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(54) Title: CAR T CELL THERAPY AND IFN GAMMA

(57) Abstract: Provided herein are, *inter alia*, compositions comprising chimeric antigen receptor (CAR)-engineered immune cells, methods of formulating, and methods useful for treating cancer and leukemia.



CAR T CELL THERAPY AND IFN GAMMA

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Application Serial No. 63/168,210, filed on March 30, 2021. The entire contents of the foregoing are incorporated
5 herein by reference.

TECHNICAL FIELD

This disclosure concerns chimeric antigen receptor (CAR)-engineered immune cells, methods of formulating, and methods of use.

BACKGROUND

10 Glioblastoma (GBM) is among the deadliest cancers with very limited therapeutic options (1, 2, 3). Despite aggressive standard-of-care therapies, tumor recurrence is almost inevitable and uniformly lethal, with most patients not surviving beyond two years from diagnosis. Advances in immunotherapy have inspired efforts to develop therapeutic strategies for eliciting anti-tumor immune responses in GBM, including adoptive transfer of chimeric antigen receptor (CAR) T
15 cells. Clinical studies evaluating CAR T cells in GBM have demonstrated early evidence of safety and bioactivity in selected patients; nevertheless, the responses have been limited. Challenges for productive CAR T cell therapy for solid tumors such as GBM are multifactorial. Tumor heterogeneity and cellular plasticity allows for outgrowth of antigen loss tumor variants, leading to treatment failure. The tumor microenvironment, for GBM tumors, are myeloid-rich
20 with scant T cell population, which also poses specific challenges to CAR T cells.

IL13R α 2-CAR T therapy has shown some promise in treating GBM despite the non-uniform expression of IL13R α 2 by tumor cells (4). The response was associated with increase in CNS inflammatory cytokines and infiltration of endogenous immune cells (4). In line with this observation, a recent longitudinal analysis of immune-monitoring after HER2-CAR T cell
25 therapy showed evidence of endogenous immune reactivity which may have contributed to the patient's favorable response (5).

Pro-inflammatory cytokines secreted by CAR T cells, such as IFN γ , may play an important role in activation and programming of the immune infiltrates in GBM TME. IFN γ can activate macrophage (6) and microglia (7), recruit and activate cytotoxic T cells, polarize CD4 $^{+}$ T cells into Th1 effector cells and impair tumor-promoting Treg development and function (8, 9, 10).

- 5 IFNs can additionally act as a key signal (30) to facilitate the activation and priming of tumor reactive T cells (11).

SUMMARY

Described herein are immune system cells, e.g., T cells or NK cells, that express both a CAR targeted to a tumor antigen and human IFN γ that is encoded by a nucleic acid molecule

- 10 (“recombinant human IFN γ ”), e.g., immune cells harboring a nucleic acid molecule that encodes both a CAR and human IFN γ . Without being bound by any theory it appears that the co-expression increases one or more of activation of the immune cells, proliferation of the immune cells and tumor cell killing by endogenous cells that recognize tumor cells. The CAR can include a targeting domain that is an scFv targeted to a tumor antigen (e.g., an scFv targeted to CD19) or
15 a ligand (e.g., IL-13 or a variant thereof) that binds a receptor on tumor cells. Thus, the cells can harbor a nucleic acid molecule that encodes a CAR and human IFN γ . Expression of the CAR and the human IFN γ can be under the control of the same expression control sequences or under the control of different expression control sequences. The cells can harbor a nucleic acid molecule that encodes a single amino acid sequence that includes a CAR and human interferon gamma.
20 For example, the amino acid sequence of the CAR can be followed by a ribosomal skip sequence and then an amino acid sequence that includes human IFN γ . The amino acid sequence can include at least one signal sequence for secretion of a protein (e.g., a signal sequence for secretion of the CAR and a signal sequence for expression of the human IFN γ). In some embodiments, a nucleic acid of the disclosure can be a non-endogenous nucleic acid.

- 25 Immune cells that express a CAR and interferon can target and kill cancer cells expressing the target of the CAR. In addition, they can activate killing of cancer cells that do not express the target of the CAR by, for example, activating innate and adaptive immune subsets in tumor microenvironment. In this manner, they are useful for treating tumors that include both

cancer cells expressing the target of the CAR and cancer cells that do not express the target of the CAR or have very low expression of the target of the CAR.

The human IFN γ can comprise the following amino acid sequence:

QDPYVKEAENLKKEYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFYFKLFKNFKDDQSIQKSVETIKEDMNV
5 KFFNSNKKKRDDFEKLTNYSVTDLNVQRKAIHELIQVMAELSPAAGTGRKRKRSQMLFRGRRASQ (SEQ ID NO: 1)

The human IFN γ amino acid sequence can be preceded by a signal sequence that directs secretion of the human interferon gamma from a eukaryotic cell, e.g., a human cell. Thus, human interferon gamma precursor can be used (signal sequence underlined):

MKYTSYILAFQLCIVLGLSLGCYCQDPYVKEAENLKKEYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFYFKLFK
10 NFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTNYSVTDLNVQRKAIHELIQVMAELSPAAGTGRKRKRSQMLFR
GRRASQ (SEQ ID NO: B)

The CAR can be targeted to a tumor antigen, not limiting examples of which include:

A suitable IL-13 CAR comprises a variant of human IL-13 comprising the following amino acid sequence:

15 GPGPVPPSTALRYLIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALESINLVSGCSAIEKTQRMLSGFCPHKVSAG
QFSSLHVRDTKIEVAQFVKDLLLLHLKKLFREGFRN (SEQ ID NO: C)

Sequence of wild-type human IL13 (signal sequence underlined):

20 MHPLLNPILLALGLMALLTTVIALTCLGGFASPGPVPPSTALRELIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAA
LESINLVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLLHLKKLFREGFRN (SEQ ID NO:D)

The IL-13 CAR can include a variant IL13 comprising, for example, SEQ ID NO:C; a spacer (e.g., comprising any of SEQ ID NOs: 2-12); a transmembrane domain (e.g., comprising any of
25 SEQ ID NOs: 13-20); a co-stimulatory domain (comprising any of SEQ ID NOs: 22-25); optionally a linker of 3-15 amino acids (e.g., GGG); and a CD3 zeta cytoplasmic domain (SEQ ID NO: 21 or a variant thereof comprising any of SEQ ID NOs: 50-56). A useful CAR can comprise any of SEQ ID NO: 70-76.

GPVPPSTALRYLIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALESLINVSG
 CSAIEKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLHLKCLFREGR
 FNESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPE
 VQFNWYVDGVEVHNAKTKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSR
 5 NKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVE
 WESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHIALH
 NHYTQKSLSLGKMLIVLGGVAGLLLFIGLGIFFKRGRKKLLYIFKQPFMRPV
 QTTQEEDGCSCRFPEEEEGGCELGGGRVKFSRSADAPAYQQGQNQLYNELNLGR
 REEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGER
 10 RRGKGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID NO:76

Described herein is a nucleic acid molecule comprising a nucleotide sequence encoding a
 chimeric antigen receptor (CAR), wherein the chimeric antigen receptor comprises: a targeting
 domain comprising the amino acid sequence of SEQ ID NO:C; a spacer, a transmembrane
 15 domain; a co-stimulatory domain; and a CD3 ζ signaling domain. In various embodiments: the
 transmembrane domain is selected from: a CD4 transmembrane domain or variant thereof having
 1-5 amino acid modifications, a CD8 transmembrane domain or variant thereof having 1-5 amino
 acid modifications, a CD28 transmembrane domain or a variant thereof having 1-5 amino acid
 modifications; the wherein the IL13 receptor targeting domain comprises or consists of the
 20 amino acid sequence of SEQ ID NO: C with up to 3 single amino acid substitutions (in some
 cases the Y at position 13 is not substituted); the costimulatory domain is selected from: a 41BB
 costimulatory domain or variant thereof having 1-5 amino acid modifications, a CD28
 costimulatory domain or variant thereof having 1-5 amino acid modifications; a CD28gg
 costimulatory domain or variant thereof having 1-5 amino acid modifications wherein the
 25 costimulatory domain is a 41BB costimulatory domain; the 41BB costimulatory domain
 comprises the amino acid sequence of SEQ ID NO: 24 or a variant thereof having 1-5 amino acid
 modifications; the CD3 ζ signaling domain comprises the amino acid sequence of SEQ ID NO:21
 or a variant thereof comprising any of SEQ ID NOs: 50-56; a linker of 3 to 15 amino acids is
 located between the 4-1BB costimulatory domain and the CD3 ζ signaling domain or variant
 30 thereof; the CAR comprises the amino acid sequence of SEQ ID NOs: 70-76 or a variant thereof

having 1-5 amino acid modifications; the CAR comprises or consists of an amino acid sequence that is least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to any of SEQ ID NOs: 70-76; the CAR comprises an amino acid sequence that has no more than 5, 4, 3, 2, or 1 single amino acid substitutions and or deletions compared to any of SEQ ID NOs: 70-76. Also described is an expression vector comprising any of the forgoing nucleic acid molecules. Also described is a viral vector comprising any of the forgoing nucleic acid molecules.

The CAR can comprise an scFv targeted to any cancer cell antigen, e.g., CD19, MUC16, MUC1, tMUC1, CAIX, CEA, CD20, CD22, CD30, HER-2, MAGEA3, p53, PSCA, BCMA, CD123, CD44V6, Integrin B7, ICAM-1, CD70, CEA, GD2, PSMA, B7H3, CD33, Flt3, CLL1, folate receptor, EGFR, CD7, EGFRvIII, glypican3, CD5, ROR1, CS1, AFP, CD133, and TAG-72. The CAR can comprise a ligand, e.g., an IL-13 or a variant thereof, a chlorotoxin or a variant thereof, etc.

Thus, useful CAR for co-expression include those described in: WO 2016/044811, WO 2017/079694, WO 2017/066481, and WO 2017/062628.

Also described is a population of human T cells, NK cells, myeloid cells, gamma delta T cells, or iPSC-derived effector cells containing any of the forgoing nucleic acid molecules. Also described is a population of human T cells containing any of the forgoing expression vectors or viral vectors. In various embodiments, the population of human T cells comprise central memory T cells, naive memory T cells, pan T cells, or PBMC substantially depleted for CD25+ cells and CD14+ cells.

Also described is a method of treating a patient suffering from a cancer (e.g., brain cancer (glioblastoma), pancreatic, melanoma, neuroblastoma, liver, sarcoma, colorectal, gastric, ovarian carcinoma, fallopian tube, thyroid, bladder, cervical, digestive system, head and neck, osteosarcoma, renal cell carcinoma, prostate cancer, breast cancer or lung cancer), comprising administering a population of autologous or allogeneic human T cells harboring a nucleic acid described herein. In various embodiments, the cells are administered locally or systemically; and are administered by single or repeat dosing.

Also described herein is a method of preparing CAR T cells comprising: providing a population of autologous or allogeneic human T cells and transducing the T cells by a vector comprising a nucleic acid molecule described herein.

5 Also described are T cells harboring a vector or nucleic acid expressing the CAR and IFN γ . In various embodiments: at least 20%, 30%, or 40% of the transduced human T cells are central memory T cells; at least 30% of the transduced human T cells are CD4 $^{+}$ and CD62L $^{+}$ or CD8 $^{+}$ and CD62L $^{+}$. In various embodiments: the population of human T cells comprise a vector expressing a chimeric antigen receptor comprising an amino acid sequence selected from SEQ
 10 ID NOs: C or 70-76 or a variant thereof having 1-5 amino acid modifications (e.g., 1 or 2) amino acid modifications (e.g., substitutions); the population of T cells can include one or more of effector T cells, effector memory cells, central memory T cells, stem central memory cells and naïve T cells; the population of human T cells comprises central memory T cells (TCM cells) e.g., at least 20%, 30%, 40%, 50% 60%, 70%, 80% of the cells are T CM cells, or the population
 15 of T cells comprises a combination of central memory T cells, naïve T cells and stem central memory cells (TCM/SCM/N cells) e.g., at least 20%, 30%, 40%, 50% 60%, 70%, 80% of the cells are T CM/SCM/N cells. In some embodiments, the population of T cells includes effector T cells and effector memory cells. In some embodiments, the population of T cells includes both CD4 $^{+}$ cells and CD8 $^{+}$ cells (e.g., at least 20% of the CD3 $^{+}$ T cells are CD4 $^{+}$ and at least 3% of
 20 the CD3 $^{+}$ T cells are CD8 $^{+}$ and at least 70, 80 or 90% are either CD4 $^{+}$ or CD8 $^{+}$; at least 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60% of the cells CD3 $^{+}$ cells are CD4 $^{+}$ and at least 4%, 5%, 8%, 10%, 20 of the CD3 $^{+}$ cells are CD8 $^{+}$ cells). In some embodiments, the population of human T cells are autologous to the patient. In some embodiments, the population of human T cells are allogenic to the patient. In some embodiments, T cells expressing a CAR and an IFN γ are called,
 25 *inter alia*, IL13R α 2-IFN γ CAR T cells, IL13R α 2-CAR/IFN γ T cells, and IL13 CAR T-IFN γ cells, interchangeably throughout.

