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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
09 November 2017 (09.11.2017)

(10) International Publication Number
WO 2017/192536 A1

(51) International Patent Classification:
A61K 39/39 (2006.01) *C07K 16/30* (2006.01)
A61P 31/00 (2006.01) *C07K 19/00* (2006.01)
C07K 14/705 (2006.01)

(21) International Application Number:
PCT/US2017/030567

(22) International Filing Date:
02 May 2017 (02.05.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/330,499 02 May 2016 (02.05.2016) US

(71) Applicant: UNIVERSITY OF KANSAS [US/US]; 1450 Jayhawk Boulevard, 245 Strong Hall, Lawrence, KS 66045 (US).

(72) Inventors: YANKEE, Thomas; 12804 W 121st Street, Overland Park, KS 66213 (US). SZAREJKO, John; 1012 Canterbury Lane, Liberty, MO 64068 (US).

(74) Agent: GIBSON, Matthew, S.; Reed Smith LLP, 811 Main Street, 17th Floor, Houston, TX 77002 (US).

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

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

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2017/192536 A1

(54) Title: ELIMINATING MHC RESTRICTION FROM THE T CELL RECEPTOR AS A STRATEGY FOR IMMUNOTHERAPY

(57) Abstract: The present disclosure generally relates to chimeric antigen receptors, more specifically to chimeric antigen receptor compositions and methods for use of the same. The present disclosure also provides for nucleic acid molecules and expression vectors for making and using the chimeric antigen receptors and for co-receptor signaling using such chimeric antigen receptors. The present disclosure also provides methods of treatment using such compositions. The chimeric antigen receptors of the present disclosure interact with the endogenous T-cell receptor complex enabling physiological control of signaling and T-cell response and can be combined with ligands such as co-stimulatory ligands for further controlling and influencing T-cell activation and response.

ELIMINATING MHC RESTRICTION FROM THE T CELL RECEPTOR AS A STRATEGY FOR IMMUNOTHERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Provisional Application No. 62/330,499, filed on May 2, 2016 which is herein incorporated by reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been filed electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 2, 2017, is named 17-21007-WO_SL.txt and is 66,321 bytes in size.

FIELD OF THE INVENTION

[0003] The present disclosure generally relates to chimeric antigen receptors, more specifically to chimeric antigen receptor compositions and methods for use of the same. The present disclosure also provides for nucleic acid molecules and expression vectors for making and using the chimeric antigen receptors and for co-receptor signaling using such chimeric antigen receptors. The present disclosure also provides methods of treatment using such compositions.

BACKGROUND OF THE INVENTION

[0004] Chimeric antigen receptors (CARs) are used to redirect patients' T cells towards tumor cells to destroy malignant cells. Despite impressive response rates for B cell malignancies, clinical outcomes have been disappointing for the treatment of most cancers and CAR therapy is highly toxic, commonly inducing cytokine release syndrome and on-target/off-tumor effects. Some clinical limitations of CARs may be attributed to the unregulated surface expression and functionality of CARs, which lack key regulatory components inherent to the endogenous T cell receptor (TCR) complex.

[0005] Previously developed CAR designs are generally monomeric proteins consisting of a single chain antibody fragment (scFv) fused to linker, transmembrane and signaling domains of receptors capable of activating T cells. The signaling component in first generation CARs

(gen1-CAR) was commonly TCR ζ chain while second generation CARs added the signaling domain from a co-receptor, such as 4-1BB. Third generation CARs combined multiple co-receptors into a single construct. However, these constructs do not permit regulation of downstream signaling pathways. By contrast, the endogenous T cell receptor (TCR) is a large multimeric complex whose expression and function is tightly regulated, which restricts the kinetics and intensity of signaling. This regulation enables TCR signaling to drive distinct biological outcomes depending on antigen expression and affinity.

[0006] The TCR complex consists of at least eight proteins and ten immunoreceptor tyrosine-based activation motifs (ITAMs), which is in direct contrast to monomeric ζ chain-conatining CARs that only contain three ITAMs. Multiple signaling proteins bind endogenous CD3 chains, a feature that cannot be replicated using monomeric CARs. Although some ζ chain-based CARs can associate with the TCR and activate ZAP-70, MAPK, and NF-AT, the signalizing pathways are dysregulated and CAR-expressing cells are highly active.

[0007] In view of the foregoing, an unmet need exists for a novel platform for CAR therapy that is less toxic and more broadly applicable than other CARs and which can utilize the endogenous regulation of the TCR complex to improve sensitivity, limit toxicity, incidence of cytokine release syndrome and off-tumor effects.

SUMMARY

[0008] The present disclosure generally relates to chimeric antigen receptors, more specifically to chimeric antigen receptor compositions and methods for use of the same. The present disclosure also provides for nucleic acid molecules and expression vectors for making and using the chimeric antigen receptors and for co-receptor signaling using ligands fused to antigen-specific peptides. The present disclosure also provides methods of treatment using such compositions.

[0009] In an embodiment, a construct includes a 4-1BB ligand (4-1BBL) fused to a peptide that is capable of specifically binding a tumor-specific antigen.

[0010] In some embodiments, a nucleic acid molecule encodes the construct including 4-1BBL fused to a peptide that is capable of specifically binding a tumor-specific antigen.

[0011] In some embodiments, a modified T lymphocyte expresses a construct including 4-1BBL fused to a peptide that is capable of specifically binding a tumor-specific antigen and a chimeric antigen receptor. In some embodiments, the chimeric antigen receptor includes a T-cell receptor beta chain constant region and a T-cell receptor alpha chain constant region wherein the T-cell receptor beta chain constant region is fused to a peptide having an affinity to an antigen, and wherein the T-cell receptor beta chain constant region is complexed with the T-cell receptor alpha chain constant region. In some embodiments, the chimeric antigen receptor does not include a T-cell receptor beta chain variable region and a T-cell alpha chain variable region. In some embodiments, a modified T lymphocyte expresses a construct including a chimeric antigen receptor includes a T-cell receptor beta chain constant region and a T-cell receptor alpha chain constant region wherein the T-cell receptor beta chain constant region is fused to a peptide having an affinity to an antigen, and wherein the T-cell receptor beta chain constant region is complexed with the T-cell receptor alpha chain constant region.

[0012] In some embodiments, a recombinant expression vector is provided which includes a nucleic acid molecule encoding the ligand fused to a peptide that is capable of specifically binding a tumor-specific antigen and/or a chimeric antigen receptor including a T-cell receptor beta chain constant region and a T-cell receptor alpha chain constant region wherein the T-cell receptor beta chain constant region is fused to a peptide having an affinity to an antigen, and wherein the T-cell receptor beta chain constant region is complexed with the T-cell receptor alpha chain constant region. In some embodiments, the chimeric antigen receptor does not include a T-cell receptor beta chain variable region and a T-cell alpha chain variable region.

[0013] In some embodiments, a method is provided for treating cancer in a subject a modified T-lymphocyte of the present disclosure.

DESCRIPTION OF THE DRAWINGS AND FIGURES

[0014] The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings.

[0015] Figure 1A is a schematic representation of the cDNA constructs.

[0016] Figure 1B shows a T-cell receptor-based chimeric antigen receptor of the present disclosure complexed with the endogenous T-cell receptor subunits, a gen1-CAR and a gen2-CAR with a 4-1BB signaling domain.

[0017] Figure 2A shows a graph illustrating HEK 293T cells transfected with the indicated CAR only (top panel) or co-transfected with CD3δ, CD3ε, CD3γ, and CD3ζ (bottom panel).

[0018] Figure 2B depicts a graph illustrating the effect of transducing Primary T cells with the indicated CAR on the surface expression of FLAG and CD19.

[0019] Figure 3 depicts graphs illustrating the effect of transducing Jurkat T cells with the indicated construct and stimulating them as shown, with the percentages (mean ± S.E.M.) of CD19+ cells expressing CD69 or phosphorylated ERK ($*p < 0.05$, $****p < 0.0001$, $n = 2\text{-}3$).

[0020] Figure 4 depicts graphs illustrating the effect of transducing Primary T cells with the indicated constructs and stimulating them with the agents shown. Cells were gated on $CD8^{+}CD19^{+}$ singlets and the percentages (mean ± S.E.M.) of cells expressing CD25, CD69, and CD137 are shown ($**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, $n = 6\text{-}8$).

[0021] Figure 5 depicts graphs illustrating the effects of transducing Primary T cells with the indicated construct and culturing them with no stimulation, anti-CD3, or the indicated cell line. Cells were gated on CD8+CD19+ singlets and the percentages (mean ± S.E.M.) of cells expressing surface LAMP-1 or intracellular IFNγ and TNF shown ($*p < 0.05$, ***p M 0.001, $****p < 0.0001$, $n = 5\text{-}8$).

[0022] Figure 6 depicts graphs illustrating the concentrations of each cytokine (mean ± S.E.M.) produced after 24 hours by transducing Primary T cells with the indicated construct and culturing them with no stimulation, anti-CD3, or the indicated cell line ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, $n = 5\text{-}8$).

[0023] Figure 7A shows a graph illustrating the effect of transducing Primary T cells with each construct and incubated with M21 and after four hours, the number of remaining cells were calculated and normalized according to cytolysis by ΔCD19 cells ($n = 5$).

[0024] Figure 7B shows a graph illustrating the effect of transducing Primary T cells with each construct and incubated with HeLa and after four hours, the number of remaining cells were calculated and normalized according to cytolysis by ΔCD19 cells ($n = 5$).

[0025] Figure 7C shows a graph illustrating the effect of incubating purified, transduced $CD8^{+}$ T cells with M21 cells for 4 hours, with specific lysis calculated ($n = 3$).

[0026] Figure 7D shows a graph illustrating the effect of incubating purified, transduced $CD8^+$ T cells with M21 cells for 8 hours, with specific lysis calculated (n = 3).

[0027] Figure 7E shows a graph illustrating the effect of incubating purified, transduced $CD8^+$ T cells with M21 cells for 24 hours, with specific lysis calculated (n = 3).

[0028] Figure 7F shows a graph illustrating the effect of incubating purified, transduced $CD8^+$ T cells with HeLa cells for 4 hours, with specific lysis calculated (n = 3).

[0029] Figure 8A shows graphs illustrating the effects of transducing Primary T cells with anti-CD19 TCRβα-CAR or ΔCD34 and incubating them under the indicated stimuli. Shown are the percentages (mean ± S.E.M.) of cells expressing CD25, CD69, and CD137 (n = 4).

[0030] Figure 8B shows graphs illustrating the effects of transducing Primary T cells with anti-CD19 TCRβα-CAR or ΔCD34 and incubating them under the indicated stimuli. Shown are the percentages (mean ± S.E.M.) of cells expressing surface LAMP-1 or intracellular IFNγ and TNF (n = 4).

[0031] Figure 8C shows a graph depicting the ability of anti-CD19 TCRβα-CAR T cells to lyse Ramos or A375 cells (n = 4).

[0032] Figure 9A shows graphs depicting data and a summary (mean ± S.E.M.) of replicates for CD69 expression.

[0033] Figure 9B shows graphs depicting data and a summary (mean ± S.E.M.) of replicates for phosphor-ERK expression (***$p < 0.0001$, n=2-3).

[0034] Figure 10A shows graphs depicting GD2 expression on M21, A375, and HeLa cells, with each cell line labelled with anti-GD2 (shaded histograms) or an isotype control (dotted histograms).

[0035] Figure 10B shows a graph depicting the data from Figure 10A presented as an overlay to show the differences in expression between M21, A375, and HeLa.

[0036] Figure 11 depicts Hematoxylin and eosin staining of tumor cells in a mice lung after mice were tolerized against M21 cells in utero and reimplanted with M21 cells two weeks post-weaning.

DETAILED DESCRIPTION OF THE INVENTION

[0037] The present disclosure describes particular embodiments and with reference to certain drawings, but the subject matter is not limited thereto. The drawings described are only

schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated or distorted and not drawn to scale for illustrative purposes. Where the elements of the disclosure are designated as “a” or “an” in first appearance and designated as “the” or “said” for second or subject appearances unless something else is specifically stated.

[0038] The present disclosure will provide description to the accompanying drawings, in which some, but not all embodiments of the subject matter of the disclosure are shown. Indeed, the subject matter may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein, rather, these embodiments are provided so that this disclosure satisfies all the legal requirements.

[0039] Certain terminology is used in the following description for convenience only and is not limiting. Certain words used herein designate directions in the drawings to which reference is made. Unless specifically set forth herein, the terms “a,” “an” and “the” are not limited to one element, but instead should be read as meaning “at least one.” As used herein “another” means at least a second or more. The terminology includes the words noted above, derivatives thereof and words of similar import.

[0040] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive.

[0041] Use of the term “about”, when used with a numerical value, is intended to include +/- 10%. For example, if a number of amino acids is identified as about 200, this would include 180 to 220 (plus or minus 10%).

[0042] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

[0043] All patents and publications cited herein are incorporated by reference in their entirety, including any references cited therein.

[0044] While various embodiments and aspects of the present invention are shown and described herein, it will be obvious to those skilled in the art that such embodiments and aspects are provided by way of example only. Numerous variations, changes, and substitutions will now

occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention.

[0045] “Nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, and complements thereof. The term “polynucleotide” refers to a linear sequence of nucleotides. The term “nucleotide” typically refers to a single unit of a polynucleotide, i.e., a monomer. Nucleotides can be ribonucleotides, deoxyribonucleotides, or modified versions thereof. Examples of polynucleotides contemplated herein include single and double stranded DNA, single and double stranded RNA (including siRNA), and hybrid molecules having mixtures of single and double stranded DNA and RNA. Nucleic acid as used herein also refers to nucleic acids that have the same basic chemical structure as a naturally occurring nucleic acid. Such analogues have modified sugars and/or modified ring substituents, but retain the same basic chemical structure as the naturally occurring nucleic acid. A nucleic acid mimetic refers to chemical compounds that have a structure that is different the general chemical structure of a nucleic acid, but that functions in a manner similar to a naturally occurring nucleic acid. Examples of such analogues include, without limitation, phosphorothiolates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs).

[0046] The term “antigen” as used herein is defined as a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene.

[0047] “Single chain variable fragment” or “scFv” antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. In some embodiments, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains, which enables the sFv to form the desired structure for antigen binding.

[0048] The term "promoter" as used herein is defined as a DNA sequence recognized by the transcriptional machinery of the cell, or introduced transcriptional machinery, required to initiate the specific transcription of a polynucleotide sequence.

[0049] The present disclosure generally relates to chimeric antigen receptors, more specifically to chimeric antigen receptor compositions and methods for use of the same. The present disclosure also provides for nucleic acid molecules and expression vectors for making and using the chimeric antigen receptors and for co-receptor signaling using such chimeric antigen receptors. The present disclosure also provides methods of treatment using such compositions.

[0050] The present disclosure is based on the discovery that a T-cell receptor-based chimeric antigen receptor (TCAR) can exhibit improved properties over currently developed CARs. Specifically, such TCARs can reduce T-cell exhaustion, cytokine release syndrome (CRS), which is caused by the release of vast quantities of cytokines and may result in high fever, tachycardia, cardiac dysfunction, and end-organ failure, and off-tumor effects which are challenges with conventional chimeric antigen receptors. The incidence of CRS correlates with disease burden at the time of CAR infusion but does not correlate with clinical outcome.

[0051] The clinical limitations of previously developed CARs may be caused by several factors. First, such CARs are constitutively active and lack key regulatory components inherent to the endogenous T cell receptor complex. This unregulated surface expression and functionality can result in T-cell exhaustion and overstimulation of the T-cell response. This can result in increased cytokine production which can cause CRS. Further, because antigens are often expressed at high levels on tumor cells and low levels on healthy cells, previously developed chimeric antigen receptors cannot distinguish between cells with varying levels of antigen.

[0052] By combining the antigen recognition capacity of CARs with the functionality of the T-cell receptor complex these drawbacks of previously developed CARs can be avoided and/or reduced. The T-cell receptor-based CARs of the present disclosure demonstrate surface expression that is limited by the endogenous CD3 proteins which results in more regulated surface expression. Further, such TCARs demonstrate a higher threshold of activation which suggests that off-tumor effects can be limited and also induce cytokine production at a more physiological level than previously developed CARs, which can reduce the incidence of CRS. In

addition, the TCARs of the present disclosure can selectively lyse antigen-expressing tumor cells while sparing antigen-negative tumor cells. Thus, the TCARs of the present disclosure can be less toxic than previously developed CARs. By allowing physiological regulatory mechanisms to control the kinetics and duration of downstream signals, optimal transcription of immunologically critical genes, such as IL-2 and IL-8, can be assured.

