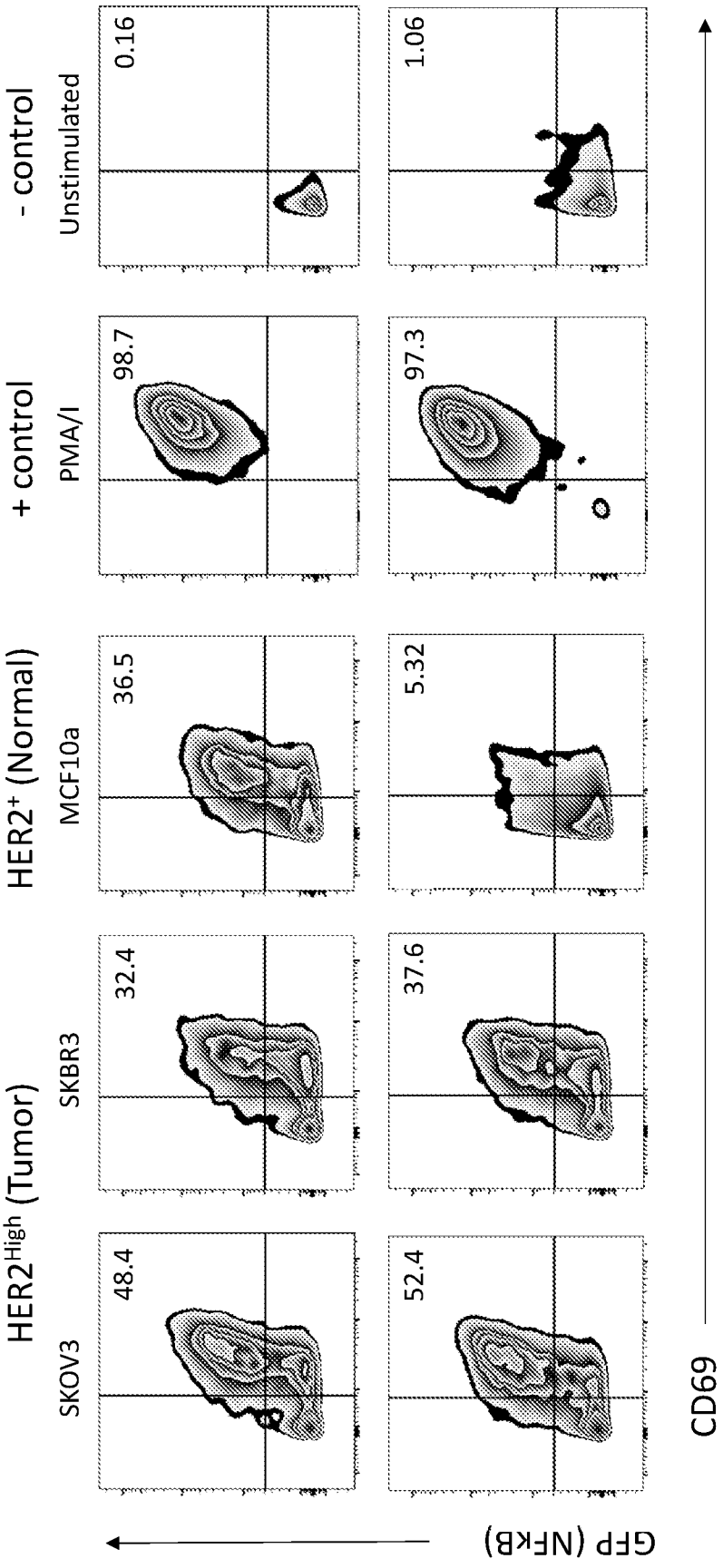

HER2High (Tumor)
HER2+ (Normal)
SKOV3
SKBR3
MCF10a
+ control
PMA/I
- control
Unstimulated
48.4
32.4
36.5
98.7
0.16
52.4
37.6
5.32
97.3
1.06
4D5
Medium
Spacer
CasMab250 H/L
Medium
Spacer
GFP (NFκB)
CD69