In various embodiments: the spacer domain is selected from the group consisting of: and IgG4(EQ) spacer domain, a IgG4(HL-CH3) spacer domain and an IgG4(CH3) spacer domain; the spacer domain comprises SEQ ID NO: 10; the spacer domain comprises SEQ ID NO: 9; the
 30 spacer domain comprises SEQ ID NO: 12; the transmembrane domain is selected from the group

consisting of: a CD4 transmembrane domain, a CD8 transmembrane domain, and a CD28 transmembrane domain; the co-stimulatory domain is selected from a CD28 costimulatory domain, and CD28gg costimulatory domain, and a 41-BB co-stimulatory domain.

Also disclosed is a nucleic molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR), wherein the chimeric antigen receptor comprises: targeting domain comprising an amino acid sequence comprising a variant IL13 domain comprising 109, 110, 111, 112, 113 contiguous amino acids of SEQ ID NO: C or the entirety of SEQ ID NO: C with 1, 2, 3, 4 or 5 single amino acid changes; a spacer domain; a transmembrane domain; a costimulatory domain and a CD3 ζ signaling domain.

10 In various embodiments: the spacer domain comprises the amino acid sequence of any of SEQ ID NOs: 2-12; the costimulatory domain comprises the amino acid sequence of any of SEQ ID NOs: 22-25; and a CD3zeta domain or a variant thereof. In some cases the CAR comprises a CD28 co-stimulatory domain and a variant CD3zeta domain.

Also disclosed is: a vector or an expression vector comprising a nucleic acid molecule described herein; a population of human T cells or NK harboring a nucleic acid molecule described herein. In various embodiments: the population of human T cells comprise central memory T cells, naive memory T cells, pan T cells, or PBMC substantially depleted for CD25+ cells and CD14+ cells.

Also described is a method of treating a patient suffering from glioblastoma, pancreatic ductal adenocarcinoma, melanoma, ovarian carcinoma, renal cell carcinoma, breast cancer or lung cancer, comprising administering a population of autologous or allogeneic cells harboring a nucleic acid molecule described herein. In various embodiments: the cells are administered locally or systemically or intraventricularly; by single or repeat dosing.

Also described is a method of preparing CAR T cells comprising: providing a population of autologous or allogeneic human T cells or NK and transducing the cells with a vector comprising a nucleic acid molecule described herein.

Also described is a polypeptide encoded by a nucleic acid described herein.

In various embodiments, the NK cells are derived from cord blood, peripheral blood or stem cells.

The CAR or polypeptide can be expressed with additional sequences that are useful for monitoring expression, for example, a T2A or P2A skip sequence and a truncated EGFR or truncated CD19 or LNGFR (can consist of or comprise the amino acid sequence of SEQ ID NO:31).

A non-endogenous or exogenous nucleic acid molecule (or polypeptide) is a nucleic acid molecule (or polypeptide) that is not endogenously present in a cell. The term includes recombinant nucleic acid molecule (or polypeptide) expressed in a cell. An exogenous nucleic acid is a nucleic acid not present in a native wild-type cell; for example, an exogenous nucleic acid may vary from an endogenous counterpart by sequence, by position/location. An exogenous nucleic acid molecule can be introduced into a cell by genetic engineering, either into the cell or a progenitor of the cell. An exogenous nucleic acid molecule encoding a polypeptide can be linked to an expression control sequence and can include a sequence encoding a signal sequence, one or both of which can be heterologous to the sequence encoding the polypeptide.

Spacer Region

The CAR or polypeptide described herein can include a spacer located between the targeting domain (i.e., IL13 or variant thereof) and the transmembrane domain. A variety of different spacers can be used. Some of them include at least portion of a human Fc region, for example a hinge portion of a human Fc region or a CH3 domain or variants thereof. **Table 1** below provides various spacers that can be used in the CARs described herein.

Table 1: Examples of Spacers

Name	Length	Sequence
a3	3 aa	AAA
linker	10 aa	GGGSSGGGSG (SEQ ID NO:2)
IgG4 hinge (S→P) (S228P)	12 aa	ESKYGPPCPPCP (SEQ ID NO:3)
IgG4 hinge	12 aa	ESKYGPPCPSCP (SEQ ID NO:4)
IgG4 hinge (S228P)+ linker	22 aa	ESKYGPPCPPCPGGGSSGGGSG (SEQ ID NO:5)
CD28 hinge	39 aa	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPFPGPSKP (SEQ ID NO:6)
CD8 hinge-48aa	48 aa	AKPTTTPAPRPPTPAPTASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO:7)
CD8 hinge-45aa	45aa	TTTPAPRPPTPAPTASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO:8)
IgG4(HL-CH3) Also called IgG4(HL-ΔCH2) (includes S228P in hinge)	129 aa	ESKYGPPCPPCPGGGSSGGGSGGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGSSFFLYSRLTVDKSRWQEGNVFSCSV MHEALHNHYTQKSLSLGLK (SEQ ID NO:9)
IgG4(L235E,N297Q)	229 aa	ESKYGPPCPSCPAPEFEGGSPVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNATKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGSSFFLYSRLTVDKSRWQEGNVFSCSV MHEALHNHYTQKSLSLGLK (SEQ ID NO:10)
IgG4(S228P,L235E,N297Q)	229 aa	ESKYGPPCPPCPAPEFEGGSPVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNATKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGSSFFLYSRLTVDKSRWQEGNVFSCSV MHEALHNHYTQKSLSLGLK (SEQ ID NO:11)
IgG4(CH3) Also called IgG4(ΔCH2)	107 aa	GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGSSFFLYSRLTVDKSRWQEGNVFSCSV MHEALHNHYTQKSLSLGLK (SEQ ID NO:12)
IgG1 Hinge	16 aa	LEPKSCDKTHTCPPCP (SEQ ID NO: 44)

Some spacer regions include all or part of an immunoglobulin (e.g., IgG1, IgG2, IgG3, IgG4) hinge region, i.e., the sequence that falls between the CH1 and CH2 domains of an immunoglobulin, e.g., an IgG4 Fc hinge or a CD8 hinge. Some spacer regions include an immunoglobulin CH3 domain (called CH3 or Δ CH2) or both a CH3 domain and a CH2 domain.

5 The immunoglobulin derived sequences can include one or more amino acid modifications, for example, 1, 2, 3, 4 or 5 substitutions, e.g., substitutions that reduce off-target binding.

The spacer region can also comprise an IgG4 hinge region having the sequence ESKYGPPCPCP (SEQ ID NO:4) or ESKYGPPCPPCP (SEQ ID NO:3). The spacer region can also comprise the hinge sequence ESKYGPPCPPCP (SEQ ID NO:3) followed by the linker

10 sequence GGGSSGGGSG (SEQ ID NO:2) followed by IgG4 CH3 sequence GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLD SDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGLGK (SEQ ID NO:12). Thus, the entire spacer region can comprise the sequence:

15 ESKYGPPCPPCPGGGSSGGGSGGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHY TQKSLSLGLGK (SEQ ID NO:11).

Transmembrane Domain

A variety of transmembrane domains can be used in the CAR. In some cases, the transmembrane domain is a CD28 transmembrane domain that includes a sequence that is at least 90%, at least

20 95%, at least 98% identical to or identical to: FWVLVVGGVLACYLLVTVAFIIFWV (SEQ ID NO:14). In some cases, the CD28 transmembrane domain has 1, 2, 3, 4 of 5 amino acid changes (preferably conservative) compared to SEQ ID NO:14. **Table 2** includes examples of suitable transmembrane domains. Where a spacer region is present, the transmembrane domain (TM) is located carboxy terminal to the spacer region.

25 **Table 2: Examples of Transmembrane Domains**

Name	Accession	Length	Sequence
CD3z	J04132.1	21 aa	LCYLLDGILFIYGVILTALFL (SEQ ID NO:13)

CD28	NM_006139	27aa	FWVLVVVGGVLACYSLLVTVAFIIFWV (SEQ ID NO:14)
CD28(M)	NM_006139	28aa	MFWWLVVVGGVLACYSLLVTVAFIIFWV (SEQ ID NO:15)
CD4	M35160	22aa	MALIVLGGVAGLLFIGLGIFF (SEQ ID NO:16)
CD8tm	NM_001768	21aa	IYIWAPLAGTCGVLLSLVIT (SEQ ID NO:17)
CD8tm2	NM_001768	23aa	IYIWAPLAGTCGVLLSLVITLY (SEQ ID NO:18)
CD8tm3	NM_001768	24aa	IYIWAPLAGTCGVLLSLVITLYC (SEQ ID NO:19)
41BB	NM_001561	27aa	IISFFLALTSTALLFLFF LTLRFVV (SEQ ID NO:20)
NKG2D	NM_007360	21aa	PFFFCFIAVAMGIRFIIMVA (SEQ ID NO:65)

Costimulatory Domain

- The costimulatory domain can be any domain that is suitable for use with a CD3 ζ signaling domain. In some cases, the co-signaling domain is a CD28 co-signaling domain that includes a sequence that is at least 90%, at least 95%, at least 98% identical to or identical to: RSKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRS (SEQ ID NO: 22). In some cases, the 4-1BB co-signaling domain has 1, 2, 3, 4 of 5 amino acid changes (preferably conservative) compared to SEQ ID NO:22.
- 10 The costimulatory domain(s) are located between the transmembrane domain and the CD3 ζ signaling domain. **Table 3** includes examples of suitable costimulatory domains together with the sequence of the CD3 ζ signaling domain.