[0053] Further, in some aspects, the use of co-receptor ligands with the T CARs of the present disclosure can allow for inducible activation of co-receptor signals which can enable tighter control of the receptors to allow for physiological regulatory mechanisms to control the kinetics and intensity of downstream signals to ensure optimal transcription of immunological critical genes. This can provide more efficient tumor destruction than constitutively active signaling and can reduce T-cell exhaustion, cytokine release syndrome (CRS) and off-tumor effects. The present disclosure combines the antigen recognition capacity of CARs with the functionality of the TCR complex. By way of example but not limitation, a modified T cell expressing a TCAR of the present disclosure can be activated upon binding to a target antigen which can stimulate expression of the 4-1BB receptor. When a secreted co-receptor ligand fused to a peptide for a tumor-specific antigen on the surface of the same cell is expressed by the modified T cell, for example the 4-1BB ligand, it can then bind to the now expressed 4-1BB receptor and result in an inducible response that is physiological rather than a constitutively active response as observed with gen2-CAR with an intracellular 4-1BB signaling domain. In certain instances, the ligand could be attached to the T-cell receptor-based CAR of the present disclosure as this would still permit an inducible response.

[0054] FIGURE 1A shows exemplary CARs of the present disclosure (TCRßα-CAR and TCRαß-CAR), a gen1-CAR (ζ-CAR) and a gen2-CAR (BBζ-CAR). All of the constructs include a scFv with embedded FLAG tag (V_H-FLAG-V_L), 2A linker sequences and a ΔCD19 tail as discussed further in the Examples. The exemplary CARs of the present disclosure also include a T-cell receptor alpha chain constant region (cTCRα) and a T-cell receptor beta constant region (cTCRß) and an additional, in some instances different 2A linker. The gen1-CAR and gen2-CAR shown include a CD28 hinge region, a transmembrane domain, and the CD3ζ chain. gen2-CAR also includes a 41BB signaling domain.

[0055] The T-cell receptor-based CARs of the present disclosure are able to associate with other endogenous subunits of the TCR as shown in FIGURE 1B. FIGURE 1B shows the assembly of TCRαß-CAR or TCRßα-CAR (TCR-CAR) with the endogenous TCR complex as well as the gen1-CAR and gen2-CAR in the cellular membrane. Each CAR includes a scFv sequence (**1**). The scFv in the TCR-CAR is linked to the T-cell receptor beta constant region which is complexed with the T-cell receptor alpha constant region and includes a conserved antigen receptor transmembrane motif (**2**) which is located in the TCRß transmembrane domain and involved in TCR polarization and NF-κB recruitment to the immunological synapse and required for the formation of memory cells. The CD3ε subunit includes a basic rich stretch (**3**) which modulates TCR signaling and recruitment to the immunological synapse through ITAM accessibility and a proline rich sequence (**4**) which is involved in actin cytoskeleton reorganization and formation of the immunological synapse. ITAMs (**5**) of the CDγ ,CDε, CDδ and CDζ subunits can be phosphorylated which recruits Zap-70 which activates signaling pathways important for T cell survival, proliferation, differentiation, and effector functions. The degree of ITAM accessibility modulates TCR signaling.

[0056] In contrast, the gen1-CAR (ζ-CAR) shown in FIGURE 1B includes a scFv sequence (**1**), a CD28 hinge/transmembrane domain (**6**) which acts as a scaffold for scFv surface expression and attachment of intracellular signaling domains and a CD3ζ signaling domain (7) which allows for signaling through TCR pathways. The gen2-CAR (41BBζ-CAR) includes these features as well as an intracellular 41BB signaling domain (**8**) which provides signals necessary for sustained survival and effector functions. As can be observed, the T-cell receptor-based CARs of the present disclosure allow the constructs to associate with other components of the TCR complex which can limit the surface expression of the TCAR by the availability of CD3 proteins. By comparison, the surface expression of gen1-CAR and gen2-CAR are only limited by gene copy number and promoter strength.

[0057] By restricting the surface expression of the scFv-TCRß/TCRα complex, it is believed without being bound by theory that this limits the tonic signaling associated with the high surface expression of gen1-CAR and gen2-CARs.

[0058] In some embodiments a construct includes a ligand fused to a peptide that is capable of specifically binding a tumor-specific antigen. In some aspects, the ligand can be a co-stimulator ligand that binds a cognate co-stimulatory molecule, *.e.g.* a receptor, on a T cell that stimulates the T cell to effect a response.

[0059] In some embodiments, the ligand is the 4-1BB ligand (4-1BBL). In some embodiments, the amino acid sequence of the ligand may be modified, *e.g.* by deletion, insertion, addition, substitution, or truncation, as compared to its native amino acid sequence. For example, the 4-1BBL used may have an amino acid sequence of SEQ ID NO: 1, amino acids 85-254 of the full-length, native 4-1BBL amino acid sequence, where there is a mutation of E254D and addition of H255 to the native amino acid sequence of 4-1BBL, which is encoded by SEQ ID NO: 2. In some embodiments, the ligand is void of its transmembrane domain. For example, the 4-1BBL of SEQ ID NO: 1 does not contain the transmembrane domain, amino acids 29-49, of the full-length, native amino acid sequence of 4-1BBL. In some embodiments, the ligand has a mutation in its trimerization domain. For example, for 4-1BBL or a variant thereof such as that of SEQ ID NO: 1, the amino acids corresponding to positions 94, 142, 144, 199, 204, 234 and/or 238 of the full-length, native amino acid sequence may be mutated. The ligand may be selected based on the co-receptor target. By way of example but not limitation, in certain alternative embodiments the ligand can be 4-1BBL, CD80, CD58, OX40L, MICA, ICAM-1, CD5 ligand or CD48 which bind to the 4-1BB, CD28, CD2, OX40, NKG2D, LFA-1, CD5 and 2B4 receptors, respectively. In some embodiments, the intracellular domain can be fused to the C-terminus of the scFv-TCRß via a lengthy, flexible linker domain that will extend the ligand further than the membrane than through the endogenous CD3 and ζ chains.

[0060] The peptide can be any amino acid sequence that can specifically bind to an antigen, in some aspects, a tumor-specific antigen. In some embodiments, the peptide is a single chain variable fragment (scFv). In some embodiments the peptide is an antibody or other protein with an affinity for binding an antigen. The tumor-specific antigen can be any antigen which is specifically expressed by or more highly expressed by tumor cells as compared to non-tumor cells. By way of example but not limitation, the tumor-specific antigen can be selected from the group consisting of disialoganglioside GD2 (GD2), mucin 1 (MUC1), prostate-specific membrane antigen (PSMA), human epidermal growth factor receptor 2 (Her2), mucin 16

(MUC16), melanoma-associated antigen 1(MAGE-A1), carbonic anhydrase 9 (CAIX), b-lymphocyte surface antigen CD19 (CD19), prominin-1 (CD133), CD33 antigen (CD33), CD38 antigen (CD38), neural cell adhesion molecule (CD56), interleukin-3 receptor (CD123), and b-lymphocyte antigen CD20 (CD20). By way of example but not limitation, the peptide can be anti-GD2 scFv of SEQ ID NO: 16 encoded by SEQ ID NO: 17.

[0061] In some embodiments a chimeric antigen receptor is provided which includes a T-cell receptor beta chain constant region and a T-cell alpha chain constant region, wherein the T-cell receptor beta chain constant region is fused to a peptide having affinity to an antigen, wherein the chimeric antigen receptor does not include a T-cell receptor beta chain variable region and a T-cell alpha chain variable region, and wherein the T-cell receptor beta chain constant region is complexed with the T-cell receptor alpha chain constant region. In some embodiments, the peptide having affinity to an antigen is a peptide as described in the foregoing embodiments and the antigen is any antigen including those described in the foregoing embodiments, in some aspects, a tumor-specific antigen. In some embodiments the peptide is a scFv as described in the foregoing embodiments. The peptide fused to the T-cell beta constant region can be the same or different from the peptide fused to the ligand in the foregoing embodiments.

[0062] In some embodiments, a nucleic acid molecule encodes a construct of any of the foregoing embodiments. In some embodiments, the nucleic acid molecule encodes a chimeric antigen receptor of any of the foregoing embodiments. In some embodiments, the nucleic acid molecule further includes a promoter region. The promoter region can be responsive to signaling through a T cell receptor. By way of example but not limitation, the promoter region can be derived from the human NF-AT promoter or from the Elk-1 pathway. For example, the promoter region can have the sequence of SEQ ID NO: 7, which includes a NF-AT response element encoded by SEQ ID NO: 8 and a mini promoter encoded by SEQ ID NO: 9. By way of example but not limitation, the nucleic acid molecule could have the sequence of SEQ ID NO: 10 encoding SEQ ID NO: 11, which encodes restriction sites, a NF-AT response element, mini promoter, anti-GD2 scFv, a linker (SEQ ID NO: 14) encoded by SEQ ID NO: 15, HA tag (SEQ ID NO: 12) encoded by SEQ ID NO:13, and 4-1BBL. By way of example but not limitation, the nucleic acid molecule could have the sequence of SEQ ID NO: 41 which encodes anti-GD2 scFv-T-cell receptor ß constant region-P2A linker-T-cell receptor alpha constant region-T2A

linker-ΔCD19-NF-AT Response Element-mini promoter-anti-GD2 scFv-HA tag-41BBL. By way of further example but not limitation, the nucleic acid molecule can include SEQ ID NO: 27 which encodes T-cell receptor ß constant region-P2A linker-T-cell receptor alpha leader sequence-T-cell receptor alpha constant region which includes the T-cell receptor alpha leader sequence which can be used to ensure that the construct is transported to the cell membrane. Other leader sequences known to those of skill in the art can be used to direct the construct to the cell membrane. It should be understood that all of the constructs and nucleic acid molecules within the scope of the present disclosure can be modified to include a leader sequence.

[0063] In some embodiments, a modified T lymphocyte is provided which expresses a construct including a ligand fused to a peptide that is capable of specifically binding a tumor-specific antigen and a chimeric antigen receptor. In some embodiments, a modified T lymphocyte is provided which expresses a construct including a chimeric antigen receptor of any of the foregoing embodiments. In some embodiments, the chimeric antigen receptor includes a T-cell receptor beta chain constant region and a T-cell receptor alpha chain constant region, where the beta chain constant region is fused to a peptide having affinity to an antigen, and wherein the T-cell receptor beta chain constant region is complexed with the T-cell receptor alpha chain constant region. In some embodiments, the peptide having affinity to an antigen is a peptide as described in the foregoing embodiments and the antigen is any antigen including those described in the foregoing embodiments, in some aspects, a tumor-specific antigen. In some embodiments the peptide is a scFv as described in the foregoing embodiments. In some embodiments, the modified T lymphocyte expresses a chimeric antigen receptor of any of the foregoing embodiments. The peptide fused to the T-cell beta constant region can be the same or different from the peptide fused to the ligand in the foregoing embodiments. In some aspects, the construct including a ligand fused to a peptide that is capable of specifically binding a tumor-specific antigen can be fused to the chimeric antigen receptor, for example, by a 2A linker.

[0064] In some embodiments, a method is provided for treating cancer in a subject which includes the step of administering to the subject a modified T lymphocyte of any of the foregoing embodiments.

[0065] In some embodiments a recombinant expression vector comprises a nucleic acid molecule of any of the foregoing embodiments.

[0066] In any of the foregoing embodiments, the T-cell receptor alpha chain constant region can be the full-length, native T-cell receptor alpha chain constant region or any modified portion thereof. By way of example but not limitation, the T-cell receptor alpha chain constant region can have the sequence of SEQ ID NO: 5 encoded by SEQ ID NO: 6 which has a mutation of T42C to promote pairing with the T-cell receptor beta chain constant region. This modified sequence, when used in conjunction with a similar modification (S57C) to the T-cell receptor beta constant region helps to avoid mispairing between endogenous and exogenous T-cell receptor proteins by introducing a second disulfide bond between the exogenous T-cell receptor alpha constant region and T-cell receptor beta constant region.

[0067] In any of the foregoing embodiments, the T-cell receptor beta chain constant region can be the full-length, native T-cell receptor beta chain constant region or any modified portion thereof. By way of example but not limitation, the T-cell receptor beta chain constant region can have the sequence of SEQ ID NO: 3 encoded by SEQ ID NO: 4 which has a mutation of S57C to promote pairing with the T-cell receptor alpha chain constant region. This modified sequence, when used in conjunction with a similar modification (T42C) to the T-cell receptor beta constant region helps to avoid mispairing between endogenous and exogenous T-cell receptor proteins by introducing a second disulfide bond between the exogenous T-cell receptor alpha constant region and T-cell receptor beta constant region.

[0068] Linkers may be included in the TCARs of the present disclosure. By way of example but not limitation, such linkers can include SEQ ID NO: 18 and SEQ ID NO: 20, encoded by SEQ ID NO: 19 and SEQ ID NO: 21, respectively.

[0069] In some embodiments, alternative mechanisms by which 4-1BB can be activated can be used. For example, the intracellular domain of 4-1BB can be fused to the C-terminus of the constant region of TCRα, TCRβ, or other chains of the TCR complex (such as CD3δ) via a PRK linker. For example, SEQ ID NOs: 37, 35 and 33 which encode SEQ ID NOs: 36, 34 and 32, respectively, can be used to express TCRßα-PRK-41BB, TCRß-PRK-41BB and CD3δ-PRK-41BB, respectively. In preliminary experiments, it has been found that direct fusion of the

intracellular portion of 4-1BB to TCRα or TCRß (i.e. without a linker), the TCAR is not expressed on the cell surface. Further preliminary data suggests that TCRßα-PRK-41BB and TCRß-PRK-41BB possess enhanced function over CD3δ-PRK-41BB (as measured by increased expression of Bcl-xL).

[0070] Alternatively, two 4-1BB intracellular motifs can be expressed as a single protein in which the N-terminus has a membrane-localization sequence, trimerization motif, and a calmodulin switch motif is inserted in between the two 4-1BB domains. In this way, CAR-induced calcium mobilization would induce dimerization of 4-1BB on individual proteins and heximerization of the trimeric protein complex. For example, SEQ ID NO: 39 which encodes 41-BB-Calmodulin Switch (SigSeq-HA tag-TM-Trimerization Domain-41BB-NCaM-CKKp-CCaM-41BB could be used.

EXAMPLES

[0071] The present invention is demonstrated in the following examples, it being understood that these are for illustrative purposes only, and the invention is not intended to be limited thereto.

MATERIALS AND METHODS

[0072] For all examples herein, the following materials and methods were used:

Plasmid construction

[0073] Transgenes encoding CAR constructs were cloned into the pSFG retroviral transfer plasmid. The pSFG-T2A-ΔCD19 control plasmid containing cDNA encoding a 2A viral linker (SEQ ID NO: 21 encoding by SEQ ID NO: 20) and a ΔCD19 gene (SEQ ID NO: 23 encoded by SEQ ID NO: 22) was generated by Gibson Assembly of a T2A-ΔCD19 gene fragment (Life Technologies) between the NcoI and XhoI restriction sites of pSFG. The gene fragment included a 5' multiple cloning site (NcoI, SphI, NsiI, AfeI, XhoI, SalI, SacII, BamHI) for cloning upstream of T2A and a 3' multiple cloning site (BsaBI, MluI, BsiWI, SnaBI, BstZ17I, MfeI, AleI) for cloning downstream of ΔCD19. A gene fragment encoding the anti-GD2 scFv (SEQ ID NO: 17) was cloned between the NcoI and SalI sites in pSFG-T2A-ΔCD19 and the subsequent plasmid used for generation of CAR constructs. For TCRβα-CAR (SEQ ID NO: 24, GD2 scFv-TCRßc-P2A-TCRαc-T2A-ΔCD19), gene fragments encoding human TCRβ (SEQ ID

NO: 4) and P2A-TCRα (P2A of SEQ ID NO: 19 and TCRα of SEQ ID NO: 6) were cloned into the BamHI site. TCRβα-CAR19 was constructed by replacing the anti-GD2 scFv with anti-CD19 scFv (SEQ ID NO: 30 encoded by SEQ ID NO: 31) following NcoI/SalI restriction digest. The ΔCD19 transduction marker was subsequently replaced with ΔCD34 (SEQ ID NO: 28 encoded by SEQ ID NO: 29) using RsrII/MfeI digest. Gen1-CAR and gen2-CAR were generated by cloning gene fragments between SalI and BamHI sites.