FIG. 7

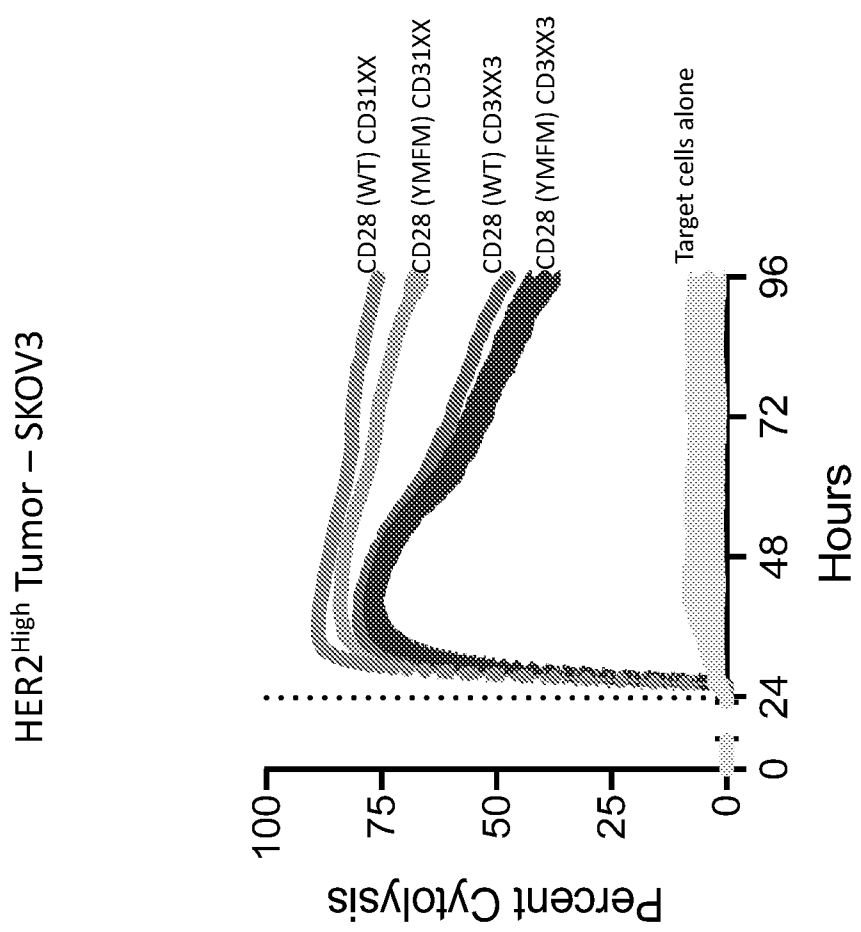

HER2High Tumor – SKOV3
Percent Cytolysis
100
75
50
25
0
0
24
48
72
96
Hours
CD28 (WT) CD31XX
CD28 (YMFM) CD31XX
CD28 (WT) CD3XX3
CD28 (YMFM) CD3XX3
Target cells alone

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2023/065537

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2023/065537

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. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☑ Claims Nos.: **16**
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:

As claim 16 refers to an unsearchable claim which does not comply with PCT Rule 6.4(a), this claim is unclear (PCT Article 6).

3. ☑ Claims Nos.: **15**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2023/065537

A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/32(2006.01)i; **C07K 14/725**(2006.01)i; **C07K 14/705**(2006.01)i; **A61K 39/00**(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)

C07K 16/32(2006.01); A61K 39/395(2006.01); A61P 35/00(2006.01); C07K 14/725(2006.01); C07K 16/28(2006.01); C07K 16/30(2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: chimeric antigen receptor (CAR), HER2 antigen, heavy chain variable (VH) domain, light chain variable (VL) domain, transmembrane domain, signaling domain

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2020-0399397 A1 (GREEN CROSS LAB CELL CORPORATION) 24 December 2020 (2020-12-24) claim 1; and paragraphs [0012], [0018], [0019], [0021]	1-14
A	US 2020-0102366 A1 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 02 April 2020 (2020-04-02) paragraphs [0012], [0108]	1-14
A	US 2021-0139605 A1 (AGENCY FOR SCIENCE, TECHNOLOGY AND RESEARCH) 13 May 2021 (2021-05-13) claim 1	1-14
A	WO 2020-191434 A1 (OLIVIA NEWTON-JOHN CANCER RESEARCH INSTITUTE) 01 October 2020 (2020-10-01) the whole document	1-14
A	US 2022-0089750 A1 (NOVARTIS AG et al.) 24 March 2022 (2022-03-24) the whole document	1-14

☐ 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
"D" document cited by the applicant in the international application
"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
07 August 2023	**07 August 2023**

Name and mailing address of the ISA/KR	Authorized officer
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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2023/065537

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		EP 3752536 A1	23 December 2020
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WO 2020-191434 A1	01 October 2020	AU 2020-247828 A1	18 November 2021
		CA 3134363 A1	01 October 2020
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		US 2022-0169747 A1	02 June 2022
US 2022-0089750 A1	24 March 2022	US 11161907 B2	02 November 2021
		US 2018-044424 A1	15 February 2018
		WO 2016-126608 A1	11 August 2016