Table 3: CD3 ζ Domain and Examples of Costimulatory Domains

Name	Accession	Length	Sequence
CD3 ζ	J04132.1	113 aa	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLYNELOKDKMAEAYSEIGMKGERR RGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:21) ITAMS 1-3 underlined
CD3 ζ variant		113 aa	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLFNELOKDKMAEAFSEIGMKGERR RGKGHDGLFQGLSTATKDTFDALHMQALPPR (SEQ ID NO:50)
CD3 ζ variant		113 aa	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLFNELOKDKMAEAFSEIGMKGERR RGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:51)
CD3 ζ variant		113 aa	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLYNELOKDKMAEAYSEIGMKGERR RGKGHDGLFQGLSTATKDTFDALHMQALPPR (SEQ ID NO:52)
CD3 ζ variant		113 aa	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLYNELOKDKMAEAFSEIGMKGERR RGKGHDGLFQGLSTATKDTFDALHMQALPPR (SEQ ID NO:53)
CD3 ζ variant		113 aa	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLFNELOKDKMAEAYSEIGMKGERR RGKGHDGLFQGLSTATKDTFDALHMQALPPR (SEQ ID NO:54)
CD3 ζ variant		113 aa	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLFNELOKDKMAEAFSEIGMKGERR RGKGHDGLYQGLSTATKDTFDALHMQALPPR (SEQ ID NO:55)
CD3 ζ variant		113 aa	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLFNELOKDKMAEAFSEIGMKGERR RGKGHDGLFQGLSTATKDTYDALHMQALPPR (SEQ ID NO:56)
CD28	NM_006139	42aa	RSKRSRLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS (SEQ ID NO: 22)

CD28gg*	NM_006139	42aa	R5KRSRGGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYR S (SEQ ID NO:23)
41BB	NM_001561	42 aa	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO:24)
OX40	NM_003327	42 aa	ALYLLRRDQRLPPDAHKKPPGGGSFRTPIQEEQADAHSTLAKI (SEQ ID NO:25)
2B4	NM_016382	120 aa	WRRKRKEKQSETSPKEFLTIYEDVKDLKTRRNHEQEQTFFGG GSTIYSMIQSQSSAPTSQEPAYTLYSLIQPSRKSGSRKRNHSPS FNSTIYEVIGKSQPKAQNPRLSRKELENFDVYS (SEQ ID NO:66)

In various embodiments: the costimulatory domain is selected from the group consisting of: a costimulatory domain depicted in **Table 3** or a variant thereof having 1-5 (e.g., 1 or 2) amino acid modifications, a CD28 costimulatory domain or a variant thereof having 1-5 (e.g., 1 or 2) amino acid modifications, a 4-1BB costimulatory domain or a variant thereof having 1-5 (e.g., 1 or 2) amino acid modifications and an OX40 costimulatory domain or a variant thereof having 1-5 (e.g., 1 or 2) amino acid modifications. In certain embodiments, a 4-1BB costimulatory domain or a variant thereof having 1-5 (e.g., 1 or 2) amino acid modifications is present. In some embodiments there are two costimulatory domains, for example a CD28 co-stimulatory domain or a variant thereof having 1-5 (e.g., 1 or 2) amino acid modifications (e.g., substitutions) and a 4-1BB co-stimulatory domain or a variant thereof having 1-5 (e.g., 1 or 2) amino acid modifications (e.g., substitutions). In various embodiments the 1-5 (e.g., 1 or 2) amino acid modification are substitutions. The costimulatory domain is amino terminal to the CD3 ζ signaling domain and a short linker consisting of 2 – 10, e.g., 3 amino acids (e.g., GGG) is can be positioned between the costimulatory domain and the CD3 ζ signaling domain.

CD3 ζ Signaling Domain

The CD3 ζ signaling domain can be any domain that is suitable for use with a CD3 ζ signaling domain. In some cases, the CD3 ζ signaling domain includes a sequence that is at least 90%, at least 95%, at least 98% identical to or identical to:

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLKRRGRDPEMGGKPRRKNPQEG
LYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR
(SEQ ID NO:21). In some cases, the CD3 ζ signaling domain has 1, 2, 3, 4 of 5 amino acid

changes (preferably conservative) compared to SEQ ID NO:21. In some case the CD3 ζ signaling domain comprises any of SEQ ID NOs: 50-56. These variant CD3 ζ signaling domains have Y to F mutations in one or more ITAM domains. In some cases it is preferable to use a variant with mutations that inactive ITAMs 2 and 3.

5 Human IFN γ

The IFN γ domain is a domain that includes at least a functional portion of mature human IFN γ (e.g., amino acids 24-161 human IFN γ ; GenBank NP_000610) or a functional portion of mature human IFN γ .

Mature human IFN γ has the sequence: QDPYVKE AENLKKEYFNA GHSDVADNGT

10 LFLGILKNWKEESDRKIMQS QIVSFYFKLF KNFKDDQSIQ KSVETIKEDM

NVKFFNSNKK KRDDFEKLTNYSVTDLNVQR KAIHELIQVM AELSPAAGTG

KRKRSQMLFR GRRASQ (mature IFN γ ; SEQ ID NO:1). Immature human IFN γ (includes a signal sequence) has the sequence: MKYTSYILAF QLCIVLGSLG CYCQDPYVKE

AENLKKEYFNA GHSDVADNGT LFLGILKNWK EESDRKIMQS QIVSFYFKLF

15 KNFKDDQSIQ KSVETIKEDM NVKFFNSNKK KRDDFEKLTN YSVTDLNVQR

KAIHELIQVM AELSPAAGTG KRKRSQMLFR GRRASQ (SEQ ID NO: B). In some embodiments, a human IFN γ comprises the sequence:

QDPYVKEAENLKKEYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFYFKLFLK

NFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTNYSVTDLNVQRKAIHELIQVM

20 AELSPAAGTGKRKRSQ (SEQ ID NO:Z)

In some cases, the IFN γ domain has 1, 2, 3, 4 or 5 amino acid changes (preferably conservative) compared to SEQ ID NO:1 or SEQ ID NO:B or SEQ ID NO: Z. For example, 1, 2 or all 3 of the following amino acid changes can be made in SEQ ID NO: 1 or SEQ ID NO:Z: K74A, E75Y

and N83R. In some embodiments, an IFN γ domain provided herein comprise an amino acid

25 sequence having at least 95% identity to SEQ ID NO: 1 or SEQ ID NO:B or SEQ ID NO: Z. In

some embodiments, an IFN γ comprises at least one amino acid substitution at a position

corresponding to an amino acid residue selected from Q1, D2, P3, K6, Q64, Q67, K68, E71, T72, K74, E75, D76, N78, V79, K80, N83, S84, K86, R89, D90, and any combination thereof of SEQ ID NO: 1 or SEQ ID NO:Z.

In some embodiments, an IFN γ domain provided herein comprise an amino acid sequence having at least 95% identity to SEQ ID NO: 1 or SEQ ID NO: Z, and further including at least one amino acid substitution at a position corresponding to an amino acid residue selected from Q1, D2, P3, K6, Q64, Q67, K68, E71, T72, K74, E75, D76, N78, V79, K80, N83, S84, K86, R89, D90, and any combination thereof of SEQ ID NO: 1 or SEQ ID NO: Z.

In some embodiments, a variant of IFN γ can also be used. A number of IFN γ variants are known in the art and can be useful (Mendoza JL, et. al., (2019) *Nature* 567:56; WO 2020/028275).

Truncated EGFR or truncated CD19 or LNGFR

In some embodiments, a CAR or peptide described herein can comprise a ribosomal skip sequence (e.g., LEGGGEGRGSLLTCGDVEENPGPR; SEQ ID NO:27) and a truncated EGFR having a sequence that is at least 90%, at least 95%, at least 98% identical to or identical to: LVTSLLLCELPHPAFLIPRKVCNGIGIGEFKDSLSINATNIKHFKNCTISISGDLHILPVAFR GDSFTHTPPLDPQELDILKTVKEITGFLLIQAWPENRTDLHAFENLEIIRGRTKQHGGQFSL AVVSLNITSLGLRSLKEISDGDVIISGNKNLCYANTINWKKLFGTSGQKTKIISNRGENSC KATGQVCHALCSPEGCWGPEDRDCVSCRNVSRGECVDKCNLLEGEPREFVENSECIQC HPECLPQAMNITCTGRGPDNCIQCAHYIDGPHCVKTCPAGVMGENNTLVWKYADAGH VCHLCHPNCTYGCTGPGLEGCPNTPKIPSIATGMVGALLLLLVVALGIGLFM (SEQ ID NO:28). In some cases, the truncated EGFR has 1, 2, 3, 4 of 5 amino acid changes (preferably conservative) compared to SEQ ID NO:28.

In some embodiments, a CAR or peptide described herein can comprise a ribosomal skip sequence (e.g., LEGGGEGRGSLLTCGDVEENPGPR; SEQ ID NO:27) and a truncated CD19R (also called CD19t) having a sequence that is at least 90%, at least 95%, at least 98% identical to or identical to: MPPPRLLFFLLFLTPMEVRPEEPLVVKVEEGDNAVLQCLKGTSDGPTQQLTWSRESPLKP FLKLSLGLPGLGIHMRPLAIWLFIFNVSSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSG ELFRWNVSDLGGLGCGLKNRSSEGPSSPSGKLMSPKLYVWAKDRPEIWEGEPPCVPPRD SLNQSLSQDLTMAPGSTLWLSGCVPPDSVSRGPLSWTHVHPKGPKSLLSLELKDDRRPAR DMWVMETGLLLPRATAQDAGKYCHRGNTMSFHLEITARPVLWHWLLRTGGWKVS AVTLAYLIFCLCSLVGILHLQRALVLRKR (SEQ ID NO:26). In some cases, the truncated

CD19t has 1, 2, 3, 4 of 5 amino acid changes (preferably conservative) compared to SEQ ID NO:26.

In some embodiments, a CAR or peptide described herein can comprise a ribosomal skip sequence (e.g., LEGGEGGRGSLLTCGDVEENPGPR; SEQ ID NO:27) and tEGFR having a sequence that is at least 90%, at least 95%, at least 98% identical to or identical to:

MLLLVTSLLLCELPHPAFLIPRKVCNGIGIGEFKDSLSINATNIKHFKNCTSISGDLHILPV
AFRGDSFTHTPPLDPQELDILKTVKEITGFLLIQAWPENRTDLHAFENLEIIRGRGTKQHGGQ
FSLAVVSLNITSLGLRSLKEISDGDVHISGNKNLCYANTINWKKLFGTSGQKTKIISNRGEN
SCKATGQVCHALCSPEGCWGPPEPRDCVSCRNVSRGRECVDKCNLLEGEPREFVENSECI
QCHPECLPQAMNITCTGRGPDNCIQCAHYIDGPHCVKTCAPAGVMGENNTLVWKYADA
GHVCHLCHPNCTYGCTGPGLEGCPNGPKIPSIATGMVGALLLLLVALGIGLFM (SEQ
ID NO:45).

In some embodiments, a CAR or peptide described herein can comprise a ribosomal skip sequence and a truncated LNGFR having a sequence that is at least 90%, at least 95%, at least 98% identical to or identical to:

MGAGATGRAMDGPRLLLLLLLLVSLGGAKEACPTGLYTHSGECCKACNLGEGVAQPC
GANQTVCEPCLDSVTFSDVVSATEPCKPCTECVGLQSMSAPCVEADDAVCRCAYGYQQ
DETTGRCEACRVCEAGSGLVFSCQDKQNTVCEECPDGTYSDEANHVDPCLPCTVCEDT
ERQLRECTRWADAEECEIPGRWITRSTPPEGSDSTAPSTQEPEAPPEQDLIASTVAGVTT
VMGSSQPVVTRGTTDNLIPVYCSILAAVVVGLVAYIAFKRWNSCKQNK (SEQ ID
NO:CC). In some cases, the truncated LNGFR has 1, 2, 3, 4 of 5 amino acid changes (preferably conservative) compared to SEQ ID NO:CC.

Other ribosomal skip sequences useful in a CAR or peptide described herein include T2At having a sequence that is at least 95% identical to: EGRGSLLTCGDVEENPGP (SEQ ID NO:46) or P2A having a sequence that is at least 95% identical to:

GSGATNFSLLKQAGDVEENPGP (SEQ ID NO:47). In some cases, the ribosomal skip sequence has 1, 2, 3, 4 of 5 amino acid changes (preferably conservative) compared to SEQ ID NO:46 or 47.

An amino acid modification refers to an amino acid substitution, insertion, and/or deletion in a protein or peptide sequence. An "amino acid substitution" or "substitution" refers to replacement of an amino acid at a particular position in a parent peptide or protein sequence with another amino acid. A substitution can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. The following are examples of various groupings of amino acids: 1) Amino acids with nonpolar R groups: Alanine, Valine, Leucine, Isoleucine, Proline, Phenylalanine, Tryptophan, Methionine; 2) Amino acids with uncharged polar R groups: Glycine, Serine, Threonine, Cysteine, Tyrosine, Asparagine, Glutamine; 3) Amino acids with charged polar R groups (negatively charged at pH 6.0): Aspartic acid, Glutamic acid; 4) Basic amino acids (positively charged at pH 6.0): Lysine, Arginine, Histidine (at pH 6.0). Another grouping may be those amino acids with phenyl groups: Phenylalanine, Tryptophan, and Tyrosine.

In some cases, the CAR can be produced using a vector in which the CAR open reading frame is followed by a ribosome skip sequence and a truncated EGFR (EGFRt), which lacks the cytoplasmic signaling tail, or a truncated CD19R or a LNGFR. In this arrangement, co-expression of EGFRt provides an inert, non-immunogenic surface marker that allows for accurate measurement of gene modified cells, and enables positive selection of gene-modified cells, as well as efficient cell tracking of the therapeutic NK cells *in vivo* following adoptive transfer. Efficiently controlling proliferation to avoid cytokine storm and off-target toxicity is an important hurdle for the success of NK cell immunotherapy. The EGFRt, CD19t, or LNGFR incorporated in the CAR lentiviral or retroviral vector can act as suicide gene to ablate the CAR+ cells in cases of treatment-related toxicity.