Cell lines and antibodies

[0074] The M21 and A375 melanoma cell lines, HeLa adenocarcinoma cells, and HEK 293T cells were maintained in DMEM 10-013-CV (Corning Life Sciences, Corning, NY) supplemented with 10% fetal calf serum (Atlanta Biologicals, Flowery Branch, GA), 1x MEM non-essential amino acids (Corning Life Sciences), and 1x pen/strep (Corning Life Sciences). Jurkat T cell leukemia and Ramos B cell lymphoma cell lines were cultured in RPMI1640 (Corning Life Sciences) supplemented with 10% fetal calf serum and 1x pen/strep. M21 cells were generously provided by Dr. Susan Knox (Stanford University). A375 cells and Ramos cells were obtained from ATCC (Manassas, Virginia). Anti-CD3-APC-Cy7, anti-CD3-PerCP, anti-CD4-PE-Cy7, anti-CD8-BV785, anti-CD8-APC-Cy7, anti-CD19-BV421, anti-CD19-APC-Cy7, anti-CD25-PerCP-Cy5.5, anti-CD34-BV421, anti-CD69-BV650, anti-CD137-PE, anti-DYKDDDDK (FLAG)-PE, anti-DYKDDDDK (FLAG)-APC, anti-IFNγ-PB, anti-GD2-PE, mouse IgG2a,κ-PE isotype, and anti-rabbit IgG-PE were purchased from Biolegend (San Diego, CA) or BD Biosciences (San Diego, CA). Anti-TNFα-PE was purchased from eBioscience (San Diego, CA).

Blood donors and PBMC isolation

[0075] Blood was collected from consented donors in accordance with University of Kansas Medical Center IRB approved protocols. PBMCs where isolated using SepMate tubes (STEMCELL Technologies, Cambridge, MA) and Ficoll-Paque PLUS (GE Healthcare Life Sciences, Pittsburgh, PA) according to the manufactures' instructions. Cells were maintained in AIM V media (Thermo Fischer Scientific, Waltham, MA) supplemented with 2% human serum (Atlanta Biologicals, Atlanta, GA) and 20ng/mL rhIL-2 (PeproTech, Rocky Hill, NJ).

Retroviral production

[0076] Retrovirus was produced by co-transfecting HEK 293T cells with CAR-encoding transfer plasmid, Peg-Pam-e plasmid encoding the MoMLV gag-pol, and the RDF plasmid encoding the

RD114 envelope protein, in the presence of FuGENE HD Transfection Reagent (Promega, Madison, WI). Retroviral supernatants were collected at 48 and 72 hours, purified through 0.45μM filters, flash frozen in liquid nitrogen, and stored at -80°C.

T cell transduction and isolation

[0077] T cells from freshly isolated PBMCs were activated for 3 days using 10ng/mL anti-CD3ε clone OKT3 (BioXCell, Lebanon, NH) in complete AIM V media as previously described in Lamers CH, Willemsen RA, Luider BA, Debets R, Bolhuis RL. Protocol for gene transduction and expansion of human T lymphocytes for clinical immunogene therapy of cancer. Cancer Gene Ther. 2002;9(7):613-23. For both primary and Jurkat T cell transduction, retroviral supernatants were bound to RetroNectin (Takara Bio, Inc.) coated 6 well plates according to the manufacturer's instructions followed by addition of $2.5x10^6$ Jurkat or activated primary T cells per well. After 48 hours, transduced cells were isolated using IMag anti-human CD19 magnetic beads (BD Biosciences, San Jose, CA) and expanded for 4 to 6 days before use in experiments. For some experiments, $CD8^+$ T cells were isolated by negative selection using anti-human CD4 IMag beads (BD Biosciences).

Phosphorylation Assay

[0078] Transduced Jurkat T Cells were washed in RPMI media and re-suspended at $5x10^6$ cells / mL in RPMI media. For each treatment, 50 μL of T cells were transferred to a 96-well-round bottom plate and rested for one hour at 37°C. T cells were stimulated by addition of 50 μL RPMI media warmed to 37°C containing either 2 μg/mL anti-CD3, $2.5x10^5$ M21 or HeLa cells, or media alone. After 15 minutes, cells were fixed by addition of 100 μL 4% paraformaldehyde and incubated 10 minutes at 37°C. Cells were then labeled with anti-CD19 and anti-FLAG, permeabilized in ice-cold 90% methanol for 30 minutes, and stained intracellularly with anti-phospho-Erk1/2 (clone D13.14.4E, Cell Signaling Technology, Danvers, MA) followed by secondary staining with anti-rabbit Ig-PE and analyzed by flow cytometry.

Activation assay

[0079] RPMI or AimV media were used for Jurkat or primary T cells respectively. Cells were washed in media and re-suspended at $2x10^6$ cells/mL in media. For each treatment, 100 μL of cells were transferred to a 96-well flat-bottom plate and stimulated by the addition of 100 μL media containing either 2 μg/mL anti-CD3 (clone OKT3) (BioXcell), $2.0x10^5$ tumor cells, or

media alone. Cells were then incubated for 24 hours at 37°C with 5% CO_2. Following incubation, cells were pelleted by centrifuging at 400g for 5 minutes. For primary T cells, 150µL of supernatant removed for CBA assays. Jurkat T cells were labeled with antibodies against CD3, CD69, and CD19. Primary T cells were labeled with antibodies against CD3, CD4, CD8, CD25, CD69, CD137, and either CD19 or CD34. All cells were analyzed by flow cytometry.

Lamp-1 assay and intracellular staining

[0080] Transduced T cells were washed in AIM V media and re-suspended at $5x10^6$ cells/mL in AIM V containing 40 µL/mL anti-CD107a-BV605 antibody (Biolegend, San Diego, CA). For each treatment, 50 µL of T cells ($2.5x10^5$ total) were transferred to a 96-well round-bottom plate and stimulated by addition of 50 µL AIM V media containing either 2 µg/mL anti-CD3, 20ng/mL PMA and 1µg/mL ionomycin, $2.5x10^5$ of the indicated tumor cells, or media alone. Cells were incubated one hour at 37°C with 5% CO_2 before 20 µL of AIM V media containing GolgiStop (1.33µL/mL final concentration, BD Biosciences) and Brefeldin A (3µg/mL final concentration) was added to each well. Four hours later, cells were surfaced labeled with antibodies against CD3, CD4, CD8, and either CD19 or CD34 followed by intracellular staining with anti-TNF and anti-IFNγ. Cells were analyzed by flow cytometry.

Cytokine bead array

[0081] Cytokines were measured in supernatants collected from the 24-hour activation assays using a TH1/2/17 Cytokine Bead Array kit (BD Biosciences) according to the manufacturer's instructions. Samples were collected via flow cytometry and analyzed using FCAP Array 3.0 software (Soft Flow, Hungary).

Cytotoxicity assay

[0082] Luciferase expressing M21, A375, HeLa, and Ramos tumor cells were generated by transduction with a retrovirus containing a luciferase transgene and a neomycin selection cassette. Transduced cells were selected by culturing in complete DMEM or complete RPMI supplemented with 400µg/mL Geneticin (Life Technologies). To measure cytotoxicity, $2x10^3$ target cells were co-cultured in round bottom plates with effector cells at effector to target ratios ranging from 40:1 to 1:1 in AIM V media for 4, 8, and 24 hours. At the indicated time points, cells were gently mixed, pelleted at 400g for 5 minutes, supernatants discarded, and re-suspended in luciferin lysis buffer, a process described in Siebring-van Olst E, Vermeulen C, de

Menezes RX, Howell M, Smit EF, van Beusechem VW. Affordable luciferase reporter assay for cell-based high-throughput screening. J Biomol Screen. 2013;18(4):453-61. After five min, luciferase activity was measured on a Synergy H1 plate reader and analyzed using Gen5 software (Biotek). Percent lysis was calculated using the formula: (Target Cells Plated – Target Cells Remaining)/Target Cells Plated x 100 and percent specific lysis was normalized to ΔCD19 or ΔCD34 controls.

Flow cytometry

[0083] Flow cytometry was performed using an LSRII (BD Biosciences). Unless otherwise noted, surface labeling was done in FACS buffer (2% FBS in PBS) in the dark at 4°C for 20 minutes. Cells were then quenched in ice cold FACS buffer followed by two washes in FACS buffer and then fixed in 1% paraformaldehyde before analysis. For intracellular staining, surface labeled and fixed cells were permeabilized in 1x Permeabilization Buffer (eBioscience, San Diego, CA) followed by staining in Permeabilization Buffer at room temperature for 30 minutes. Cells were then washed three times in Permeabilization Buffer and re-suspended in FACS buffer for analysis.

Statistical Analysis

[0084] Statistical Analysis was performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). Significance was determined using a one-way analysis of variance with post-hoc Tukey Test. Outliers were eliminated using the ROUT method (Q = 0.1%).

EXAMPLE 1

TCR-based CAR constructs

[0085] To generate a novel CAR that merges the antigen recognition capacity of currently used CARs with the functionality of the endogenous TCR complex, a scFv was fused to the constant region of TCRβ or TCRα. The scFv was based on the humanized anti-disialoganglioside (GD2) clone 3F8 and contained an embedded FLAG epitope tag to allow for detection of CAR surface expression. Each scFv-TCR chain was co-expressed with the constant region of the opposing TCR chain to create TCRαβ-CAR and TCRβα-CAR (Fig. 1A). To avoid mispairing with endogenous TCR chains, a second disulfide bond was added between the TCRα and TCRβ chains, which can be achieved by adding a single cysteine on each receptor chain to promote the formation of an additional interchain disulfide bond. For comparison, first and second generation CARs were also generated (Fig. 1A). As a marker for transduction, each construct

also contained cDNA encoding the extracellular and transmembrane domains of CD19 (ΔCD19) attached via a 2A linker sequence.

EXAMPLE 2

Surface expression of TCR-based CARs

[0086] The biochemical structure of the TCR complex prohibits the surface expression of individual chains, so each CAR was tested to determine whether it was dependent on CD3 for surface expression. HEK 293T cells were transfected with cDNA encoding each CAR alone or co-transfected with cDNA encoding CD3ε, CD3δ, CD3γ, and CD3ζ (Fig. 2A). Gen1-CAR and gen2-CAR could be detected on the cell surface independently of the CD3 chains. By contrast, TCRαβ-CAR and TCRβα-CAR could only be detected on the cell surface if the CD3 chains were co-expressed, demonstrating that the TCR-based CARs are incorporated into the endogenous TCR complex.

[0087] Since T cells tightly regulate CD3 expression, surface expression of TCR-based CARs was hypothesized to be constrained by the availability of CD3, whereas surface expression of both gen1-CAR and gen2-CAR would be unrestricted in T cells. Indeed, primary human T cells transduced with the TCR-based CARs had significantly lower levels of CAR expression than T cells expressing gen1-CAR or gen2-CAR, despite similar surface expression of ΔCD19 (Fig. 2B).

EXAMPLE 3

T cell activation by TCR-based CARs

[0088] To test whether the TCR-based CARs could be used to activate T cells, TCRβα-CAR and TCRαβ-CAR were expressed in Jurkat T cells. As a negative control, a cell line was generated expressing the ΔCD19 empty vector. Cells were stimulated with anti-CD3ε, $GD2^{hi}$ M21 melanoma cells, $GD2^{-}$ HeLa cells, or left unstimulated. M21 cells could induce both CD69 expression and ERK1/2 phosphorylation in TCRβα-CAR T cells, but not TCRαβ-CAR T cells or ΔCD19 T cells (Fig. 3). Because directly fusing the scFv to the TCR could constrain the orientation of the scFv, constructs were generated in which a flexible $(Gly_4Ser)_3$ linker was inserted between the scFv and the TCR constant region. Addition of the linker did not affect T cell activation for either TCR-CAR construct (Figs. 9A and 9B). These data indicate that TCR-

CAR functionality is dependent on the scFv being fused to TCRβ and the presence of the $(Gly_4Ser)_3$ linker did not affect activity. Based on these results, the remaining studies were limited to the TCRβα-CAR construct.

EXAMPLE 4

TCRβα-CAR-induced activation of primary $CD8^+$ T cells

[0089] To test whether TCRβα-CAR T cells could discriminate between cells expressing varying levels of antigen, primary human T cells were transduced with TCRβα-CAR, gen1-CAR, gen2-CAR, or ΔCD19 and stimulated by co-culturing with $GD2^{hi}$ M21, $GD2^{lo}$ A375, or $GD2^-$ HeLa cells (Figs. 10A and 10B). M21 cells induced expression of CD25, CD69, and CD137 in T cells expressing each CAR (Fig. 4), indicating that all three CARs could become activated in response to a $GD2^{hi}$ tumor cell line. A375 cells induced robust activation of gen1-CAR T cells and gen2-CAR T cells, but not TCRβα-CAR T cells. Even without stimulation, more gen1-CAR T cells and gen2-CAR T cells expressed CD25 and CD137 than TCRβα-CAR and ΔCD19 T cells ($p < 0.05$). Collectively, these data indicate that TCRβα-CAR T cells can be activated in the presence of $GD2^{hi}$ tumor cells, but not $GD2^{lo}$ or $GD2^-$ tumor cells. Further, gen1-CAR and gen2-CAR result in non-specific T cell activation.

[0090] The finding that TCRβα-CAR T cells, but not gen1-CAR T cells or gen2-CAR T cells, could discriminate between cells expressing high and low levels of antigen is significant because tumor antigens are rarely expressed exclusively on tumor cells. Instead, tumor antigens are often highly expressed on tumor cells and at low levels on healthy cells. The on-target/off-tumor destruction of healthy tissue by CAR T cells is a common problem and can be lethal. Thus, the selectivity of TCRβα-CAR T cells for high antigen expression could prove to be a valuable safety feature.

EXAMPLE 5

TCRβα-CAR $CD8^+$ T cell functionality

[0091] $CD8^+$ T cells expressing each CAR were stimulated with OKT3, M21, A375, or HeLa cells with non-stimulated (No Stim) cells as a control and assessed for their potential to

degranulate and produce TNF and IFNγ (Fig. 5). After co-culturing with M21 cells, 28 ± 3.1% of TCRβα-CAR $CD8^+$ T cells expressed surface LAMP1, 29 ± 3.3% expressed TNF, and 18 ± 3.3% expressed IFNγ ($p < 0.05$, compared to M21-stimulated ΔCD19 T cells). As with the activation markers, TCRβα-CAR T cells did not express LAMP1, TNF, or IFNγ after culturing with A375 or HeLa cells.

[0092] After culturing with M21 and A375 cells, 75 ± 9.7% and 57% ± 9.9%, of gen1-CAR T cells expressed surface LAMP1 respectively ($p < 0.0001$, as compared to similarly activated ΔCD19 cells). Similarly, 89 ± 3.8% and 60 ± 6.4% of gen2-CAR T cells expressed surface LAMP1 after culturing with M21 and A375 cells, respectively ($p < 0.0001$, as compared to ΔCD19 cells). Even in the presence of HeLa cells, 27% ± 4.3% of gen1-CAR T cells and 39% ± 5.2% of gen2-CAR T cells expressed LAMP1 as compared to 9.1 ± 0.78% for TCRβα-CAR T cells ($p < 0.01$) (Fig 5). Similar trends were observed for both TNF and IFNγ expression by gen1-CAR and gen2-CAR T cells. Additionally, unstimulated gen2-CAR T cells exhibited significantly higher basal expression of LAMP1 (p = 0.0002), TNF (p = 0.0015), and IFNγ (p = 0.0068) compared to ΔCD19 T cells.

EXAMPLE 6

Cytokine production by TCRβα-CAR T cells

[0093] Primary human T cells were transduced with each CAR, stimulated overnight with M21, A375, or HeLa cells, and analyzed for the production of the Th1 cytokines (IL-2, IFNγ, and TNF), Th2 cytokines (IL-4 and IL-10), and IL-17 (Fig. 6). In response to M21 cells, T cells expressing each CAR produced significantly more IL-2 compared to unstimulated cells, but gen1-CAR and gen2-CAR T cells produced 10-fold and 48-fold more IL-2 than TCRβα-CAR T cells. None of the CAR T cells produced IL-2 after co-culturing with A375 or HeLa cells.

[0094] TCRβα-CAR T cells produced IFNγ after co-culturing with M21 cells, but not with A375 or HeLa cells. By contrast, gen1-CAR and gen2-CAR T cells produced IFNγ in response to both M21 and A375 cells. The quantity of IFNγ produced by M21-stimulated gen2-CAR T cells was nearly 6-fold more than TCRβα-CAR T cells ($p < 0.0001$). M21-stimulated gen1-CAR and gen2-CAR T cells also produced 25- and 47-fold more TNF, respectively, than M21-stimulated TCRβα-CAR T cells ($p < 0.05$). Basal IFNγ and TNF production was similar between TCRβα-

CAR and ΔCD19 T cells, but resting gen2-CAR T cells produced 12-fold more IFNγ and 15-fold more TNF than ΔCD19 T cells ($p < 0.01$).

[0095] IL-4 production mirrored that of TNF for all CARs tested with only M21 stimulated gen1-CAR and gen2-CAR T cells resulting in statistically significant increases in IL-4 ($p = 0.0027$ and $p < 0.0001$, compared to unstimulated cells). Moreover, co-culture with M21 cells resulted in 6-fold ($p < 0.0595$) and 5-fold more ($p = 0.1444$) IL-4 produced by gen1-CAR T cells and gen2-CAR T cells, respectively, than M21 stimulated TCRβα-CAR T cells. No significant differences in IL-10 were observed for any of the CAR T cells for any stimulation though all CAR T cells produce IL-10 following M21 stimulation. Lastly, each CAR induced IL-17A production after co-culture with M21 cells, but was nearly 6-fold greater in gen1-CAR T cells (p = 0.012) and 4-fold greater in gen2-CAR T cells (p = 0.0975) than TCRβα-CAR T cells.

[0096] In summary, TCRβα-CAR T cells secreted IL-2, IFNγ, and IL-17 upon stimulation with $GD2^{hi}$ tumor cells, but not $GD2^{lo}$ or $GD2^{-}$ cells. Gen1-CAR T cells and gen2-CAR T cells also produced these cytokines, but secreted significantly greater quantities and responded to both $GD2^{hi}$ and $GD2^{lo}$ cells. Additionally, stimulated gen1-CAR T cells and gen2-CAR T cells secreted TNF and IL-4 in significantly greater quantity than TCRβα-CAR.

[0097] In addition to being selectively activated by cells expressing high levels of antigen, another major difference between TCRβα-CAR and the other CARs was in the quantity of cytokines generated, particularly the pro-inflammatory cytokines (Fig. 6). In each case, production of cytokines by gen1-CAR T cells and gen2-CAR T cells greatly exceeded that of TCRβα-CAR T cells. While the production of large quantities of pro-inflammatory cytokines might be considered a positive attribute for anti-tumor immunity, it can be detrimental. IFNγ, TNF, and IL-17 can all be linked to CRS, a common phenomenon observed in patients receiving CAR therapy. Additionally, these cytokines can drive tumor proliferation and differentiation in the tumor microenvironment. Lastly, high expression of IL-2 by CAR T cells has been linked to the recruitment of regulatory T cells, which suppress CAR T cell activity by supporting the immunosuppressive tumor micro-environment. Thus, while the overall cytokine profile generated by all CARs we tested was skewed toward a Th1-like response, the more physiologic cytokines levels produced by TCRβα-CAR T cells is preferable in the treatment of solid tumors while also reducing the risk of CRS in patients receiving CAR immunotherapy.

EXAMPLE 7

Antigen-specific cytotoxicity of TCRβα-CAR CD8⁺ T cells

Total T cells were co-cultured expressing each CAR with M21 (Fig. 7A) or HeLa cells (Fig. 7B). All CAR constructs induced cytotoxicity of M21 cells in a dose-dependent manner and none of the constructs targeted the HeLa cells. Next, the cytotoxic potential of purified CAR-expressing $CD8^+$ T cells were examined at three time points (Fig. 7C-E). At each time point, TCRβα-CAR, gen1-CAR, and gen2-CAR T cells induced cytolysis of M21 cells and the cytolysis was complete for all CARs within 24 hours. Gen1-CAR $CD8^+$ T cells and gen2-CAR $CD8^+$ T cells also targeted antigen negative HeLa cells whereas TCRβα-CAR $CD8^+$ T cells did not (Fig. 7F). These data demonstrate that TCRβα-CAR T cells could induce tumor cell lysis in an antigen-specific-manner.

EXAMPLE 8

Targeting other antigens with TCRβα-CAR

[0098] To test whether the novel CAR could induce T cell activity for other antigens, the anti-GD2 scFv were replaced with anti-CD19 scFv (as described in Nicholson IC, Lenton KA, Little DJ, Decorso T, Lee FT, Scott AM, et al. Construction and characterisation of a functional CD19 specific single chain Fv fragment for immunotherapy of B lineage leukaemia and lymphoma. Mol Immunol. 1997;34(16-17):1157-65.) to create TCRβα-CAR19. To avoid the anti-CD19 CAR from targeting ΔCD19-expressing CAR T cells, the ΔCD19 transduction marker were switched with a truncated CD34 (ΔCD34). After co-culturing with $CD19^+$ Ramos cells, but not $CD19^-$ A375 cells, TCRβα-CAR19 T cells expressed CD25, CD69, and CD137 (Fig. 8A). TCRβα-CAR19 $CD8^+$ T cells also had antigen-specific increases in intracellular TNF and surface LAMP1 expression (Fig. 8B). Lastly, TCRβα-CAR19 T cells could induce cytolysis of Ramos cells but demonstrated no cytotoxicity against antigen negative targets (Fig. 8C). Collectively, these data demonstrate that TCRβα-CAR is a flexible platform capable of targeting multiple antigen targets.

EXAMPLE 9

Comparing the functionality of T cells expressing TCR-CAR and gen1-CAR

[0099] Human PBMCs will be transduced with TCR-CAR, gen1-CAR, or ΔCD19 empty vector control, by transfection with SFG plasmids containing cDNA encoding each CAR construct along, pRDF (contains RD114 retroviral envelope), and pPEG-Pam3 (which contains MoMLV gag-pol).. Because the phenotype of the T cells changes over time, all assays will be performed within one week of transduction. Cells will be stimulated with GD2^{hi} M21 cells, GD2^{lo} A375 cells, GD2^{-} HeLa cells, or anti-CD3ε.

[0100] At various time points ranging from 0-30 min after stimulation, cells will be fixed, surface labeled with anti-CD19 (to identify the transduced cells), anti-CD4, and anti-CD8. Then, cells will be permeabilized and labeled intracellularly with antibodies that recognize the phosphorylated forms of ZAP-70 (Tyr-319), LAT (Tyr-226), PLCγ1 (Tyr-783), and ERK (Thr-202/Tyr-204). As controls, we will use non-specific antibodies of the same isotype and antibodies against non-phosphorylated forms of the proteins.

[0101] cDNA were generated in which expression of Luc2P (Promega Corporation, Madison, WI) is under control of either the NF-AT or the NF-κB promoter. CAR-expressing T cells will be transduced with NFAT-Luc2P or NFκB-Luc2P and cultured with target cells for 0, 4, 8, or 18 hours and luciferase activity will be assessed using a microplate reader. The short half-life (~1h) of Luc2P allow meaningful data to be generated from this time course.

[0102] CAR-expressing cells will be cultured with target cells for 0, 24 or 48 hours. Tissue culture supernatant will be collected to quantify the production of Th1, Th2, and Th17 cytokines (IL-2, IL-4, IL-17A, IFNγ, and TNFα) by ELISA. In addition, cells will be assessed for their expression of the activation markers CD69 and CD25 and the transcription factors T-bet, GATA-3, and RORγt.

EXAMPLE 10:

Comparing the functionality of TCR/41BBL-CAR and a gen2–CAR.

[0103] cDNA will be generated encoding a secreted form of 4-1BBL fused to the same scFv as TCR-CAR and gen2-CAR. Expression of scFv-4/1BBL will be under control of the NF-AT promoter. For example, in some experiments, SEQ ID NO: 26 encoding SEQ ID NO: 25 can be used which encodes anti-GD2 scFv-HA-4-1BBL. The complete construct will be TCRα-2A-

scFv/TCRβ-STOP-NFAT promoter-scFv-4-1BBL (SEQ ID NO: 41, TCR/41BBL-CAR) and will be used as follows:

[0104] Using the NF-AT promoter to express scFv-4-1BBL: Jurkat T cells will be transduced with TCR/41BBL-CAR and stimulated with anti-CD3ε. One and two days later, tissue culture supernatant will be used to label M21 cells or HeLa cells. The ability of secreted scFv-4-1BBL to bind tumor cells will be assessed by flow cytometry using antibodies against an HA tag inserted into the cDNA encoding scFv-4-1BBL.

[0105] Inducing 4-1BBL-mediated T cell activation: TCR-CAR and TCR/41BBL-CAR-expressing primary T cells will be co-cultured with M21, A375, or HeLa cells for 0, 24, 48, and 72 hours. As a positive control, agonistic anti-4-1BBL antibodies will be added to the cells. Because 4-1BB promotes the expression of Bcl-xL, we will measure Bcl-xL expression in CAR-expressing T cells using intracellular staining and flow cytometry.

[0106] Comparing cytotoxicity and exhaustion in TCR/41BBL-CAR- and gen2-CAR-T cells: Cytotoxicity will be assessed by culturing CAR-expressing T cells with GFP+ M21, GFP+ A375, or GFP+ HeLa cells at effector to target ratios ranging from 0.5 to 8. Target cell lysis will be measured by loss of GFP+ cells, for example, by inducing cell death by a variety of apoptotic stimuli in various GFP-expressing mammalian cell lines, including those routinely used in anti-cancer drug screening. A decrease in fluorescence may be assessed either by flow cytometry (and compared with other apoptotic markers) or by a fluorescence microplate reader. Parallel cultures will be incubated for 0, 2, 4, or 7 days and monitored for expression of the activation markers CD25 and CD127 and the exhaustion markers and PD-1, LAG-3, and TIM-3.

EXAMPLE 11

Efficacy of TCR/41BBL-CAR in an (NOD-SCID-$\gamma c^{-/-}$) NSG mouse tumor mode

[0107] NSG mice will be inoculated sc with 10^6 firefly luciferase-expressing M21 cells. Tumor burden will be monitored with calipers and using *in vivo* imaging by injecting mice with D-luciferin and visualizing the tumor with an IVIS Imaging System (PerkinElmer, Waltham, MA). When tumors reach an approximate volume of 100-200 mm^3, mice will be injected with 10^6 T cells expressing TCR/41BBL-CAR, gen2-CAR, or ΔCD19 alone. Tumor growth will continue to be monitored bi-weekly for at least six weeks or until the tumor burden reaches 1.5 cm diameter. In addition, the number of CAR-expressing T cells in the blood will be monitored

weekly by obtaining a droplet of blood and labeling the cells with antibodies against CD3, CD4, CD8, CD19, and FLAG and analyzing the cells using flow cytometry.

[0108] After euthanasia, lungs, liver, kidneys, and spleens will be weighed, harvested, and paraffin embedded for histopathology. In addition to bioluminescence imaging, tumor burden will be determined as was done previously. In brief, the area (in square micrometers) occupied by the tumor will be calculated from 15 200x randomly selected fields using the MetaMorph 7 image analysis system. Immunohistochemical staining with FLAG antibodies will be carried out to estimate numbers of infiltrating CAR cells. A portion of the spleen and lymph nodes will be collected so the T cells can be analyzed by flow cytometry for expression of the activation markers CD25 and CD69, the differentiation markers CD45RA, CD45RO, CD27, and CD11a, and the exhaustion markers PD-1, LAG-3, and TIM-3.

[0109] TCR/41BBL-CAR is hypothesized to destroy the tumor as completely as gen2-CAR, albeit with slower kinetics because of the more physiologic manner in which T cell activation will occur. Slower kinetics will likely lessen the cytokine release syndrome often observed when large numbers of T cells release cytokines simultaneously. Another advantage of slower activation kinetics is that the IFNγ and TNFα concentrations produced within the tumor are likely to be more physiologic, resulting in the conversion of tumor-infiltrating macrophages and neutrophils from pro-tumorigenic cells to pro-inflammatory cells. The activation of innate cells will be critical for complete tumor destruction in patients

[0110] Despite the clinical success observed using 4-1BBL, it remains prudent to directly compare 4-1BBL to other co-receptors and combinations of co-receptors

[0111] If TCR/41BBL-CAR does not reduce the tumor burden to the level observed with gen2-CAR, this would suggest that T cell activation is not being adequately induced by either signal 1 (TCR) or signal 2 (4-1BB). To test whether TCR ligation is sufficient to induce scFv-4-1BBL expression *in vivo*, the tumor model described here will be repeated except the scFv-4-1BBL cDNA will be replaced with cDNA encoding click beetle red luciferase. Green tumor cells and red T cells can be visualized simultaneously. If red luciferase are not detected in the tumors, then the TCR-CAR-induced signal is too weak to induce scFv-4-1BBL expression. Such a result would indicate that a different promoter is needed. In addition to the ideas discussed in Example 10, signals generated in the tumor microenvironment, such as those generated by IL-10 or TGFβ will also be exploited. IL-10 activates the STAT3 and TGFβ activates the SMAD complex, so

the red luciferase assay will be repeated to determine whether activation can be detected of the either the STAT3 or SMAD transcriptional response elements in the tumor.

[0112] If red cells are observed in the tumor, the original experiment in this aim will be repeated and the tumor will be excised at a time at which CAR-expressing T cells infiltrate. The tumor will be examined for scFv-4-1BBL expression using anti-HA and immunohistochemistry to determine if the co-receptor ligand is expressed. The findings may show that scFv-4-1BBL is expressed adequately, but does not function *in vivo*. An alternate strategy for inducing 4-1BB signaling would be to drive expression of "constitutively active" 4-1BB through the NF-AT promoter. Because of the high level of constitutive activity of gen2-CAR, an option would be to drive expression of a gen2-CAR using the NF-AT promoter.

EXAMPLE 12

TCR/41BBL-CAR inducement of tumor destruction in a novel immunocompetent murine tumor model

[0113] In a paper currently in revision, immunocompetent mice were injected *in utero* with M21 cells to promote tolerance (Fig. 11). Postnatally, mice were reimplanted with luciferase-expressing M21 cells and tumors were found in the lung, a common site of metastasis in cancer patients. The ability of TCR/41BBL-CAR- and gen2-CAR-expressing murine T cells to destroy the tumors will be compared. Timed pregnant inbred FVB mice will be injected with Luc2-expressing M21 cells (2.5×10^4) into the amniotic cavity. Two weeks post-weaning, mice will be transplanted with 10^6 Luc2-expressing M21 cells via the tail vein. Fifty days later, splenocytes from syngeneic mice will be transduced with TCR/41BBL-CAR or gen2-CAR and infused into the tumor-bearing mice via retroorbital injection or via the lateral tail vein. Mice will be analyzed as described in aim 2a.

[0114] If TCR/41BBL-CAR does not reduce the tumor burden in immunocompetent mice, this would indicate that further CAR development is warranted. In particular, pro-inflammatory innate immune cells would need to be recruited. Adding components to the CARs that would manipulate the tumor microenvironment and skew macrophage and neutrophil differentiation will be investigated.

EXAMPLE 13

Targeting one antigen with the scFv fused to TCR-CAR and a different antigen with the scFv fused to 4-1BBL

[0115] Stable M21 melanoma cell lines have been generated that express CD19 and luciferase. M21 cells also express high levels of the GD2 disialoganglioside. $CD19^{+}GD2^{+}$ M21 cells will be used to test the model that it is possible to target one antigen with the scFv fused to TCR-CAR and a different antigen with the scFv fused to 4-1BBL. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice will be implanted with 2 x 106 M21 melanoma cells in Matrigel® subcutaneously into the flank. M21 cells express high levels of the GD2 antigen. An M21 variant cell line that expresses CD19 and luciferase was generated. One week after implantation, 5 x 106 TCR-CAR T cells will be injected retroorbitally into the mice. Mice will be imaged using an IVIS in vivo imaging system prior to injection of CAR T cells and weekly thereafter. For in vivo imaging, mice will be injected intraperitoneally with luciferin, anesthetized, and scanned. The relative light units emitted from each mouse will be calculated at each time point. After 30 days, mice will be euthanized and the any remaining tumor will be excised and weighed. Mice will be withdrawn from study if the tumor reaches more than two cm3 or the body conditioning score (as defined in Lab Animal Sciences 49: 319) drops below two.