In some cases, a nucleic acid molecule described herein comprises a promoter that controls expression of both the CAR and human interferon gamma. In other cases, a nucleic acid molecule described herein comprises a first promoter controls expression of the CAR and a

second promoter controls expression of human interferon gamma. In some cases, the first and second promoters are identical and in some cases they are different. In some embodiments, the first promoter is a strong constitutive promoter or an inducible promoter. In some embodiments, the second promoter is a weaker promoter than the first promoter or is an inducible promoter.

- 5 Useful promoters are well-known in the art. For example, synthetic NFAT promoter can be used in a nucleic acid encoding a CAR construct. Useful promoters can comprise one or more of CMV, EF1, SV40, PKG1, PKG100, Ubc, Tetracycline, Doxycycline, NFAT, and any other constitutive or inducible promoter. In some embodiments, a NFAT recognition element can be used (TGGAGGAAAACTGTTTCATACAGAAGGCG; SEQ ID NO: X). In some
- 10 embodiments, a useful promoter comprises one, two, three, four, five, six, seven, eight, nine, ten, or eleven repeats of the NFAT recognition element. In some embodiments, a useful promoter comprises any one or more of SEQ ID NO: X, X2, X3, X4, X5, X6, X7, X8, X9, X10 and X11.

TGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAA
GGCG; SEQ ID NO: X2

- 15 TGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAA
GGCGTGGAGGAAAACTGTTTCATACAGAAGGCG; SEQ ID NO: X3

TGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAA
GGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATAC
AGAAGGCG; SEQ ID NO: X4

- 20 TGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAA
GGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATAC
AGAAGGCG TGGAGGAAAACTGTTTCATACAGAAGGCG; SEQ ID NO: X5

TGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAA
GGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATAC
25 AGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTC
ATACAGAAGGCG; SEQ ID NO: X6

TGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAA
GGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATAC
AGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTC
30 ATACAGAAGGCG TGGAGGAAAACTGTTTCATACAGAAGGCG; SEQ ID NO: X7

TGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAA

GGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATAC
AGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTC
ATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACT
GTTTCATACAGAAGGCG; SEQ ID NO: X8

5 TGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAA
GGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATAC
AGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTC
ATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACT
GTTTCATACAGAAGGCG TGGAGGAAAACTGTTTCATACAGAAGGCG; SEQ ID NO:
10 X9

TGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAA
GGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATAC
AGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTC
ATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACT
15 GTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAA
AACTGTTTCATACAGAAGGCG; SEQ ID NO: X10

TGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAA
GGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATAC
AGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACTGTTTC
20 ATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAAACT
GTTTCATACAGAAGGCGTGGAGGAAAACTGTTTCATACAGAAGGCGTGGAGGAAA
AACTGTTTCATACAGAAGGCG TGGAGGAAAACTGTTTCATACAGAAGGCG; SEQ
ID NO: X11

The CAR or polypeptide described herein can be produced by any means known in the art,
25 though preferably it is produced using recombinant DNA techniques. Nucleic acids encoding the
several regions of the chimeric receptor can be prepared and assembled into a complete coding
sequence by standard techniques of molecular cloning known in the art (genomic library
screening, overlapping PCR, primer-assisted ligation, site-directed mutagenesis, etc.) as is
convenient. The resulting coding region is preferably inserted into an expression vector and used
30 to transform a suitable expression host cell line, preferably an immune cell (e.g., a T cell), and
most preferably an autologous T cell.

The CAR or polypeptide can be transiently expressed in a cell population by an mRNA encoding the CAR or polypeptide. The mRNA can be introduced into the immune cells by electroporation (Wiesinger et al. 2019 *Cancers (Basel)* 11:1198).

5 In some embodiments, described herein is a method of increasing survival of a subject having cancer comprising administering a composition comprising a CAR immune cell described herein.

In some embodiments, described herein is a method of treating a cancer in a patient comprising administering a composition comprising a CAR immune cell described herein.

10 In some embodiments, described herein is a method of reducing or ameliorating a symptom associated with a cancer in a patient comprising administering a composition comprising a CAR immune cell described herein.

In some embodiments, a composition comprising CAR NK cells or CAR T cells described herein is administered locally or systemically. In some embodiments, a composition comprising CAR immune cells described herein is administered by single or repeat dosing. In some embodiments, 15 a composition comprising CAR immune cells described herein is administered to a patient having a cancer, a pathogen infection, an autoimmune disorder, or undergoing allogeneic transplant.

In some embodiments, the cancer is a solid tumor. In some embodiments, the cancer is melanoma.

20 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 to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, 25 sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety for any and all purposes.

Other features and advantages of the described compositions and methods will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

FIGS. 1A-1D: mIL13BB ζ generation and phenotypic characterization of mIL13BB ζ CAR T cells. 1A, Schematic depicting the murine IL13R α 2-CAR T (mIL13BB ζ CAR T) construct. 1B and 1C, Flow cytometry and graph depicting phenotypic changes of murine CAR T cell from day 0 to 4. 1D, In vitro killing of mIL13BB ζ CAR T cells against IL13R α 2⁺ and IL13R α 2⁻ K-Luc cells.

FIGS. 2A-2I: Murine IL13BB ζ CAR T cells have potent antitumor activity. 2A, Flow cytometry (left panel) and bar graph summarizing percent CAR⁺ T cells demonstrating transduction efficiency (right). 2B and 2C, Immunofluorescent and flow cytometry staining confirmed transduction of mIL13R α 2 in KR158 (K-Luc) glioma cells. 2D, *In vitro* killing of mIL13BB ζ CAR T cells against K-Luc cells (E:T, 1:3). 2E, Luminex detected IFN γ and TNF α levels. 2F, Schematic depicting *in vivo* experimental design. 2G, Images of hematoxylin and eosin (H&E) showed invasive K-Luc in untreated and CAR T treated brains. 2H, Survival curve of mice bearing K-Luc-mIL13R α 2⁺ tumors in untreated and CAR T-treated groups. 2I, Bioluminescent images (BLI; top) and flux values (bottom) show tumor growth in untreated and CAR T-treated groups. Data of at least three independent experiments were presented as means $\pm$ s.e.m. (2D and 2E) and were analyzed by two-tailed, unpaired Student's t-test. Differences between survival curves were analyzed by log-rank (Mantel-Cox) test (2H). *p < 0.05, **p < 0.01, and ***p < 0.001 for indicated comparison.

FIGS. 3A-3F: mIL13BB ζ CAR T cells have potent antitumor activity and induced endogenous memory immune response against GL261-Luc tumors. 3A, Images of hematoxylin and eosin (H&E) showed morphology of GL261-Luc tumor. 3B, Immunofluorescent and flow cytometry staining confirmed transduction of IL13R α 2 in GL261-Luc glioma cells. 3C, *In vitro* killing of mIL13BB ζ CAR T cells against IL13R α 2⁺ GL261-Luc glioma cells (E:T, 1:3). 3D, Luminex ELISA detected IFN γ and TNF α levels. 3E, Survival curve of mice bearing IL13R α 2⁺ GL261-Luc glioma tumors in untreated and CAR T-treated groups. 3F, Survival of mice cured by the CAR T therapy and rechallenged with IL13R α 2 negative GL261-Luc. Data of at least two

independent experiments were presented as means $\pm$ s.e.m. (3C) and were analyzed by two-tailed, unpaired Student's *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0001 for indicated comparison. Differences between survival curves (3E) were analyzed by log-rank (Mantel–Cox) tested by log-rank (Mantel–Cox) test.

5 **FIGS. 4A-4E:** mIL13BBζ CAR T cells have a superior antitumor activity in immunocompetent host. 4A, Schema depicting *in vivo* experimental design with CAR administration at day 4 or 7. 4B, Bioluminescent images (BLI; top) and flux values (bottom) showed tumor growth in untreated and CAR T-treated group in 4-day old tumor model. 4C, Survival curve of mice bearing 4-day old K-Luc IL13Rα2+ tumors in untreated and CAR T-treated groups. 4D,
10 Bioluminescent images (BLI; top) and flux values (bottom) showed tumor growth in untreated and CAR T-treated group in 7-day old tumor model. 4E, Survival curve of mice bearing 7-day old K-Luc IL13Rα2+ tumors in untreated and CAR T-treated groups. Differences between survival curves (4C, 4E) were analyzed by log-rank (Mantel–Cox) test.

FIG. 5a-5f: CAR T cells induce endogenous memory immune response and generation of
15 antigen specific T cells. a, Survival of mice cured by CAR T therapy and rechallenged with IL13Rα2 negative K-Luc tumors. b, Bioluminescent (BLI) images (top) and flux values (bottom) show tumor growth in naïve controls and survivors of CAR T therapy groups. c, Overview of experimental design. d, In vitro killing and e, expansion of endogenous T cells isolated from untreated or CAR T-treated mice against K-Luc-mIL13Rα2+ cells (E:T, 10:1). f, Assessment of
20 in vivo killing capacity of isolated CAR T cells and endogenous T cells from untreated or CAR T-treated cells in tumor (K-Luc) bearing mice. Data are inclusive of at least two independent experiments. Each symbol represents one individual. Data are presented as means $\pm$ s.e.m. (d and e) and were analyzed by two-tailed, unpaired Student's *t*-test. Differences between survival curves were analyzed by log-rank (Mantel–Cox) test (a). **p* < 0.05, ***p* < 0.01, and ****p* <
25 0.001 for indicated comparison.

FIG. 6a-6b: mIL13BBζ CAR T cells have a potent antitumor activity but are unable to induce endogenous memory immune response in small tumor model. a, Survival curve of mice bearing 4 day-old K-Luc IL13Rα2+ tumors in untreated and CAR T-treated groups. b, Survival of mice

cured by the CAR T therapy and rechallenged with IL13R α 2 negative K-Luc tumors.

Differences between survival curves (a) were analyzed by log-rank (Mantel–Cox) test.

FIG. 7a-7e: Comparison of survival in mice bearing mixed antigen tumors. a, Schema of day 4 and 7 in vivo experimental design. b, Flow cytometry showing different levels of IL13R α 2. c, Survival curve of mice bearing day 4 K-Luc IL13R α 2+ tumors in untreated and CAR T-treated groups. d, Survival curve of mice bearing day 7 K-Luc IL13R α 2+ tumors in untreated and CAR T-treated groups. e, Quantification of CD11b and CD3 cells after flow cytometry sort of untreated, K-Luc tumor bearing mice. Each symbol represents one individual. Data are presented as means $\pm$ s.e.m. (e) and were analyzed by two-tailed, unpaired Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 for indicated comparison. Differences between survival curves (c, d) were analyzed by log-rank (Mantel–Cox) test.

Fig. 8: Flow Cytometry sort of Endogenous and CAR T cells. A representation of flow cytometry sort of endogenous (CD3+CD19-) and CAR T (CD3+CD19+) populations.

Fig. 9a-9g: CAR T cells activate endogenous T cells in glioma tumor microenvironment. a, Nanostring analysis shows global changes in gene expression of intratumoral T cells (CD3+) isolated from untreated or CAR T-treated mice 3 days post-therapy. b, UMAP plots depict changes in lymphoid compartments in the glioma TME after CAR T therapy. c, Feature plots demonstrate phenotypic characterization of T cell subclusters and enriched pathways within CD8 and CD4 T cell subclusters post therapy. d, Heatmap of enrichment scores (GSEA analysis) shows enriched pathways in T cell subclusters. e, Experimental design demonstrating the adoptive transfer of CD45.1+ mock or CAR T cells (top) and flow cytometry analysis show frequency of endogenous (CD3+CD45.2+) or adoptively transferred T cells (CD3+CD45.1+) in glioma TME (bottom). f, Bar graphs compare adoptively transferred mock (CD3+CD45.1+) or CAR T cell (CD3+CD45.1+CD19+) number and phenotypic characterization (CD69, Ki67 and GZMB). g, Bar graphs compare endogenous T cell (CD3+CD45.2+) numbers and phenotypic characterization (CD69, Ki67 GZMB, and IFN γ) in untreated, mock or CAR T treated mice (n=5 per group). Data are inclusive of at least two independent experiments. Data are presented as means $\pm$ s.e.m. (F and G) and were analyzed by two-tailed, unpaired Student's t-test. *p < 0.05, **p < 0.01, and ***p < 0.001 for indicated comparison.