Table 1: CAR constructs used in the melanoma model		
Construct	TCR portion	4-1BB portion
1	Anti-CD19 TCR-CAR	
2	Anti-CD19 TCR-CAR	Secreted Anti-GD2 scFv-41BBL fusion protein
3	Anti-CD19 TCR-CAR	4-1BB signaling motif fused to the intracellular tail of truncated TCRα
4	Anti-CD19 TCR-CAR	4-1BB signaling motif fused to the intracellular tail of truncated scFv-TCRβ
5	Anti-CD19 TCR-CAR	4-1BB signaling motif fused to the intracellular tail of CD3δ
6	Anti-CD19 Gen1-CAR	
7	Anti-CD19 Gen1-CAR	Secreted Anti-GD2 scFv-41BBL fusion protein
8	Anti-CD19 Gen1-CAR	4-1BB signaling motif fused to the intracellular tail of CD3δ
9	Anti-CD19 Gen2-CAR	
10	No CAR negative control	

EXAMPLE 14

Cytotoxic T lymphocyte (CTL) activity assay

[0116] Luciferase-expressing M21 cells described above will be used in an in vitro CTL assay. Briefly, the scFv used for each construct listed in table 2 will target the GD2 antigen. Total T cells expressing each CAR will be incubated with M21 cells at T cell to M21 cell ratios of 5:4, 5:2, 10:1, 20:1, and 40:1. After four hours of co-culture, luciferase activity remaining in each well will be quantified and compared to the no CAR control. Specific CTL activity will be calculated by subtracting the luciferase activity of M21 cells plated with CAR T cells from the luciferase activity of M21 cells plated. The result will be divided by the luciferase activity of M21 cells plated, normalized to wells with CAR-deficient T cells and multiplied by 100.

Table 2: Constructs tested *in vitro*	
TCR-CAR	scFv directly fused to the constant region of TCRβ co-expressed with the constant region of TCRα
TCRβc-CAR	scFv directly fused to the constant region of TCRβ
TCRαc-CAR	scFv directly fused to the constant region of TCRα
TCRβc/TCRαc-CAR	scFv directly fused to the constant region of TCRβ co-expressed with scFv directly fused to the constant region of TCRα
TCRβ-CAR	scFv directly fused to full-length TCRβ
TCRα-CAR	scFv directly fused to full-length TCRα
TCRβ/TCRα-CAR	scFv directly fused to full-length TCRβ co-expressed with scFv fused to full-length TCRα
TCRβLL-CAR	scFv directly fused to full-length TCRβ via three consecutive gly-ser linker regions
TCRαLL-CAR	scFv directly fused to full-length TCRα via three consecutive gly-ser linker regions
TCRβLL/TCRαLL-CAR	scFv directly fused to full-length TCRβ via three consecutive gly-ser linker regions co-expressed with scFv fused to full-length TCRα via three consecutive gly-ser linker regions
No CAR control	

What is claimed is:

1. A construct comprising 4-1BB ligand (4-1BBL) fused to a peptide that is capable of specifically binding a tumor-specific antigen.

2. The construct of claim 1, wherein the amino acid sequence of 4-1BBL is SEQ ID NO: 1.

3. The construct of claim 1, wherein the 4-1BBL is void of its transmembrane domain.

4. The construct of claim 1, wherein the 4-1BBL has a mutation in its trimerization domain.

5. The construct of claim 1, wherein the peptide is a single chain variable fragment (scFv).

6. The construct of claim 5, wherein the tumor-specific antigen is selected from the group consisting of disialoganglioside GD2 (GD2), mucin 1 (MUC1), prostate-specific membrane antigen (PSMA), human epidermal growth factor receptor 2 (Her2), mucin 16 (MUC16), melanoma-associated antigen 1(MAGE-A1), carbonic anhydrase 9 (CAIX), b-lymphocyte surface antigen CD19 (CD19), prominin-1 (CD133), CD33 antigen (CD33), CD38 antigen (CD38), neural cell adhesion molecule (CD56), interleukin-3 receptor (CD123), and b-lymphocyte antigen CD20 (CD20).

7. A nucleic acid molecule encoding the construct of any one of claims 1-6.

8. The nucleic acid molecule of claim 7 further encoding a promoter region, wherein the promoter region is responsive to signaling through a T cell receptor.

9. The nucleic acid of claim 8, wherein the promoter region is derived from the [human] NF-AT promoter.

10. The nucleic acid of claim 8, wherein the promoter region comprises the nucleic acid sequence of SEQ ID NO: 7.

11. The nucleic acid of claim 7 comprising the nucleic acid sequence of SEQ ID NO: 10.

12. A modified T lymphocyte that expresses the construct of any of claims 1-6 and a chimeric antigen receptor.

13. The modified T lymphocyte of claim 12, wherein the chimeric antigen receptor comprises: T-cell receptor beta chain constant region and T-cell receptor alpha chain constant region, wherein the T-cell receptor beta chain constant region is fused to a peptide having affinity to an antigen, and wherein the T-cell receptor beta chain constant region is complexed with the T-cell receptor alpha chain constant region.

14. The modified T lymphocyte of claim 13, wherein the peptide is a single chain variable fragment (scFv).

15. The modified T lymphocyte of claim 14, wherein the antigen is selected from the group consisting of disialoganglioside GD2 (GD2), mucin 1 (MUC1), prostate-specific membrane antigen (PSMA), human epidermal growth factor receptor 2 (Her2), mucin 16 (MUC16), melanoma-associated antigen 1(MAGE-A1), carbonic anhydrase 9 (CAIX), b-lymphocyte surface antigen CD19 (CD19), prominin-1 (CD133), CD33 antigen (CD33), CD38 antigen (CD38), neural cell adhesion molecule (CD56), interleukin-3 receptor (CD123), and b-lymphocyte antigen CD20 (CD20).

16. A method of treating cancer in a subject comprising administering to the subject a modified T lymphocyte of claim 12.

17. A method of treating cancer in a subject comprising administering to the subject a modified T lymphocyte of claim 13.

18. A method of treating cancer in a subject comprising administering to the subject a modified T lymphocyte of claim 14.

19. A method of treating cancer in a subject comprising administering to the subject a modified T lymphocyte of claim 15.

20. A method for enhancing the activity of a chimeric antigen receptor expressed in a modified T lymphocyte against a cancer cell comprising adding to the T lymphocyte a nucleic acid molecule encoding the constructs of any of claims 1-6.

21. A recombinant expression vector comprising the nucleic acids of any of claims 7-11.

22. A chimeric antigen receptor comprising: a T-cell receptor beta chain constant region and a T-cell receptor alpha chain constant region, wherein the T-cell receptor beta chain constant region is fused to a peptide having affinity to an antigen, wherein the chimeric antigen receptor does not include a T-cell receptor beta chain variable region and a T-cell alpha chain variable region, and wherein the T-cell receptor beta chain constant region is complexed with the T-cell receptor alpha chain constant region.

23. The chimeric antigen receptor of claim 22, wherein the T-cell receptor beta chain constant region comprises an amino acid sequence of SEQ ID NO: 3.

24. The chimeric antigen receptor of claim 22, wherein the T-cell receptor alpha chain constant region comprises an amino acid sequence of SEQ ID NO: 5.

25. The chimeric antigen receptor of claim 22, wherein the peptide is a single chain variable fragment (scFv).

26. The chimeric antigen receptor of claim 22, wherein the antigen is a tumor-specific antigen.

27. The chimeric antigen receptor of claim 22 wherein the tumor specific antigen is selected from the group consisting of disialoganglioside GD2 (GD2), mucin 1 (MUC1), prostate-specific membrane antigen (PSMA), human epidermal growth factor receptor 2 (Her2), mucin 16 (MUC16), melanoma-associated antigen 1(MAGE-A1), carbonic anhydrase 9 (CAIX), b-lymphocyte surface antigen CD19 (CD19), prominin-1 (CD133), CD33 antigen (CD33), CD38 antigen (CD38), neural cell adhesion molecule (CD56), interleukin-3 receptor (CD123), and b-lymphocyte antigen CD20 (CD20).

28. A nucleic acid molecule encoding the chimeric antigen receptor of claim 22.

29. A nucleic acid molecule encoding the chimeric antigen receptor of claim 23.

30. A nucleic acid molecule encoding the chimeric antigen receptor of claim 24.

31. A nucleic acid molecule encoding the chimeric antigen receptor of claim 25.

32. A nucleic acid molecule encoding the chimeric antigen receptor of claim 26.

33. A nucleic acid molecule encoding the chimeric antigen receptor of claim 27.

34. A modified T lymphocyte that expresses a chimeric antigen receptor, wherein the chimeric antigen receptor is that of claim 22.

35. A modified T lymphocyte that expresses a chimeric antigen receptor, wherein the chimeric antigen receptor is that of claim 23.

36. A modified T lymphocyte that expresses a chimeric antigen receptor, wherein the chimeric antigen receptor is that of claim 24.

37. A modified T lymphocyte that expresses a chimeric antigen receptor, wherein the chimeric antigen receptor is that of claim 25.

38. A modified T lymphocyte that expresses a chimeric antigen receptor, wherein the chimeric antigen receptor is that of claim 26.

39. A modified T lymphocyte that expresses a chimeric antigen receptor, wherein the chimeric antigen receptor is that of claim 27.

40. The modified T lymphocyte of any of claims 34-39, wherein the chimeric antigen receptor forms a complex with one or more components of the endogenous T cell receptor.

41. A recombinant expression vector comprising the nucleic acid molecule of any of claims 28-33.

42. A method of treating cancer in a subject comprising administering to the subject the modified T lymphocyte of any of claims 34-39.

Fig. 1A

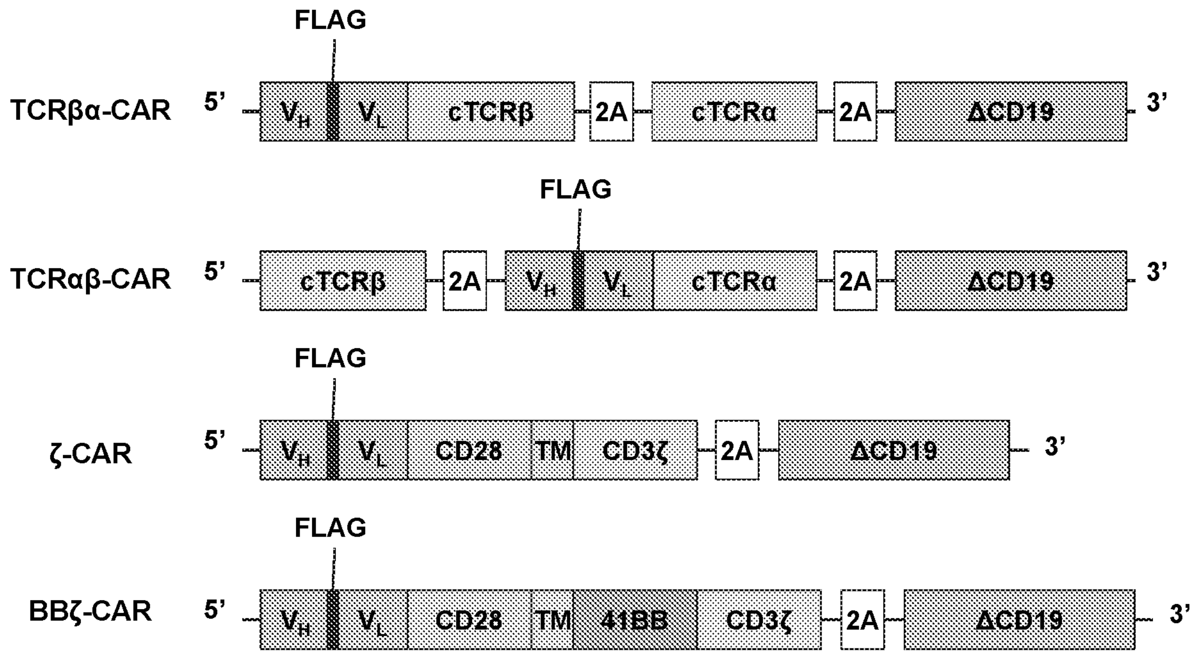


Fig. 1B

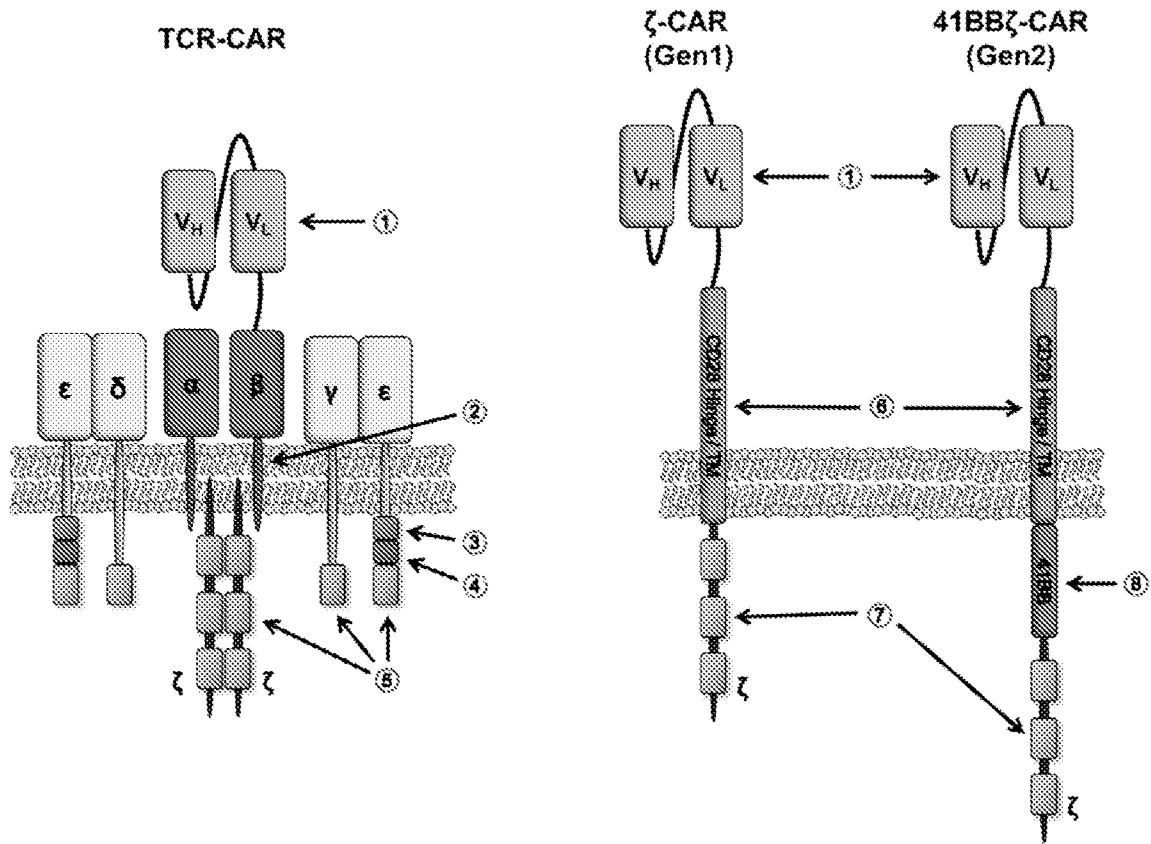


FIG. 2A

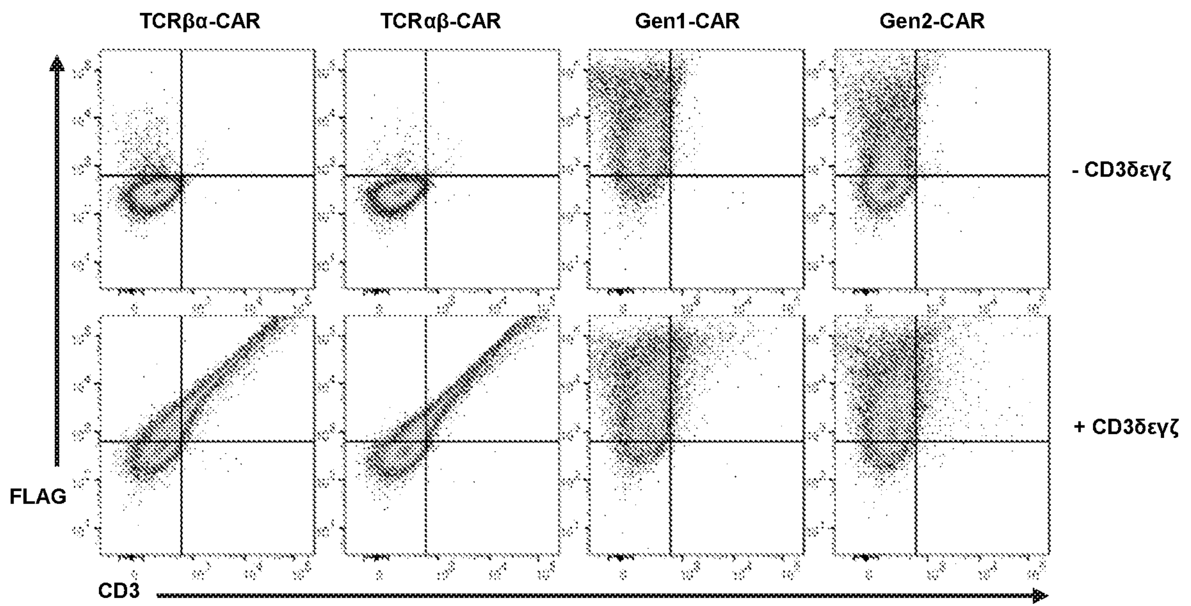


FIG. 2B

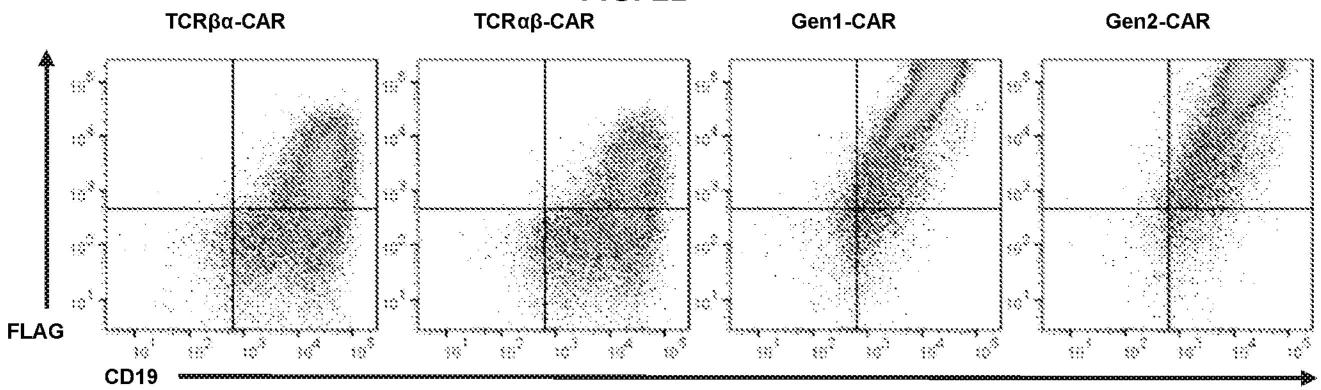


Fig. 3

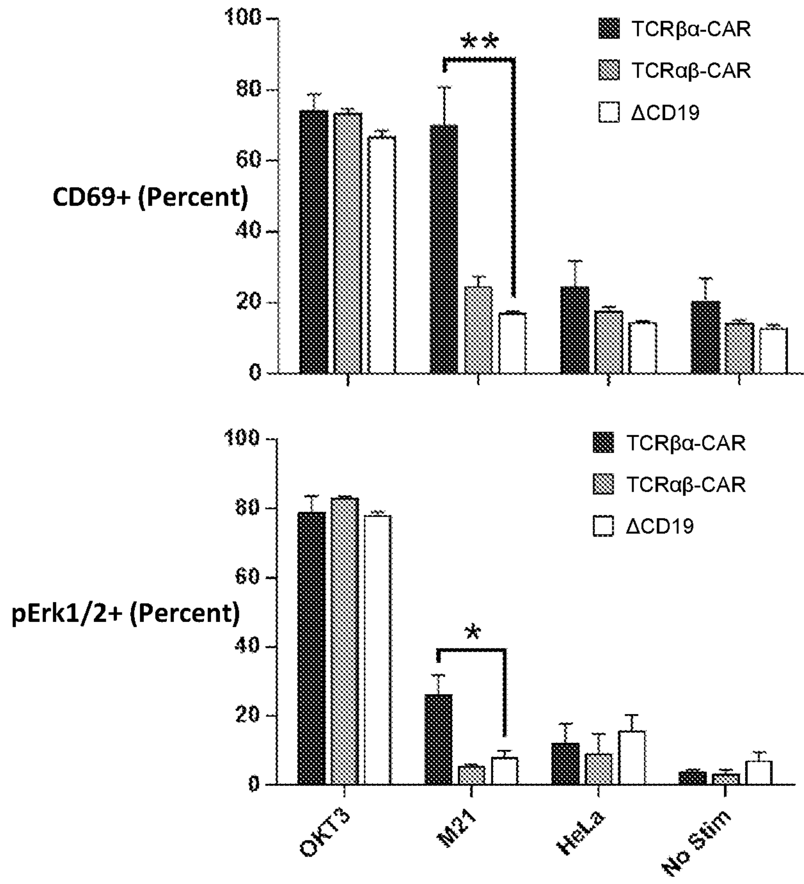


Fig. 4

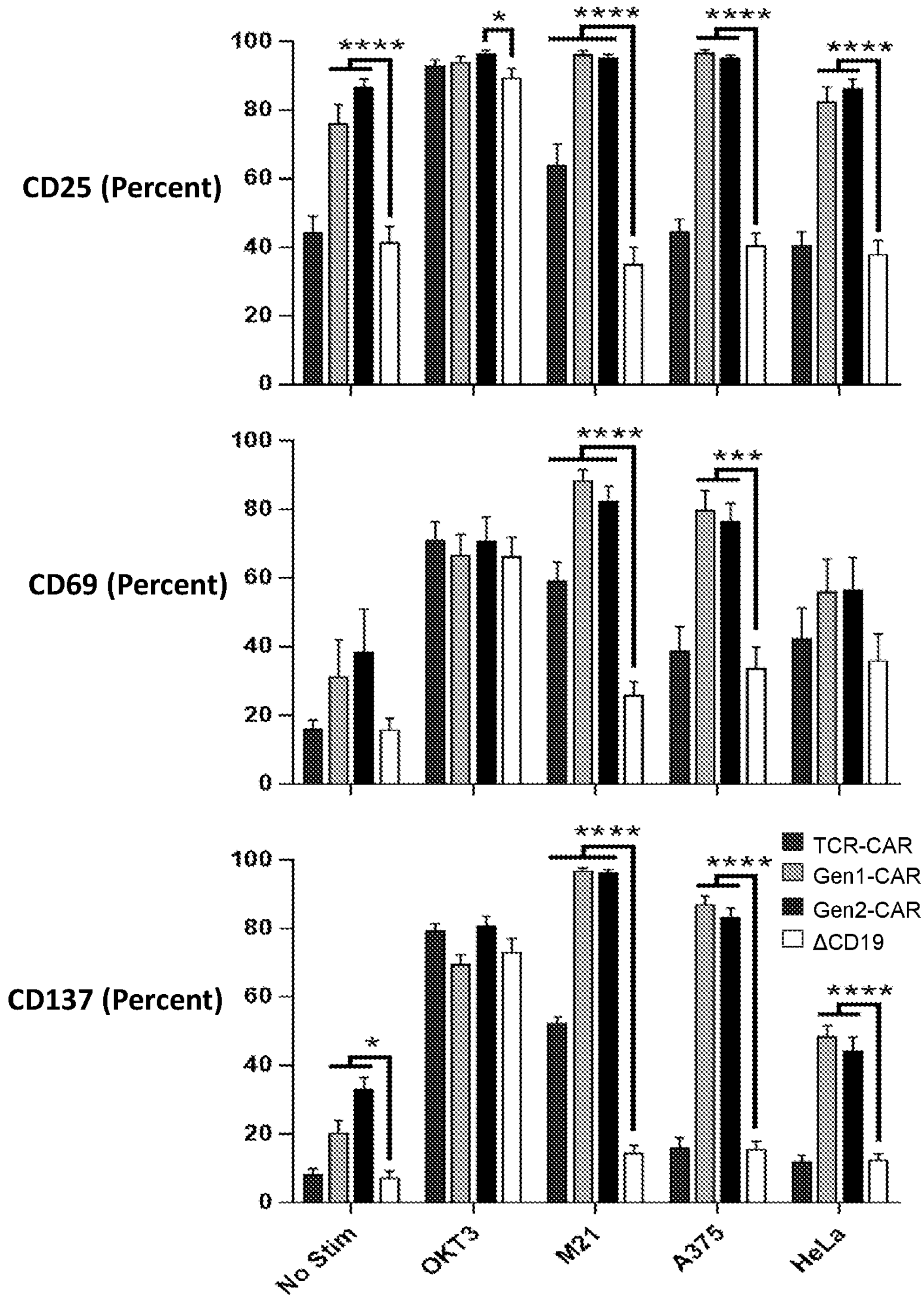


Fig. 5

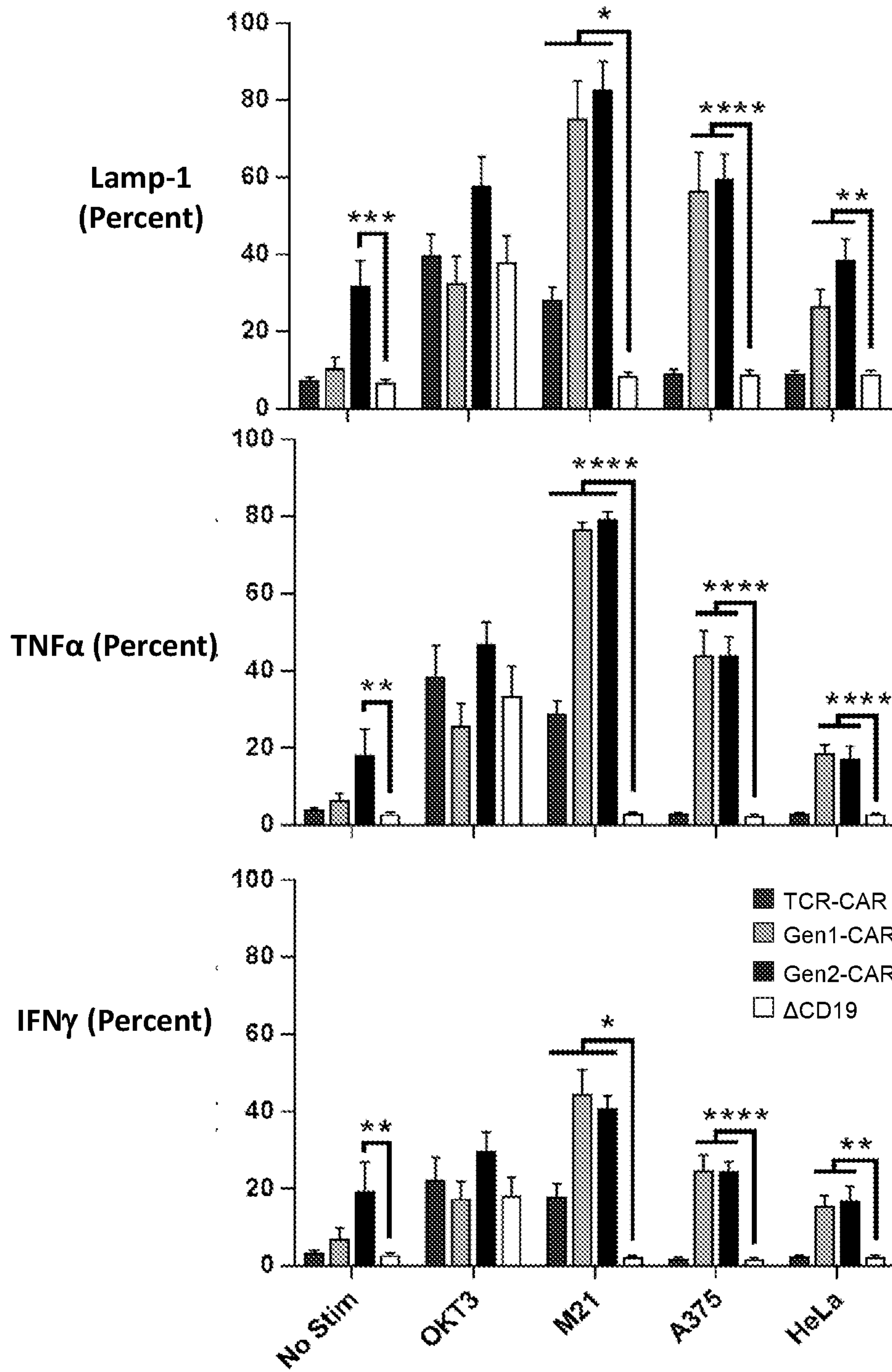


Fig. 6

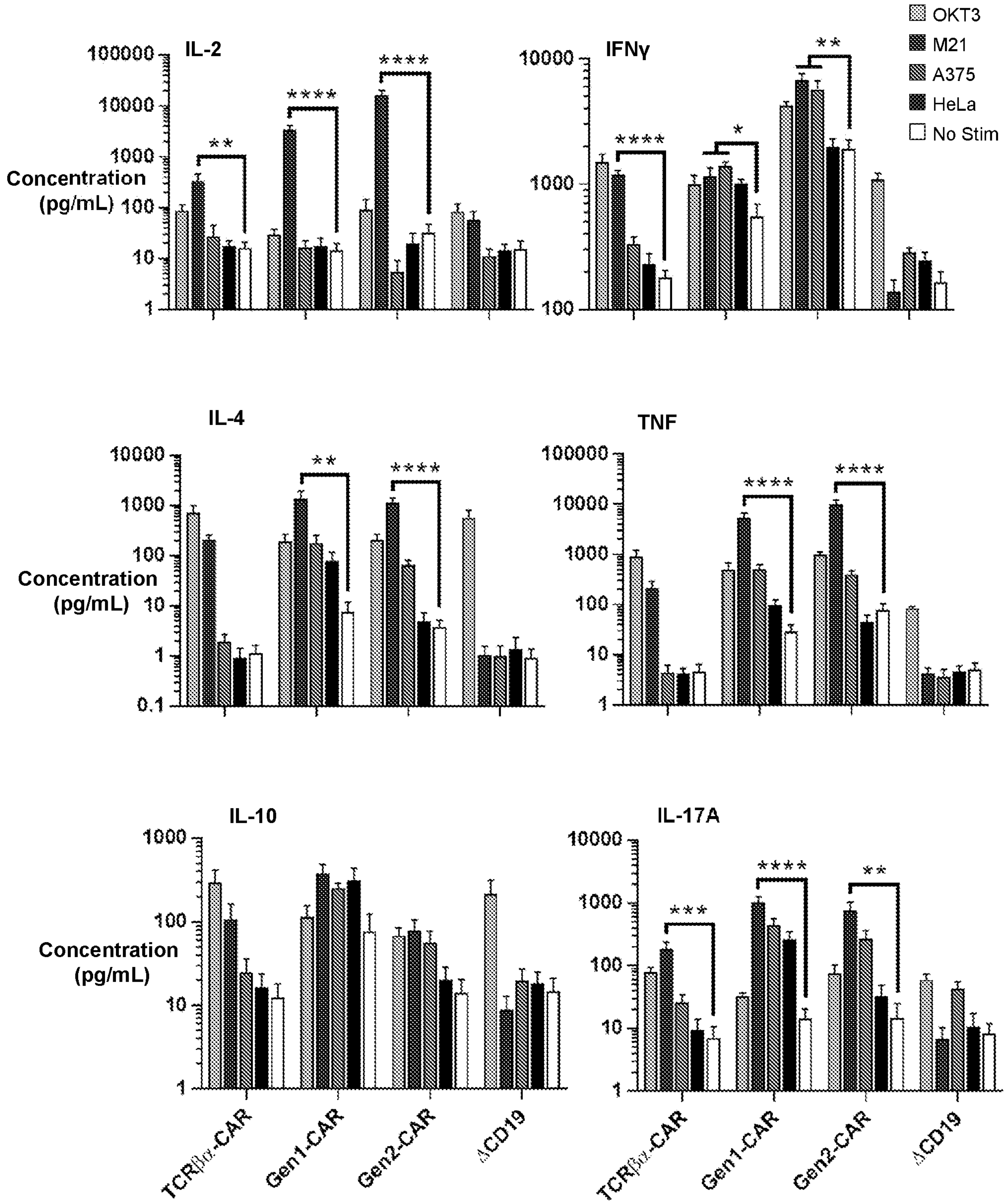


Fig. 7A

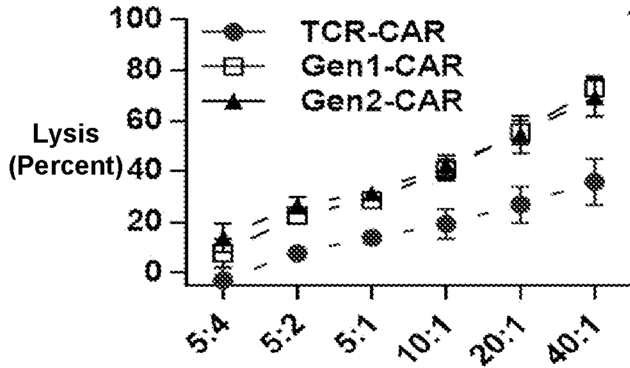