Fig. 10a-10d: Single cell RNA sequencing of intratumoral immune cells. a, UMAP plot from merged untreated and CAR T-treated data of exclusively intratumoral immune cells. b, Feature plot of immune subset-specific marker-gene expression. c, Changes in frequency of lymphoid subclusters (top) and violin plots (bottom) depict lymphoid specific markers. d, Bar graph demonstrates changes in frequency of myeloid subclusters (top) and violin plots (bottom) depict myeloid specific markers.

Fig. 11a-11b: Single-cell RNA sequencing identifies phenotypes of intratumoral T cells. a, The UMAP plots demonstrate enhancement of genes associated with memory stem like T cells after CAR T therapy. b, The UMAP plots demonstrate reduction in expression of genes associated with T regulatory cells after CAR T therapy.

Fig. 12: Expression of genes associated with T cell activation in intratumoral T cells. qPCR analysis shows genes associated with T cell activation. Data are presented as means $\pm$ s.e.m. and were analyzed by two-tailed, unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ for indicated comparison.

Fig. 13a-13f: CAR T cells activate the resident myeloid population in glioma tumor microenvironment. a, UMAPs depict changes in intratumoral myeloid cells from CAR T-treated or untreated mice. b, Enrichment plot of IFN γ signaling pathways in intratumoral macrophage and microglia cells in CAR T-treated compared with untreated, as identified by the GSEA computational method. c, GSEA analysis reveals upregulation of population specific activation pathways in myeloid subclusters (MP: macrophage; MG: microglia; DC: dendritic cells; Neu: neutrophils). d, Nanostring analysis show global changes in gene expression of myeloid cells (CD11b+) isolated from CAR T-treated vs untreated mice. e, UMAPs indicate relative expression levels of antigen presentation gene signatures at a single-cell level within the myeloid compartment. f, Histograms (left) and bar graphs (right) show intratumoral CD11b+CD45.2+ cells expressing MHCII, MHCI, CD86, and IFN γ . Data are presented as means $\pm$ s.e.m. (f) and were analyzed by two-tailed, unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ for indicated comparison.

Fig. 14a-14j: Lack of IFN γ in CAR T cells impairs antitumor activity and activation of host immune cells. a, Schematic of the experimental design. b, Comparison of percent CAR

positivity, viability, expansion, and CD4:CD8 ratio in CAR Twt and CAR TIFN γ ^{-/-}. c, In vitro killing of CAR Twt and CAR TIFN γ ^{-/-} against K-Luc-IL13R α 2⁺ cells (E:T, 1:1). d, A flow cytometry depicts intracellular cytokine levels (TNF α , GZMB and IFN γ) in wt and IFN γ ^{-/-} CAR T cells. e, Bioluminescent (BLI) images (top) and flux values (bottom) show tumor growth in untreated, CAR Twt or CAR TIFN γ ^{-/-}. f, Survival curve of mice bearing K-Luc-IL13R α 2⁺ tumors in untreated, CAR Twt and CAR TIFN γ ^{-/-} groups. g, Heatmap indicates normalized expression of genes associated with immune activation and suppression in the tumor. h, Bar graphs (left) and flow cytometry plots (left) comparing CAR T cell (CD3+CD19⁺) number and activation phenotype (CD69). i, Bar graphs (left) and flow cytometry plots (right) comparing endogenous T cell (CD3+CD19) number and activation phenotype (CD69). j, Histograms (left) and bar graphs (right) showing phenotype in myeloid (CD11b⁺) compartment. Data are inclusive of at least two independent experiments. Each symbol represents one individual. Data are presented as means $\pm$ s.e.m. (h, i, and j) and were analyzed by two-tailed, unpaired Student's t-test. Differences between survival curves (f) were analyzed by log-rank (Mantel-Cox) test. *p < 0.05, **p < 0.01, and ***p < 0.001 for indicated comparison.

Fig. 15A-15J: Lack of IFN γ production by CAR T cells impairs antitumor activity and activation of host immune cells. A, Schematic of the experimental design. B, Comparison of percent CAR positivity, viability, expansion, and CD4:CD8 ratio in CAR T^{wt} and CAR T^{IFN γ ^{-/-}}. C, *In vitro* killing of CAR T^{wt} and CAR T^{IFN γ ^{-/-}} against K-Luc-mIL13R α 2⁺ cells (E:T, 1:1). D, Representative flow cytometry plot depicts intracellular cytokine levels (TNF α , GZMB and IFN γ) in wt and IFN γ ^{-/-} CAR T cells after exposure to K-Luc-mIL13R α 2⁺ tumors. E, Representative bioluminescent (BLI) images (top) and flux values (bottom) show tumor growth in untreated, CAR T^{wt} or CAR T^{IFN γ ^{-/-}}. Individual mice are represented with dotted lines and median flux is shown in thick line. F, Survival curve of mice bearing K-Luc-mIL13R α 2⁺ tumors in untreated, CAR T^{wt} treated and CAR T^{IFN γ ^{-/-}} treated groups. G, Heatmap indicates normalized expression of genes associated with immune activation and suppression in the TME. H, Bar graphs (left) and representative flow cytometry plots (right) comparing CAR T cell (CD3+CD19⁺) number and activation phenotype (CD69). I. Bar graphs (left) and representative flow cytometry plots (right) comparing endogenous T cell (CD3+CD19⁻) number and activation phenotype (CD69). J, Representative histograms (left) and bar graphs (right) showing phenotype in myeloid (CD11b⁺) compartment. Data are representative of at least two

independent experiments. Each symbol represents one individual (H, I, and J). Data are presented as means $\pm$ s.e.m and were analyzed by two-tailed, unpaired Student's *t*-test. Differences between survival curves (F) were analyzed by log-rank (Mantel–Cox) test. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 for indicated comparison.

5 **Fig. 16a-16d:** CAR T cells promote monocyte differentiation and generation of M1 type macrophages. a, Schema of experimental design. b, Flow cytometry (left) and bar graphs (right) depict phenotypic changes in monocytes after incubation with different conditioned media. c, Microscopy images demonstrate morphological change in monocytes after incubation with different conditioned media. d, qPCR analysis of genes associated with M1 macrophage
10 phenotype. Data are presented as means $\pm$ s.e.m. (d) and were analyzed by two-tailed, unpaired Student's *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0001 for indicated comparison.

Fig. 17a-17k: CAR T cells can activate GBM patient immune cells. a, Schema of experimental design. b, Flow cytometry, c, microscopy images and d, bar graph summary of phenotypic
15 changes of patient macrophages after incubation in conditioned media. e, Flow cytometry and f, summary of phenotypic changes in patient T cells after incubation in conditioned media. g, Schematic of trial design for patients receiving CAR T therapy. h, Flow cytometry demonstrates intracellular IFN γ levels in patient T cells obtained before CAR T therapy (Pre-CAR), and during response to CAR T therapy (Post-CAR) after coculture with irradiated autologous tumor
20 followed by 4 hour stimulation. i, T cell count after incubation with autologous irradiated (Irr.) patient tumor. j, In vitro killing by T cells against autologous (UPN109) or nonspecific tumor line (K562) at 10:1, E:T ratio. k, Flow cytometry demonstrates the IL13R α 2 expression of the patient autologous (UPN109) tumor. Each symbol represents one replicate. Data are presented as means $\pm$ s.e.m. (d, f, i and j) and were analyzed by two-tailed, unpaired Student's *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0001 for indicated comparison.
25

Fig. 18: List of primers used in the studies described herein.

Fig. 19: List of antibodies used in the studies described herein.

FIGS. 20A-20D: Molecular design and generation of different IL13R α 2-IFN γ CAR T cell constructs and depicts aspects of the preparation of CAR T cell co-expressing interferon gamma. 20A. Construct design for IL13 CAR T co-expressing IFN γ compared to standard IL13 CAR T. 20B. Schema of transduction and IL13 CAR T cell production. T cells are isolated and activated in the presence of CD3/CD28 antibodies (1:1), followed by IL13 CAR transduction. 20C. Flow cytometry showed the percentage CAR positive cells using IL13 as a marker of CAR expression. 20D. IFN γ levels in various IL13 CAR T cells during production (left panel) and ELISA confirmed IFN γ expression and secretion in CAR T cells confirming transduction and expression of IFN γ within the construct.

FIGS. 21A-21E: Depicts the results of functional and phenotypic assessment of murine IL13 CAR T cells and IL13R α 2-IFN γ CAR T cells. Murine IL13 CAR T cells or IL13 CAR T cells co-expressing IFN γ were co-cultured with murine glioma tumors at 1 effector :3 target ratio. 21A. Microscopy images demonstrated killing capacity of different IL13 CAR T constructs (untransduced, mock T cells, IL13 CAR T only, and IL13 CAR IFN γ T cells). 21B. T cell count after 24 hours of co-culture. 21C. Tumor cell count after 24 hours of co-culture. 21D. Bar graph showing percent T cell activation as measured by CD69 expression after 24 hours of co-culture. 21E. Flow cytometry analysis demonstrated comparable exhaustion (PD-1+Tim3+) and differentiation (CD62L+CD45RA+) phenotype in murine IL13R α 2-IFN γ CAR T cells and IL13R α 2-IFN γ CAR T cells.

FIGS. 22A-22C: Functional and phenotypic assessment of human IL13 CAR cells and IL13R α 2-IFN γ CAR T cell. Human IL13 CAR T or IL13 CAR T-IFN γ cells were co-cultured with patient-derived glioma tumors (1 effector:25 target). 22A. T cell count after 24 hours of co-culture. 22B. Tumor cell count measured after 24 hours of co-culture. 22C. Flow cytometry analysis demonstrated comparable exhaustion (PD-1+Tim3+) and differentiation (CD62L+CD45RA+) phenotype in human IL13R α 2-IFN γ CAR T cells and IL13R α 2-IFN γ CAR T cells.

FIG. 23: IL13R α 2-IFN γ CAR T cells induced abscopal effect in multi-lesion metastatic melanoma model. Tumors were injected in both flanks. Once tumors reached a predetermined size, IL13R α 2 CAR T cells or IL13R α 2-IFN γ CAR T cells were administered locally to one

tumor. Both tumors were measured for antitumor activity. The line graph demonstrated tumor volume changes in the tumor opposite the side of treatment.

FIG. 24: Amino acid sequence of various human IL13 CAR (SEQ ID NO: 70-72) with the various domains indicated.

5 **FIG. 25:** Amino acid sequences of various human IL13 CAR (SEQ ID NO: 73-75) with the various domains indicated.

FIGS. 26A-26B: IL13R α 2-IFN γ CAR T cell can reprogram the macrophages. 26A. Schematic depiction of transduction and CAR T cell production. 26B. Bar graphs demonstrated reprogramming of macrophages with IL13R α 2-IFN γ CAR T cells. Bar graphs showed qPCR
10 analysis of genes associated with proinflammatory and metabolically active macrophages when incubated with supernatant collected during manufacturing of the CAR products or exposed to exogenous IFN γ . Each data point is indicative of one replicate.

FIGS. 27A-27C: Development of the inducible IL13R α 2-IFN γ CAR T cells through a synthetic NFAT promoter. 27A. A schematic depicting the molecular design of the inducible IFN γ
15 expression upon CAR T activation. 27B. An illustration depicting experimental design, briefly, cells were transduced with an NFAT-eGFP-CAR T cell construct and co-cultured with IL13R α 2⁺ and IL13R α 2⁻ tumors. Upon stimulation with antigen positive tumors and activation, GFP is expressed and detectable. 27C. Flow cytometry demonstrated expression of GFP in cells transduced and activated by antigen positive tumors.