Fig. 7B

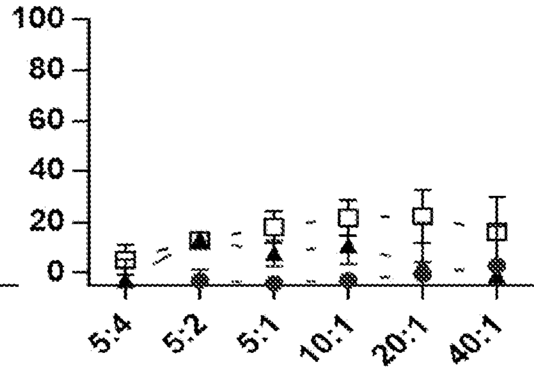


Fig. 7C

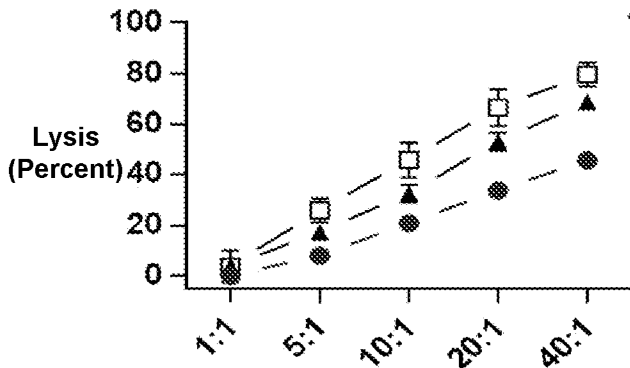


Fig. 7D

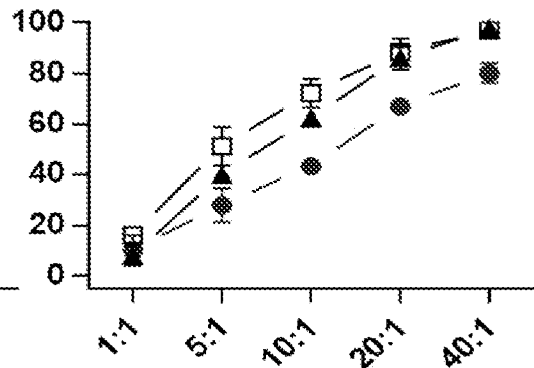


Fig. 7E

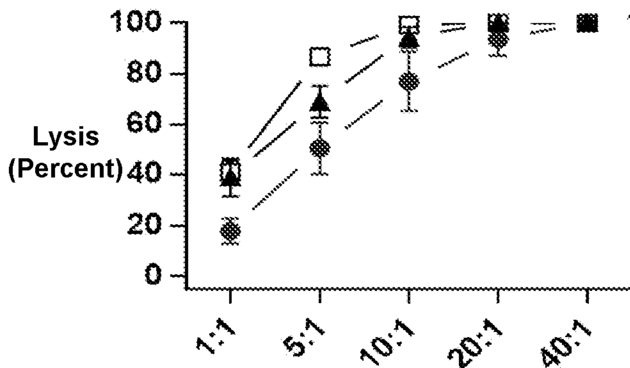


Fig. 7F

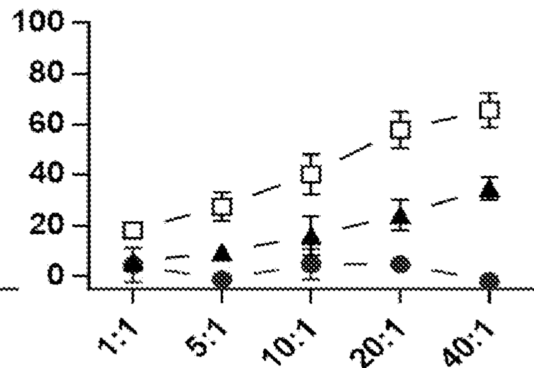


Fig. 8A

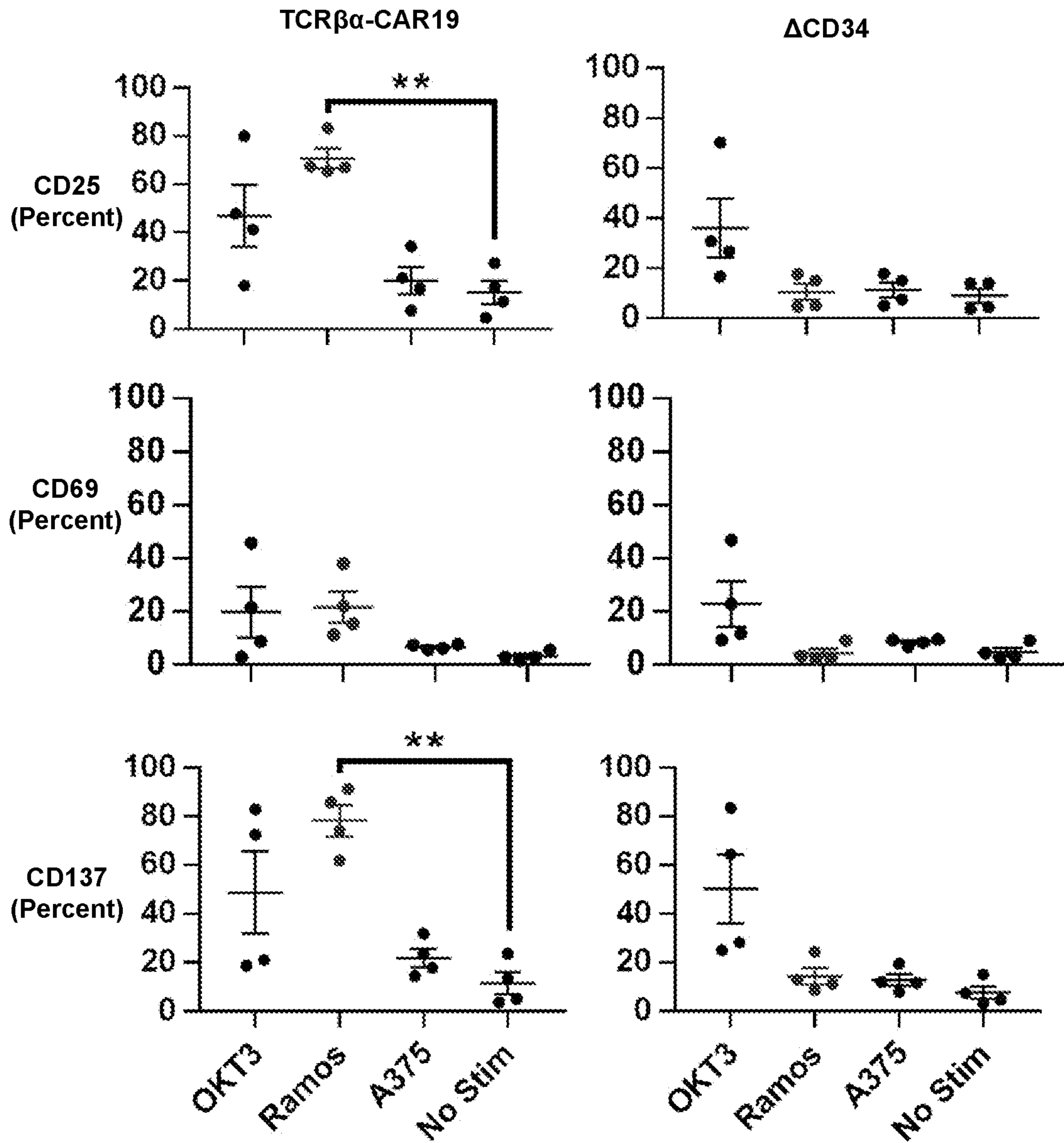


Fig. 8B

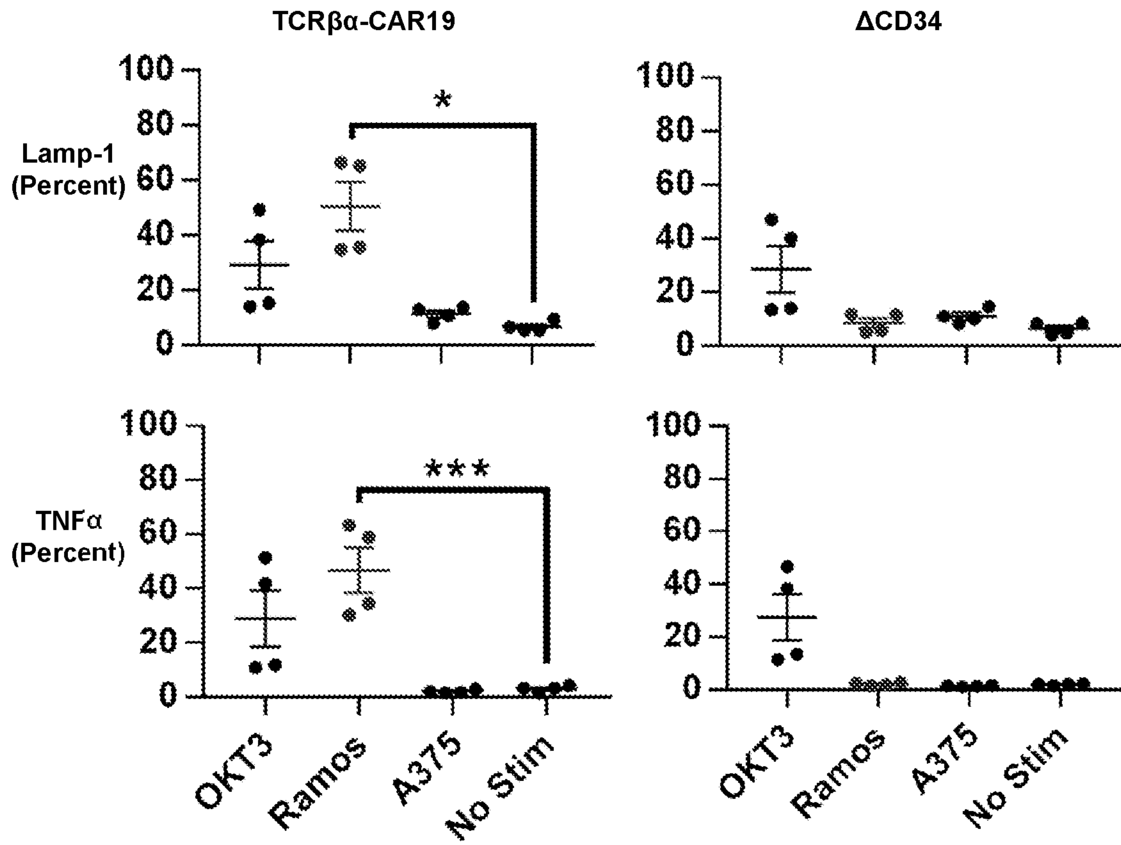


Fig. 8C

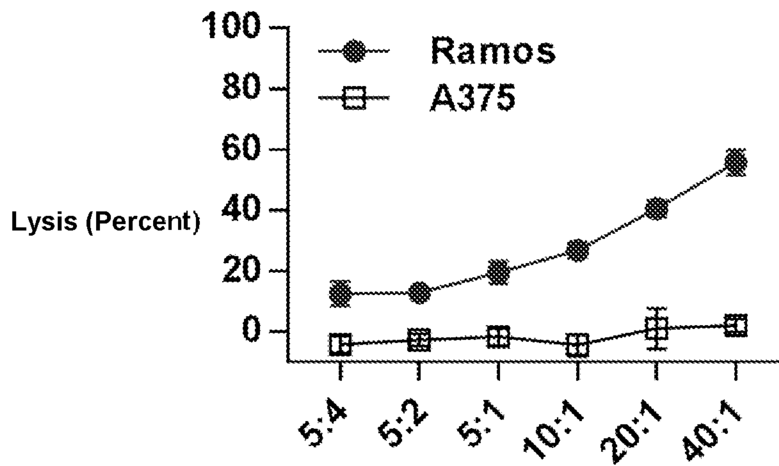
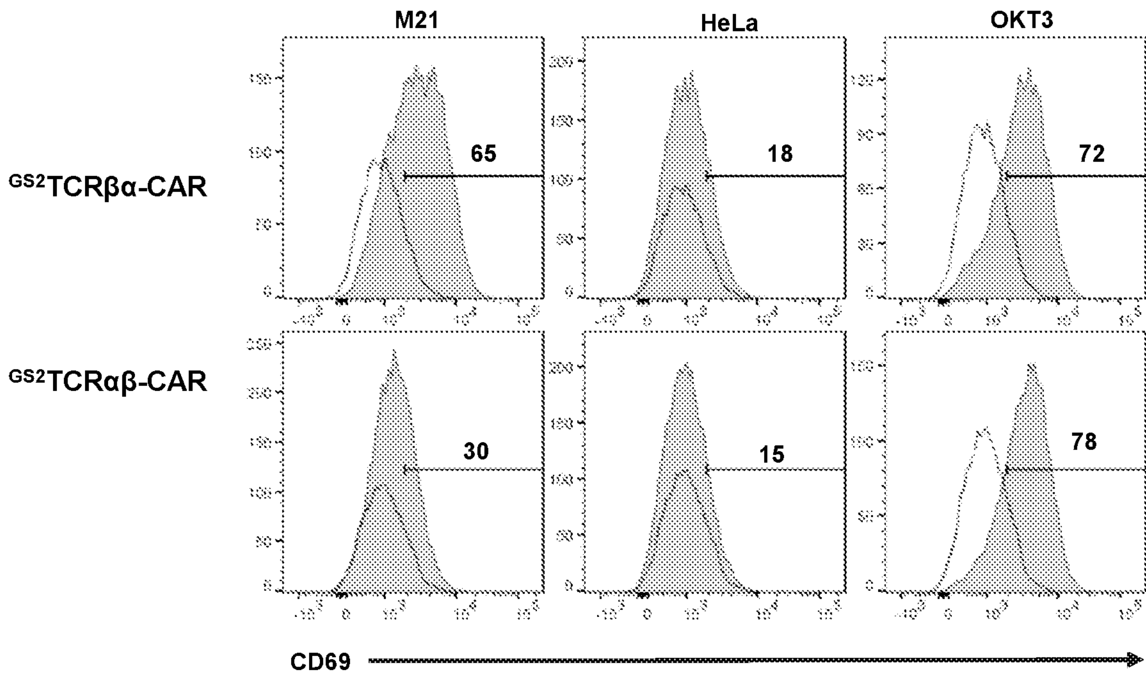