20 **FIGS. 28A-28C:** IL13R α 2-IFN γ CAR T cells were more efficacious compared to the standard IL13R α 2 CAR T cells in targeting medium/low IL13R α 2 antigen expressing tumors *in vivo*. 28A. A schematic depicting the experimental design. 28B. Bar graph showed tumor progression in mice bearing high-IL13R α 2 antigen expressing tumors after treatment with IL13R α 2 CAR T cells or IL13R α 2-IFN γ CAR T cells. 28C. Bar graph showed tumor progression in mice bearing
25 medium-IL13R α 2 antigen expressing tumors after treatment with IL13R α 2 CAR T cells or IL13R α 2-IFN γ CAR T cells.

FIGS. 29A-29D: IL13R α 2-IFN γ CAR T cells and IL13R α 2-IFN γ ^{low} CAR variants expressed different levels of IFN γ . 29A Schematics depicting the construct designs with IFN γ under the

control of different strength promoters. 29B. Schematic depicting the experimental design showing transduction and collection of supernatant. 29C. Bar graph showed IFN γ levels during *ex vivo* expansion. 29D. Bar graph showed viable tumor counts after co-culture of CAR T cells with tumor (1:50 effector:target ratio) for 5 days.

5 **FIGS. 30A-30C:** IL13R α 2-CAR T and IL13R α 2-CAR-NFAT/IFN γ T cells show comparable killing capacity. 30A. Schema of construct design demonstrates IFN γ under the control of the NFAT promoter. 30B. Schematic of the experimental design demonstrated coculture of different CARs in the presence of antigen positive tumors. 30C. Graph demonstrates cytotoxic function of IL13R α 2-CAR T compared to IL13R α 2-CAR-NFAT/IFN γ T cells.

10 **FIGS. 31A-31B:** IL13R α 2-IFN γ CAR T cells synergizes with myeloid cells for an enhanced antitumor function. 32A. Schema depicting the experimental design; briefly, T cells transduced with IL13R α 2-IFN γ CAR T cells or IL13R α 2 CAR T cells were co-cultured with macrophages and antigen positive tumor cells. 32B. Bar graph showed tumor count in the 3-way co-culture with IL13R α 2-IFN γ CAR T cells or IL13R α 2 CAR T cells with or without macrophages or
15 tumor cells.

DETAILED DESCRIPTION

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

20 **Materials and Methods**

Mice and Cell lines

C57BL/6/J, CD45.1 (B6.SJL-Ptpr^aPepc^b/BoyJ), Thy1.1 (B6.PL-Thy1a/CyJ), IFN γ R^{-/-} (B6.129S7-Ifngr1tm1Agt/J), and IFN γ ^{-/-} (B6.129S7-Ifngtm1Ts/J) mice were purchased from The Jackson Laboratory. NOD/Scid IL2R γ Cnull (NSG) mice were bred at City of Hope. All
25 mouse experiments were approved by the City of Hope Institutional Animal Care and Use Committee (IACUC).

The luciferase-expressing murine GL261 (GL261-Luc) and KR158B (K-Luc) glioma cells were transduced with lentivirus to produce murine IL13R α 2 (mIL13R α 2) expressing sublines (GL261-Luc-mIL13R α 2 and K-Luc-mIL13R α 2). These tumor lines were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 25mM HEPES (Irvine Scientific, Santa Ana, CA) and 2mM L-glutamine (Lonza). Cell surface expression of mIL13R α 2 was authenticated by flow cytometry and immunofluorescence imaging.

Patient-derived glioma cells (PBT030-2-ffLuc) were isolated from GBM patient resections under protocols approved by the COH IRB and maintained as described previously. All tumor lines were authenticated for the desired antigen/marker expression by flow cytometry and cells were tested for mycoplasma and maintained in culture for less than 1-3 months.

CAR T cell Production

Human CAR T cells: Naïve and memory T cells were isolated from healthy donors at City of Hope under protocols approved by the COH IRB (12, 32). The construct of IL13R α 2-targeted CAR and CAR transduction was described in previous studies (12, 33, 34). In brief, primary T cells were stimulated with Dynabeads Human T expander CD3/CD28 (Invitrogen) (T cells : beads = 1:2) for 24 hours and transduced with CAR lentivirus (multiplicity of infection [MOI] = 0.5). Seven days after CAR transduction, CD3/CD28 beads were removed and cells were resuspended and expanded in X-VIVO 15 media (Lonza) containing 10% FCS, 50 U/ml recombinant human IL-2, and 0.5 ng/ml recombinant human IL-15 for additional 10-15 days before proceeding to ex vivo expansion.

Murine CAR T cells

The murine IL13BB ζ chimeric antigen receptor was constructed in a MSCV retroviral backbone (Addgene), containing the extracellular murine IL13 and murine CD8 hinge, murine CD4 transmembrane domain, and intracellular murine 4-1BB costimulatory and murine CD3 ζ signals. Following a T2A ribosomal skip, a truncated murine CD19 was inserted as a transduction marker. The resulting plasmid was transfected into PlatE cells (a gift from Dr. Zuoming Sun lab) using Fugene (Promega). After 48 hours, the supernatant was collected and filtered using an 0.2 μ m filter. The retroviral supernatant was aliquoted and frozen until the time of transduction.

Murine T cells were isolated from spleens of naïve C57BL/6J mice or appropriate strain (CD45.1, Thy1.1, or IFN γ ^{-/-}) with EasySep Mouse T cell Isolation Kit (STEMCELL Technologies) and stimulated with Dynabead Mouse T-Activator CD3/CD28 beads (Gibco) at a 1:1 ratio. T cells were transduced on RetroNectin-coated plates (Takara Bio USA) using retrovirus-containing supernatants (described above). Cells were then expanded for 4 days in RPMI-1640 (Lonza) supplemented with 10% FBS (Hyclone Laboratories), 55 mM 2-mercaptoethanol (Gibco), 50 U/mL recombinant human-IL-2 (Novartis), and 10 ng/mL recombinant murine IL-7 (Peprotech). Before *in vitro* and *in vivo* experiments, T cells were debudded and CAR expression was determined by flow cytometry.

10 *qRT-PCR analysis*

RNA was isolated from myelin-removed brain tissue (either bulk tissue or flow sorted cells) using the RNeasy Mini Kit (Qiagen). cDNA was reverse transcribed using the SuperScript VILO Mastermix (Life Technologies) according to the manufacturer's instructions. qPCR reactions were performed as previously described (35). Primers are used are listed in Fig. 18.

15 *In vivo studies*

All mouse experiments were performed using protocols approved by the City of Hope IACUC. Orthotopic GBM models were generated as previously described (36). Orthotopic tumor model was established by stereotactically implanting 1×10^5 tumor cells intracranially (i.c.) into the right forebrain of 8-10 week-old C57BL/6J, IFN γ R^{-/-}, or NSG mice. Engraftment was verified by bioluminescent imaging one day prior to CAR T cell injection. Mice were randomized into groups based on bioluminescent signal. Four or seven days after tumor injection, mice were treated intracranially with 1×10^6 mIL13BB ζ -CAR T cells. Tumor burden was monitored with SPECTRAL LagoX (Spectral Instruments Imaging) and analyzed using Aura software (v2.3.1, Spectral Instruments Imaging). Survival curves were generated by GraphPad Prism Software (v8).

For rechallenge experiments, clearance of tumor was verified by bioluminescent imaging prior to tumor rechallenge, where mice were injected with 10^4 K-Luc or 5×10^4 GL261-Luc cells.

For subcutaneous studies, 1×10^6 K-Luc-mIL13R α 2 in PBS was injected into the right and left flanks of 8-10 week-old C57BL/6J donor mice. Tumors were allowed to establish for 8 days, then 1×10^6 CAR T cells were injected directly into the tumor. Three days later, the tumor mass were harvested, manually dissociated and sorted by flow cytometry into CD3+CD19-

5 (endogenous T cells) or CD3+CD19+ (CAR T cells) using the BD AriaSORP (BD Biosciences). The purified T cell populations were either used as effector cells in in vitro coculture 10:1 (effector:target) ratio as described below or reinjected back into 8 day old subcutaneous K-Luc-mIL13R α 2 tumors, which tumor volume was measured over time using calipers.

Mice were also monitored by the Center for Comparative Medicine at City of Hope for survival
10 and any symptoms related to tumor progression, with euthanasia applied according to the American Veterinary Medical Association Guidelines.

In vitro cytotoxicity

For assessment of CAR T cell proliferation and cytotoxic activity, K-Luc-mIL13R α 2 or GL261-Luc-mIL13R α 2 tumor cells were co-cultured with murine CAR T cells at 1:3 CAR+ tumor ratio
15 for 48 hours. For co-culture using effector T cells primed in vivo, T cells were plated at a 10:1 effector: tumor ratio for 72 hours. Cells were stained with anti-CD3, CD8, and CD19. Absolute number of viable tumor and CAR T cells was assessed by flow cytometry.

For the degranulation assay, CAR T cells and tumor cells were co-cultured at 1:1 effector: tumor ratio for 5 hours in the presence of GolgiStop Protein Transport Inhibitor (BD Biosciences). The
20 cell mixture was stained with anti-CD3, CD8, and CD19 followed by intracellular staining with anti-IFN γ (BD Biosciences), GZMB and TNF α (eBiosciences) antibodies and analyzed by flow cytometry.

All samples were acquired on MACSQuant Analyzer (Miltenyi Biotec) and analyzed with FlowJo software (v10.7) and GraphPad Prism (v8).

Patient sample analysis

Conditioned media was generated by seeding patient-derived glioma cells, human CAR T cells, or the combination at a 1:1 ratio for 24 hours. The supernatant was collected and centrifuged to

remove any cell debris. Peripheral blood from GBM patients (obtained from scheduled blood draws under clinical protocols approved by the City of Hope) was lysed with PharmLyse buffer (BD Biosciences). CD3 and CD14 cells were isolated using selection kits (STEMCELL Technologies). CD14 and CD3 positive cells were incubated with conditioned media, in the presence or absence of IFN γ R neutralizing antibody (R&D Systems). For macrophage differentiation, CD14 cells were incubated in the presence of M-CSF (BioLegend) for 7 days and then exposed to conditioned media, in the presence or absence of IFN γ R neutralizing antibody (R&D Systems). After 48 hours, cells were visualized using Keyence microscope and phenotyped by flow cytometry.

Assessment of endogenous response in the unique responder to CAR T therapy (ref) was conducted as previously reported (37). Briefly, T cells were isolated from total blood before and during therapy. Every two days, T cells were incubated with irradiate (40 Gy) autologous tumor cells in the presence of IL2 (50U/ml). After 14 days, T cells were purified and counted. T cells were cultured with fresh autologous tumor or irrelevant tumor line at a 10:1 (effector:target) ratio after 3 days, tumor counts were measured. IFN γ production was measure by stimulating the T cells with cell stimulation cocktail for additional 4 hours followed by flow cytometry for intracellular IFN γ .

Flow cytometry assays

Live tumor cells expanded in vitro were stained with an unconjugated goat anti-mouse IL13R α 2 (R&D Systems) followed by secondary donkey anti-goat NL637 (R&D Systems). Live murine CAR T cells were stained with CD8 (BioLegend) CD3, CD4, CD62L (eBiosciences) or CD45RA (BD Biosciences). CD19 (BD Biosciences) was used as a surrogate to detect the CAR.

Brains from euthanized mice were removed at the indicated time-points, and a rodent brain matrix was used to cut along the coronal and saggital planes to obtain a 4x4 mm section, centered around the injection site. These sections were minced manually, then passed through a 40 μ m filter. Myelin was removed using Myelin Removal Beads II and LS magnetic columns (Miltenyi Biotec) according to the manufacturer's instructions, then cells were counted. Cell were stained and analyzed using flow cytometry. For flow sorting, cells were stained with

indicated antibodies (FIG. 19) and sorted using BD AriaSORP (BD Biosciences). For gene expression analysis of TME, the remaining cells were lysed for RNA.

Immunofluorescence and Immunohistochemistry

For immunofluorescence, K-Luc and GL261-Luc parental or mIL13R α 2-transduced cells were
5 cultured on coverslip, stained with unconjugated goat anti-mouse IL13R α 2 (R&D Systems) followed by secondary donkey anti-goat NL637 (R&D Systems), and actin. Slides were imaged using confocal microscopy (Zeiss confocal microscopy) as previously described (38).

For immunohistochemistry, mice were euthanized 3 days after CAR T injection and were perfused with PBS followed by 4% PFA. Whole brains were dissected, and incubated in 4%
10 PFA for 3 days, followed by 70% ethanol for 3 days before being embedded in paraffin. 10 μ M transverse sections were cut and stained with H&E, CD3 (ab16669, Abcam) or F4/80 (ab6640, Abcam). Slides were digitized at 40x magnification using a NanoZoomer 2.0-HT digital slide scanner (Hamamatsu).

Bioplex Cytokine Analysis

15 To assess CAR T cell cytokine profile, mIL13BB ζ CAR+ T cells and tumor cells (GL261-Luc-mIL13R α 2 or K-Luc-mIL13R α 2) were incubated at 1:1 ratio for 1 day without exogenous cytokines. The cell-free supernatant was collected and assayed using the ProcartaPlex Mouse Th1/Th2 Cytokine Panel 11plex (ThermoFisher Scientific) according to the manufacturer's instructions and acquired on the Bio-Plex 3D Suspension Array System (Bio-Rad Laboratories).

Nanostring gene expression analysis

20 RNA was purified from flow-sorted CD3+ or CD11b+ sorted cells using the RNEasyPlus micro kit, following the manufacturer's instructions (Qiagen, Germantown, MD, USA). RNA samples were subsequently quantified and qualified using Nanodrop 1000 spectrophotometer (ThermoFisher, Waltham, MA, USA) and Bioanalyser Tape station (Agilent, Santa Clara, CA, USA) assays. The subsequent Nanostring analysis was performed at concentrations of 35ng/well
25 and 25ng/well respectively for CD3+ cells and CD11b+ cells.

Samples were analyzed based on the nCounter® mouse PanCancer Immune profiling gene expression panel (NanoString Technologies, Seattle, WA, USA): Hybridation reaction was performed for 18h at 65°C. Fully automated nCounter FLEX analysis system; composed of an automated nCounter® Prep station and the nCounter® Digital Analyzer optical scanner (NanoString Technologies, Seattle, WA, USA) was used. Normalization was performed by using the geometric mean of the positive control counts as well as normalization genes present in the CodeSet Content: samples with normalization factors outside of the 0.3–3.0 range were excluded, samples with reference factors outside the 0.10–10.0 range were excluded as well. Gene expression analysis was performed using the nSolver v3.0 and Advanced analysis module softwares. (NanoString Technologies, Seattle, WA, USA).

Single cell RNA-sequencing

Seven days after K-Luc-mIL13Rα2 engraftment, CAR T cells were injected or not into the tumor as described above. Brains from CAR T treated or untreated mice (n=3 per group) were harvested and pooled three days after CAR T cell injection, manually minced, and myelin removed before flow sorting on the BD AriaSORP (BD Biosciences) for live (DAPI-) CD45-PE+ (BD Biosciences) cells. Single cell suspensions were processed using the Chromium Single Cell 3' v3 Reagent Kit (10x Genomics) and loaded onto a Chromium Single Cell Chip (10x Genomics) according to the manufacturer's instructions. Raw sequencing data from each of two experiments were aligned back to mouse genome (mm10), respectively, using cellranger count command to produce expression data at a single-cell resolution according to 10x Genomics (<https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/using/count>). R package Seurat 3.9 was used for gene and cell filtration, normalization, principle component analysis, variable gene finding, clustering analysis, and Uniform Manifold Approximation and Projection (UMAP) dimension reduction. Briefly, matrix containing gene-by-cell expression data was imported to create a Seurat object individually for CAR T untreated and CAR T treated samples. Cells with <200 detectable genes and a percentage of mitochondrial genes >10% were further removed. Data were then merged and log-normalized for subsequent analysis. Principle component analysis (PCA) was performed for dimension reduction, and the first 20 principle components were used for clustering analysis with a resolution of 0.6. Clusters were visualized with UMAP embedding. In addition to the

use of Immunologic Genome Project (ImmGen)^{40, 41}, to facilitate cell type identification, the expression level of the following markers were plotted using VlnPlot. They were Itagm, Cd3e, Cd19, Cd79a, Nkh7, Cd68 and Cd8a. Upon the identification of lymphoid and myeloid parental clusters, on each of them, we followed the above-mentioned strategy for subclustering to produce daughter clusters. In concert with ImmGen, key markers for distinguishing myeloid daughter clusters were Itgam, Cd68, S100a9, Itgax, Tmem119, and P2ry12, while for lymphoid Cd3e, Cd4, Cd8a, Cd79a, and Ncr1. To further visualize the average expression of a module of genes, CD74, H2-Aa, H2-Ab1, H2-Eb1, and MARCKS, across population in myeloid daughter clusters, AddModuleScore function was employed to generate a feature that could be rendered using FeaturePlot.

Gene set enrichment analysis

Differentially expressed (DE) genes between untreated and CAR T treated in each myeloid and lymphoid parental and daughter cluster were detected with function FindAllMarkers. The analysis on Gene ontology (GO), Kyoto encyclopedia of genes and genomes pathway, and Immunologic signatures collection (ImmuneSigDB) (42) was performed with the full list of DE genes of each cluster using GSEA function implemented in clusterProfiler package (43), then being plotted with ggplot2 (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016).

Statistical analysis

Statistical significance was determined using Student t-test (two groups) or one-way ANOVA analysis with a Bonferroni (three or more groups). Survival was plotted using a Kaplan-Meier survival curve and statistical significance was determined by the Log-rank (Mantel-Cox) test. All analyses were carried out using GraphPad Prism software (v5). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Example 1: Murine IL13R α 2-CAR T cells mediate potent antitumor activity in immune competent models of GBM

We established immunocompetent mouse models of an earlier described clinical IL13R α 2-CAR T cell platform. A mouse counterpart to a human IL13R α 2-targeted CAR was constructed (12),

composed of the IL-13(E12Y) tumor-targeting domain, murine CD8 hinge (mCD8h), murine CD8 transmembrane domain (mCD8tm), murine 4-1BB costimulatory domain (m4-1BB) and murine CD3 zeta (mCD3 ζ). A T2A skip sequence separates the CAR from a truncated murine CD19 (mCD19t) used for cell tracking (Fig. 1A). The engineering process resulted in a 70-85% transduction efficiency as assessed by the frequency of CD19t+ cells (Fig. 2A). Phenotypic analyses indicated that, similar to human CAR T cells (12), the murine IL13R α 2-targeted CAR T cells (mIL13BB ζ CAR T cells) contained comparable numbers of CD4+ and CD8+ T cell subsets with mixed early memory (CD62L+) and effector (CD62L-) T cell populations on day 4 of ex vivo expansion (Fig. 1B and Fig. 1C). We designed a therapeutic setting based on K-Luc and GL261-Luc, two syngeneic, immunocompetent murine glioma models. K-Luc, a firefly luciferase engineered subline of KR15813, was used as it recapitulates the highly invasive features of GBM (Fig. 2B). This tumor line is derived from a spontaneous glioma arising from Nf1, Trp53 mutant mice, and is poorly immunogenic as indicated by its unresponsiveness to anti-PD-1 checkpoint therapy (14). As a second model, we also used GL261 engineered to express ffluc (GL261-luc), a non-invasive “bulky” glioma (Fig. 3A), which was generated by chemical induction, contains high numbers of mutations, and in contrast to K-Luc, is responsive to anti-PD-1 immunotherapy (15, 16). Both tumor lines were engineered to express murine IL13R α 2 (mIL13R α 2) (Fig. 2C and Fig. 3B). mIL13BB ζ CAR T cells specifically killed mIL13R α 2-engineered K-Luc and GL261-Luc cells (Fig. 2D and Fig. 3C), which was associated with production of inflammatory cytokines IFN γ and TNF α (Fig. 2E and Fig. 3D), and were not responsive to IL13R α 2-negative parental tumor lines in vitro (Fig. 1D).

We next evaluated CAR T cell antitumor activity against orthotopically engrafted glioma tumors in C57BL/6 immunocompetent mice. In IL13R α 2+ K-Luc and GL261-Luc tumor models, single intratumoral administration of mIL13BB ζ CAR T cells seven days after tumor injection mediated potent *in vivo* antitumor activity and conferred a significant survival benefit (Figs. 2F-2I and Fig. 3E). We compared anti-K-Luc activity in C57BL/6 mice to tumors engrafted in immunocompromised NOD-scid IL2R γ null (NSG) models, which lack adaptive immune subsets. In a smaller four-day old tumor model, antitumor activity in C57BL/6 and NSG was equivalent, indicating CAR T cell functionality is comparable in both mouse strains (Figs. 4A-4C). However, surprisingly, in the larger tumor model (day 7), with a more established immune microenvironment, the *in vivo* response to T cell therapy was superior in immunocompetent

C57BL/6 as compared to immunodeficient NSG mice ($p<0.001$) (Figs. 4D-4E). This observation raised the possibility that tumor infiltrating immune cells may enhance the antitumor effects of CAR T cell therapy in GBM.

Example 2: CAR T therapy can promote immunological memory and generation of tumor-specific T cells

To evaluate whether CAR T cells have the potential to induce endogenous antitumor immunity, cured mice following CAR T cell treatment were challenged with IL13R α 2-negative parental tumors. Indeed, in the larger engrafted tumors (7 day engraftment before CAR T therapy), cured mice in the immunocompetent C57BL/6 model successfully rejected tumor rechallenge with IL13R α 2-negative K-Luc (Fig. 5A and Fig. 5B) and GL261-Luc (Fig. 3F) parental tumor cells, demonstrating that CAR T cells can promote immunological memory in two independent tumor models with differential responsiveness to anti-PD1 immunotherapy (14, 16). The capacity of CAR T cells to induce endogenous immunity against IL13R α 2-negative tumor cells again required a more established TME, as mice cured in the small tumor model (day 4) were not capable of mounting antitumor responses following rechallenge with parental tumors (Fig. 6). This observation also suggests that tumor exposure is not sufficient to induce endogenous antitumor immunity, and instead the establishment of immunological memory requires both CAR T cell therapy and the host immune infiltrates. In a similar set of experiments, we compared the antitumor activity of mIL13BB ζ CAR T cells against IL13R α 2+ K-Luc (100% IL13R α 2+ cells) versus a mixture of IL13R α 2+ K-Luc and IL13R α 2- parental K-Luc tumor cells (1:1 ratio). Supporting the notion that CAR T cells can promote endogenous immune responses against antigen-negative tumor cells, mIL13BB ζ CAR T cells mediated comparable survival benefit against tumors with homogenous and heterogeneous IL13R α 2-antigen expression ($p<0.001$; Fig. 7A and Fig. 7B). Again, the response to IL13R α 2-negative tumors required a more established tumor microenvironment, as CAR T cell therapy was less effective against mixed antigen tumors (1:1 ratio) in the small tumor model (day 4) ($p<0.001$; Fig. 7C and Fig 7D). Together these observations suggest that the TME of GBM can be altered by CAR T cell therapy to augment antitumor responses and promote endogenous antitumor memory responses.

To more directly evaluate whether CAR T therapy can potentiate the generation of tumor-specific T cells, we isolated intratumoral endogenous (CD3+CD19-) and CAR T cells (CD3+CD19+) from untreated and CAR T cell treated mice in the IL13R α 2+ K-Luc glioma model (3 days post treatment) (Fig. 8), and either *ex vivo* co-cultured with tumor cells or adoptively transferred into tumor bearing mice (Fig. 5C). *In vitro*, endogenous T cells isolated from CAR T cell treated tumors, but not control untreated groups, exhibited enhanced killing of IL13R α 2+ K-Luc cells and T cell proliferation in co-culture assays (10:1, effector:target ratio; 72 hours) (Fig. 5D and Fig. 5E). To assess *in vivo* function, endogenous T cells from untreated and CAR T cell treated mice were isolated and adoptively transferred into IL13R α 2+ K-Luc tumor bearing mice. Measurement of tumor progression demonstrated that mice injected with endogenous T cells isolated from CAR T treated mice showed a significant reduction in tumor growth compared to the control group (Fig. 5F). Collectively, these results establish that CAR T cells have the potential to promote antigen spread and the generation of tumor-specific T cell responses.