Figure 9A



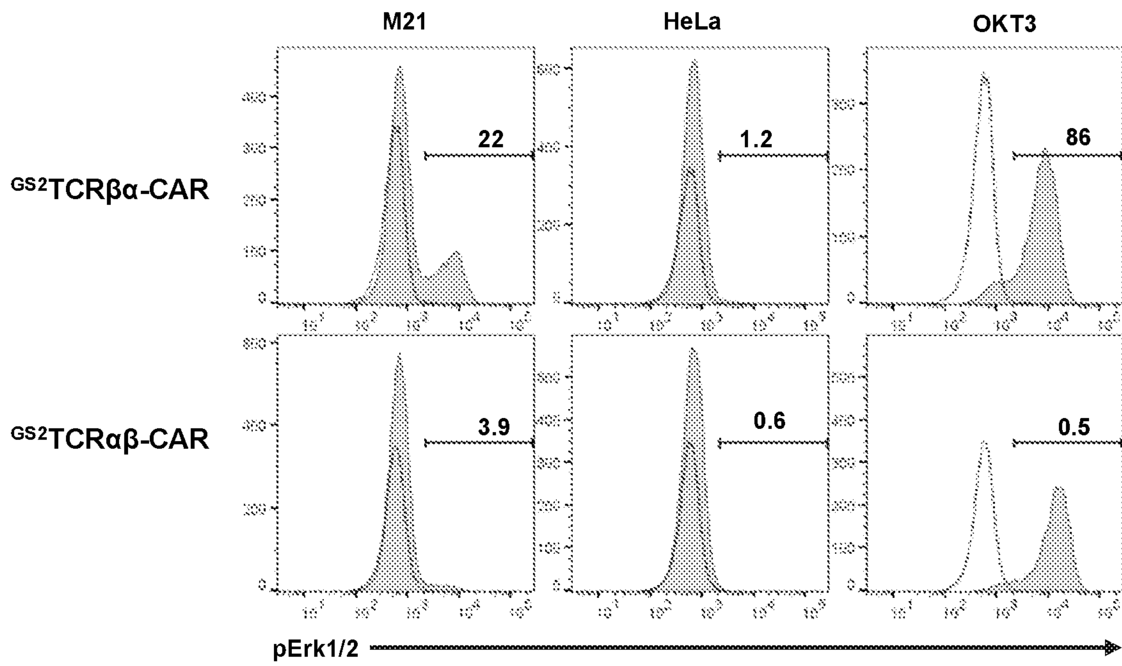


Figure 9B

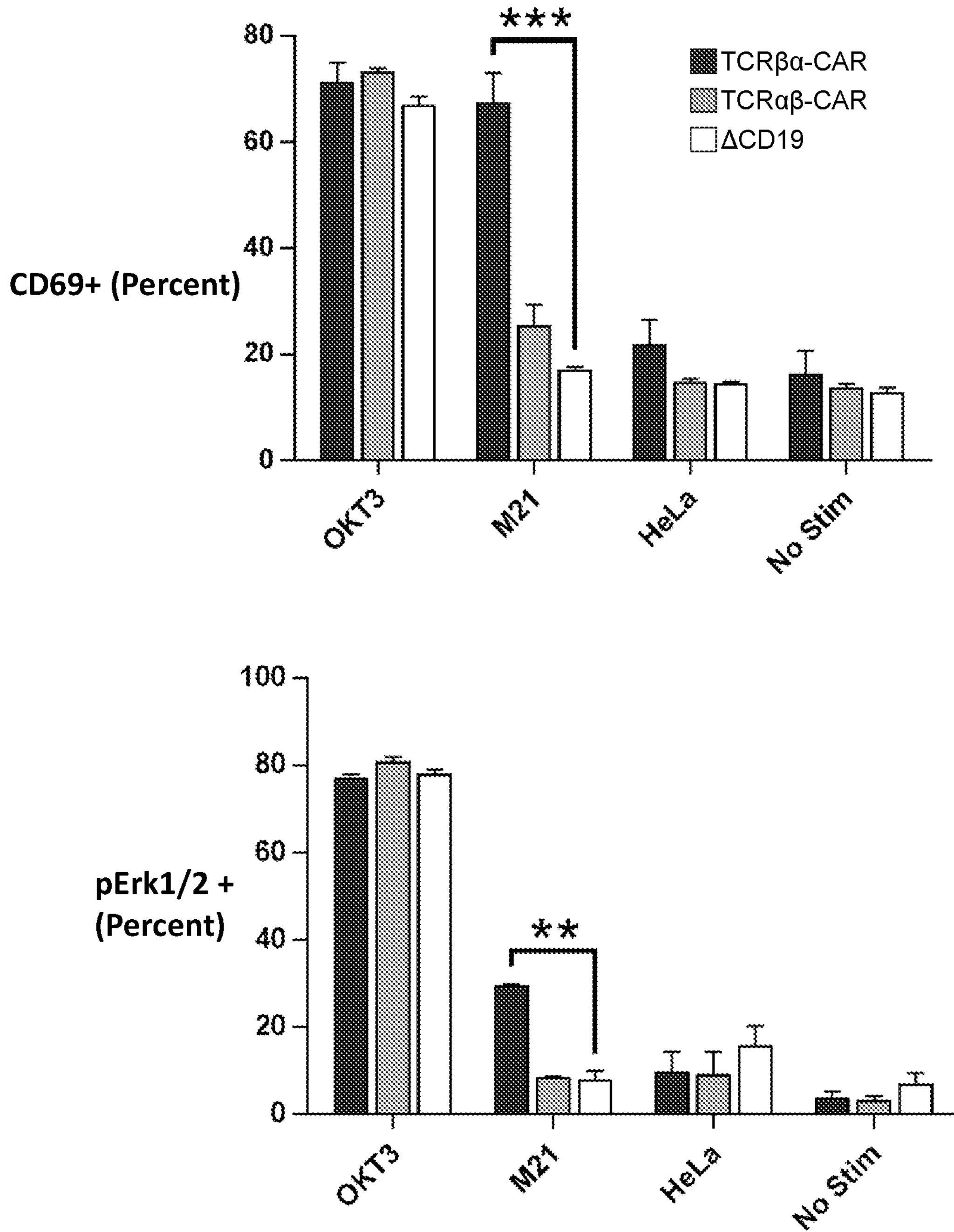


Figure 10A

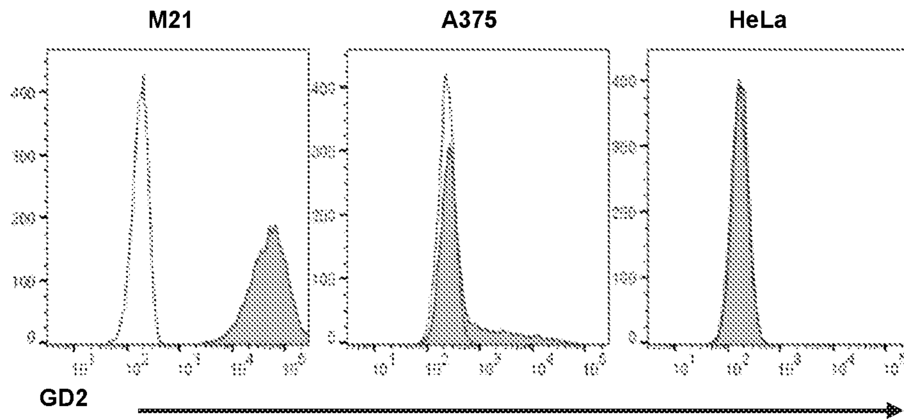


Figure 10B

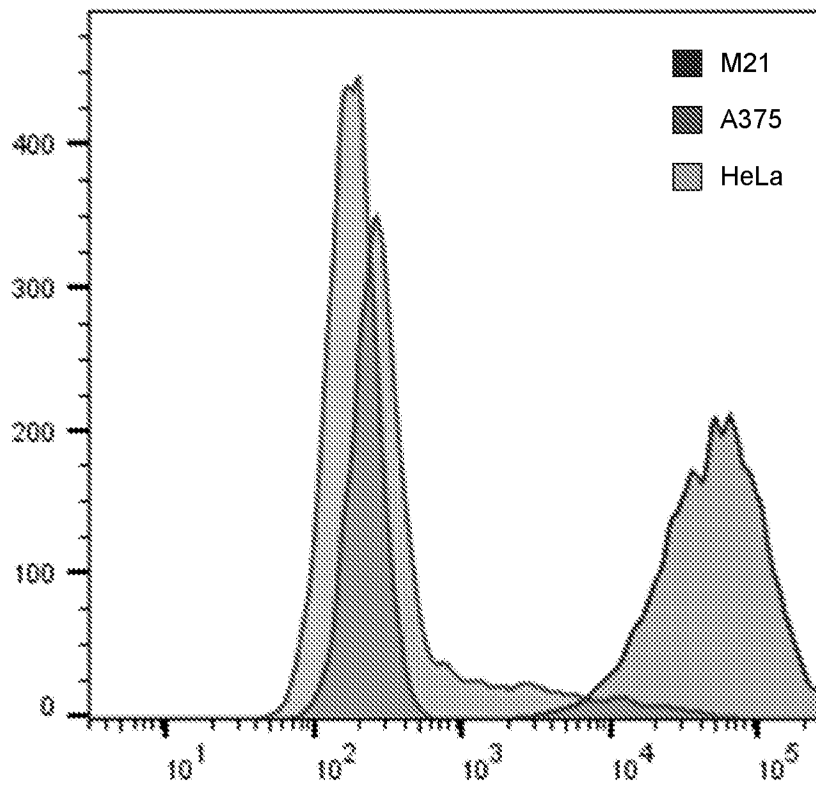
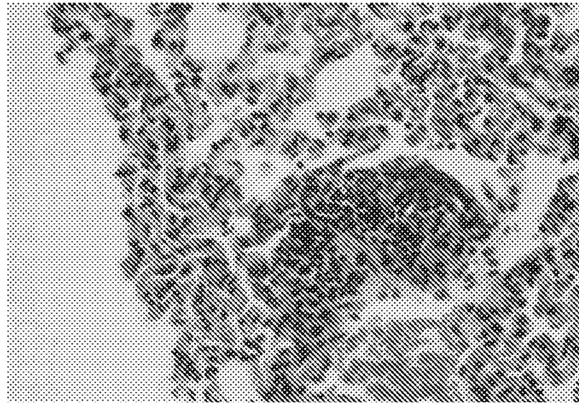


Figure 11



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/30567

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/39, A61P 31/00, C07K 14/705, C07K 16/30, C07K 19/00 (2017.01)
CPC - A61K 39/39, C07K 16/30, C12N 15/62, A61K 2039/57, C07K 2317/622, C07K 2319/75, A61K 47/6425, A61K 47/6849

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)
See Search History Document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	US 2006/0235201 A1 (KISCHEL) 19 October 2006 (19.10.2006) para [0001], [0019], [0022], [0023], [0025], [0031], [0033], [0038], [0039], [0051], [0057], [0066], [0109], [0112]	1, 3-6, 7/(1,3-6) --- (8-9,12-20)/(1,3-6) --- 2, (7-9)/2, 10-11, (12-20)/2
Y	US 2012/0114700 A1 (SAMELSON et al.) 10 May 2012 (10.05.2012) para [0009]	(8-9)/(1,3-6)
Y	WO 2015/188141 A9 (MEMORIAL SLOAN-KETTERING CANCER CENTER et al.) 10 December 2015 (10.12.2015) p 26, ln 25- 29; p 36, ln 1-4; p 48, ln 13-30; p 65, ln 32 to p 66, ln 5; p 75, ln 6-8	(12-20)/(1,3-6)
A	US 2015/0274827 A1 (Pfizenmaier et al.) 1 October 2015 (01.10.2015) SEQ ID NO: 31	2, (7-20)/2
A	US 2002/0102233 A1 to (Ashkenazi) 1 August 2002 (01.08.2002) SEQ ID NO: 9	2, (7-20)/2
A	US 8,815,247 B2 (Biocon Limited) 26 August 2014 (26.08.2014) SEQ ID NOs: 9, 24	2, (7-20)/2, 11/(1,3-6)

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
6 September 2017

Date of mailing of the international search report
25 SEP 2017

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer:
Lee W. Young
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/30567

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GenBank Accession No. DQ904462, 26 September 2006 [online]. [Retrieved on 6 September 2017]. Retrieved from the internet: <URL: https://www.ncbi.nlm.nih.gov/nuccore/DQ904462> Entire document	10/(1,3-6)
A	JP2011019467 A (KOKURITSU IYAKUHIN SHOKUHIN EISEI KENKYUSHO) 3 February 2011 (03.02.2011) SEQ ID NO: 18	10/(1,3-6)
A	US 2012/0122182 A1 (Tannous et al.) 17 May 2012 (17.05.2012) SEQ ID NO: 19	10/(1,3-6)
A	GenBank Accession No. AB590869, 25 September 2010 [online]. [Retrieved on 6 September 2017]. Retrieved from the internet: <URL: https://www.ncbi.nlm.nih.gov/nuccore/307686186?sat=3&satkey=15299530> Entire document	11/(1,3-6)
A	GenBank Accession No. AY891204, 21 March 2005 [online]. [Retrieved on 6 September 2017]. Retrieved from the internet: <URL: https://www.ncbi.nlm.nih.gov/nuccore/61368215?sat=4&satkey=9084986> Entire document	11/(1,3-6)
A	MORGAN et al., Cancer Regression in Patients After Transfer of Genetically Engineered Lymphocytes. Science. 6 October 2006, Vol 314, No 5796, pp 126-129 (author manuscript pp 1 -10). Especially p 1, para 2	11/(1,3-6)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/30567

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.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 21
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

------- please see extra sheet -------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of: Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-20, drawn to a construct comprising 4-1BB ligand (4-1BBL) fused to a peptide that is capable of specifically binding a tumor-specific antigen, nucleic acids and vectors encoding said construct, modified T lymphocytes expressing said construct, and methods of using said modified T lymphocyte to treat cancer

Group II: Claims 22-42, drawn to a chimeric antigen receptor (CAR), nucleic acids and vectors encoding said CAR, modified T lymphocytes expressing said CAR, and methods of using said modified T lymphocyte to treat cancer

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I requires a construct comprising 4-1BB ligand (4-1BBL) fused to a peptide that is capable of specifically binding a tumor-specific antigen, not required by Group II.

Group II requires a chimeric antigen receptor (CAR) comprising: a T-cell receptor beta chain constant region and a T-cell receptor alpha chain constant region, wherein the T-cell receptor beta chain constant region is fused to a peptide having affinity to an antigen, wherein the chimeric antigen receptor does not include a T-cell receptor beta chain variable region and a T-cell alpha chain variable region, and wherein the T-cell receptor beta chain constant region is complexed with the T-cell receptor alpha chain constant region, not required by Group I.

Common Technical Features

The feature shared by Groups I and II is nucleic acids and vectors encoding an anti-cancer construct, modified T lymphocytes expressing CAR, and methods of using said modified T lymphocyte to treat cancer.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by WO 2015/188141 A9 to Sloan Kettering Inst Cancer et al. (hereinafter 'Sloan Kettering').

Sloan Kettering discloses nucleic acids and vectors encoding an anti-cancer construct (p 8, ln 14-16 "The presently disclosed subject matter further provides nucleic acids encoding the presently disclosed CARs, and vectors comprising the nucleic acids. In one embodiment, the vector is a gamma-retroviral vector"; p 8, ln 30-33 "Also provided are kits for treating or preventing a neoplasia, . . . comprising nucleic acids comprising the presently disclosed CARs"; p 72, ln 13-18 "Also included in the presently disclosed subject matter are extracellular antigen-binding domains that specifically binds to human mesothelin (e.g., a scFv, such as a scFv derived from antibody m912, a Fab, or a (Fab)2), CD3.zeta., CD8, CD28, 4- IBB, 4-1 BBL, IL-12, Mz, M28z, MBBz polypeptides or fragments thereof, and polynucleotides encoding thereof that are modified in ways that enhance their antineoplastic activity when expressed in an immunoresponsive cell").

Sloan Kettering further discloses modified T lymphocytes expressing CAR (p 26, ln 25- 29 "The presently disclosed subject matter also provides immunoresponsive cells (e.g., T cell, a Natural Killer (NK) cell, a cytotoxic T lymphocyte (CTL), a regulatory T cell, a human embryonic stem cell, and a pluripotent stem cell from which lymphoid cells may be differentiated) expressing the mesothelin-targeted CARs, and methods of using such immunoresponsive cells for treating neoplasia and other pathologies"; p 65, ln 32 to p 66, ln 5 "Genetic modification of immunoresponsive cells (e.g., T cells, CTL cells, NK cells) can be accomplished by transducing a substantially homogeneous cell composition with a recombinant DNA or RNA construct. In one embodiment, the vector is a retroviral vector (e.g., gamma retroviral or lentiviral) is employed for the introduction of the DNA or RNA construct into the host cell genome. For example, a polynucleotide encoding the mesothelin-specific CAR can be cloned into a retroviral vector and expression can be driven from its endogenous promoter, from the retroviral long terminal repeat, or from an alternative internal promoter.").

Sloan Kettering further discloses methods of using said modified T lymphocyte to treat cancer (p 26, ln 25- 29 "The presently disclosed subject matter also provides immunoresponsive cells (e.g., T cell, a Natural Killer (NK) cell, a cytotoxic T lymphocyte (CTL), a regulatory T cell, a human embryonic stem cell, and a pluripotent stem cell from which lymphoid cells may be differentiated) expressing the mesothelin-targeted CARs, and methods of using such immunoresponsive cells for treating neoplasia and other pathologies"; p 75, ln 6-8 "Mesothelin-specific CARs and immunoresponsive cells expressing thereof of the presently disclosed subject matter can be provided systemically or directly to a subject for treating or preventing a neoplasia").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Groups I and II therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

NOTE:
Claim 6 is objected to for lack of antecedent basis. As drafted, claim 6 depends from claim 5, but claim 5 fails to recite a "tumor-specific antigen". For the purposes of this ISA Search and Written Opinion, claim 6 is construed as though depending from claim 1.

Claim 15 is objected to for lack of antecedent basis. As drafted, claim 15 depends from claim 14, but claim 14 fails to recite an "antigen". For the purposes of this ISA Search and Written Opinion, claim 15 is construed as though depending from claim 13.