Example 3: CAR T cells activate innate and adaptive immune subsets in tumor microenvironment

To elucidate immune-related changes in the TME that coincide with the establishment of endogenous antitumor immunity following CAR T cell therapy, we interrogated both the lymphoid and myeloid compartments by gene and protein expression profiling. Focusing first on the lymphoid compartment, we performed nanostring analysis of purified intratumoral CD3+ cells and demonstrated global changes at transcriptome level in CAR T treated mice compared to untreated (Fig. 9A). To increase resolution and more accurately define subpopulations, we performed scRNAseq on isolated CD45 cells from untreated or CAR T treated mice (Fig. 10A and Fig. 10B). We then computationally separated lymphoid and myeloid populations and reanalyzed the scRNAseq data at higher granularity. This approach yielded nine distinct lymphoid subpopulations broadly defined by the distribution of classical marker genes (Fig. 10C), including three distinct subsets of CD8+ T cells (CD8_L2, CD8_L3, and CD8_L4), two subsets of CD4+ T cells (CD4_L1, CD4_L6), one subset of NK cells, two subsets of B cells and one subset resembling $\gamma\delta$ T cells (Fig. 9B and Fig. 10C). The frequency of CD8_L2 remained unchanged, but interestingly, post CAR T therapy, increased frequency of CD8_L3 and CD8_L4

subclusters were detected Fig. 10C). Focusing on T cell subclusters, CD8_L3 is observed mainly post therapy and is characterized by expression of *Cxcr3* (Fig. 11a) which is associated with T cell trafficking and expression of *Itgae* (CD103), *Cd74* (Hladr) and *Ifitm1* (IFN-induced transmembrane protein 1) that correspond to activated resident memory CD8 T cell phenotype (Fig. 9C). CD8_L4 expanded post-therapy and was identified as highly activated, effector T cells based on higher expression of *Ki67*, *Cd74*, and *Gzma* genes (Fig. 9C). Within the CD4 subsets, the frequency of CD4_L1 cluster remained unchanged after therapy, it displayed a modest increase in expression of *Il7r*, *Tcf7*, and *Itga4* genes which is associated with effector memory CD4 T cells in CAR T treated group (Figs. 10C and 11A). Intratumoral regulatory T cells (Treg), defined by subcluster CD4_L6 based on the expression of CD4, *Foxp3*, GTR (*Tnfrsf18*) and *Ctla4*, decreased after CAR T therapy (Fig. 11B). Overall, gene set enrichment analysis (GSEA), revealed enrichment of gene signatures associated with activated and Th1 responses in most T cells subclusters (Fig. 9D). Taken together, these studies establish that CAR T cells can dramatically alter the lymphoid compartment within tumors and result in clusters of activated, memory or effector T cell populations.

To further characterize T cell populations post-CAR T cell therapy at cellular level and differentiate changes in endogenous versus adoptively transferred T cells, isogenically mismatched CD45.1 CAR T cells were used to treat IL13R α 2+ K-Luc tumors engrafted in CD45.2 mice (Fig. 9e). Following intracranial delivery, CAR T cells (CD3+ CD45.1+CD19+), but not mock-transduced T cells (CD3+CD45.1+CD19-), displayed a significant increase in T cell count and expression in markers of activation (CD69+), cytotoxic function (GZMB+) and proliferation (Ki67+) (Fig. 9f), establishing that the observed effector activity was CAR-dependent. Interestingly, only after CAR T therapy, a significant increase in the endogenous T cells (CD3+CD45.2+) count with activated (CD69+), proliferative (Ki67+), and cytotoxic phenotype (GZMB+) was observed, which was not detected in untreated or mock treated controls (Fig. 9G). This was in line with qPCR analysis demonstrating upregulation of *GZMA*, *GZMB*, and *PRF1* genes in intratumoral CD3+ T cells after CAR T therapy (Fig. 12). These results establish that CAR T cells promote endogenous T cell activation and expansion, and are consistent with our previous finding that endogenous intratumoral T cells isolated post CAR T cell therapy have antitumor activity (Figs. 5D-5F). We next evaluated changes in the innate myeloid cells, such as microglia/macrophages, as they represent a dominant immune population

in the glioma tumors and have decisive role in glioma pathogenesis (17). Analysis of intratumoral myeloid population at single cell level identified 17 distinct myeloid subpopulations which underwent a striking remodeling following CAR T therapy (Fig. 13A).

We observed an interesting complexity and dynamics of the intratumoral

monocyte/macrophage/microglia/DC compartment in glioma TME. While some macrophage/monocyte subpopulations decreased in frequency, other populations expanded and re-shaped the TME. Seven major monocyte/macrophage (*Itgam*, *Cd68*), four microglia (*Tmem119* and *P2ry12*), four DC and two clusters of neutrophils (*SI100A9*) subpopulations were identified. Gene set enrichment analysis (GSEA) revealed enrichment of genes associated with IFN γ -stimulated macrophage and microglia in CAR-treated groups (Fig. 13B). Further assessment of the main myeloid populations (macrophage, microglia, and neutrophils) identified higher expressions of genes associated with mature and IFN γ -activated macrophages as well as stimulated neutrophils (Fig. 13C), further confirming that resident innate immune cells have been exposed to IFN γ -mediated activation.

Nanostring analysis of intratumoral microglia/macrophages cells (CD11b+) from the TME 3 days post-CAR T therapy showed enrichment of genes that mediate antigen processing and presentation (e.g., *Cd74*, *H2-Ab1*, *H2-Aa*, *H2-Eb1*) (Fig. 13D). Further analyses with scRNAseq revealed that majority of macrophage/microglia subclusters may be involved in antigen processing and presentation (Fig. 13E). Assessing CAR T cell mediated changes in resident microglia/macrophage populations by flow cytometry, we found an increased frequency and number of activated brain-resident macrophage/microglia cells (CD86+, MHCII^{high}, MHCII^{high}) in CAR T-treated mice (Fig. 13F). Assessment of myeloid compartment also revealed a significant increase in the frequency of proinflammatory cytokine IFN γ + (Fig. 13F). Collectively, these data show that CAR T therapy changes the GBM immune landscape and activates the host innate and adaptive immune cells. These results also further reveal a major role for IFN γ in inducing activation of local immune cells.

Example 4: Lack of IFN γ in CAR T cells impairs *in vivo* antitumor activity and activation of host immune cells

Given that the myeloid cells constituted the largest population in the glioma TME and our scRNAseq analysis identified gene-signatures related to IFN γ -stimulation within the macrophages and microglia subclusters (Fig. 13B), we reasoned that IFN γ produced by stimulated CAR T cells may play a role in modulating the activation of resident
 5 macrophage/microglia cells and subsequent priming and induction of adaptive immune response.

IFN γ is one of the key effector cytokines abundantly produced by CAR T cells upon activation and is a prototypic macrophage activator (18). To investigate whether IFN γ secreted by CAR T cells is responsible for changes observed in phenotype and function of resident
 10 macrophages/microglia cells, CAR T cells were developed from WT (CAR T^{wt}) or IFN γ ^{-/-} (CAR T<sup>IFN γ ^{-/-}) mice (Fig. 14A) and characterized accordingly. CAR transduction efficiency, cell viability, expansion and ratio of CD4:CD8 in both CAR T cell populations (CAR T^{wt} and CAR T<sup>IFN γ ^{-/-}) showed comparable therapeutic products (Fig. 14B). We next verified the functionality of CAR T cells derived from IFN γ ^{-/-} mice by conducting an *in vitro* killing assay in comparison
 15 with CAR T cells from WT mice, which demonstrated comparable killing potency at a 1:1 effector to target ratio (Fig. 14C). Assessment of CAR T polyfunctionality demonstrated comparable production of TNF α and GZMB in both CAR T^{wt} and CAR T<sup>IFN γ ^{-/-} cells with expected lack of IFN γ production in CAR T<sup>IFN γ ^{-/-} (Fig. 14D). *In vivo*, mice that received CAR T<sup>IFN γ ^{-/-} exhibited poor overall survival compared to mice treated with CAR T^{wt}, indicating that
 20 IFN γ deficiency in CAR T cells dampens their antitumor activity and results in poor survival (Fig. 14E and Fig. 14F). Analysis of total TME showed enhanced expression in genes involved in activation and proinflammatory cytokines in mice that received CAR T^{wt} and conversely reduced expression of genes involved in suppressive phenotype and function of intratumoral immune infiltrates (Fig. 14G) indicating that lack of IFN γ secretion by CAR T cells changes the
 25 glioma TME. IFN γ is a pleiotropic cytokine that induces activation of CD8 T cells (9), promotes polarization of Th1 CD4 cells (19) and reprograms or activates macrophage/microglia cells (6, 7). Therefore, we then assessed whether lack of IFN γ secreted by CAR T cells impacted the host immune cells. Flow cytometry analysis of TME 3 days post CAR T cell therapy revealed a significant decrease in T cell number, both endogenous and CAR T cells, that correlated with a
 30 reduction in activated (CD69+) T cells (Fig. 14H and Fig. 14I). Furthermore, a significant increase in frequency of MHC I+/MHC II+ and CD86+ macrophage/microglia cell activation in</sup></sup></sup></sup></sup>

tumor bearing mice that received CAR T^{wt} compared with CAR T^{IFN γ -/-} cells (Fig. 14J) was observed. Thus, IFN γ production as a consequence of CAR T antitumor activity results in activation and reinvigoration of T cells and increase activation and the antigen presenting potential of macrophage/microglia cells.

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Example 5: Lack of IFN γ -signaling in the host results in dampen CAR T antitumor activity *in vivo*

Previous studies have reported IFN γ signaling as a signature of response to immunotherapies such as anti-PD1 treatment (20). In order to investigate whether host IFN γ signaling plays a role in the CAR T-mediated immune response, CAR T^{wt} cells were adoptively transferred into K-Luc-bearing WT or IFN γ R^{-/-} mice (Fig. 15A). IFN γ R^{-/-} mice that received CAR T^{wt} demonstrated a survival disadvantage, suggesting that lack of IFN γ signaling in the host immune cells dampens the antitumor activity of CAR T cells and the overall survival (Fig. 15B and Fig. 15C).

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Gene expression analysis of TME revealed that lack of IFN γ signaling in the host during CAR therapy resulted in reduced expression of genes involved in activation and proinflammatory responses (*CD40*, *NOS2*, *TNF α* , *GZMA*, *GZMB*, *PRF1*, and *IFN γ*) (Fig. 15D). To further investigate CAR T cell-mediated changes in the immune landscape, we adoptively transferred isogenically mismatched (Thy1.1+) CAR T^{wt} cells into K-Luc-bearing WT or IFN γ R^{-/-} mice.

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Flow cytometry analysis of TME revealed significant increase in activation of macrophage/microglia cells (CD11b+CD86+MHCI+MHCII+) in WT mice compared to IFN γ R^{-/-} mice as early as 3 days post CAR T cell therapy (Fig. 15E). Compared to WT mice, the number of endogenous T cells (Thy1.2+CD3+), activated with proliferative and effector-cytokine producing capacities was significantly lower in IFN γ R^{-/-} mice (Fig. 15F). Interestingly, relative to WT mice, the adoptively transferred CAR T cells (Thy1.1+CD3+) exhibited significantly higher counts, proliferative (Ki67+) and cytotoxic phenotype (IFN γ + and GZMB+), relative to IFN γ R^{-/-} mice (Fig. 15G) suggesting potential IFN γ positive feedback from host immune cells that promote CAR T cell activity in the TME. Collectively, these results confirm that there is an interplay between host and adoptively transferred immune cells and host IFN γ signaling in glioma TME is necessary for mounting a potent immune response during CAR T therapy.

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To investigate whether IFN γ secreted by CAR T cells is responsible for changes observed in phenotype and function of resident macrophages/microglia cells, CAR T cells were generated from wild-type (CAR T^{WT}) or IFN γ ^{-/-} (CAR T^{IFN γ ^{-/-}) mice (Fig. 15A) and characterized accordingly. CAR transduction efficiency, cell viability, expansion in both CAR T-cell populations (CAR T^{WT} and CAR T^{IFN γ ^{-/-}) showed comparable therapeutic products with some difference in ratio of CD4:CD8 T cells ($P < 0.05$; Fig. 15B). We next verified the functionality of CAR T cells derived from IFN γ ^{-/-} mice by conducting an *in vitro* killing assay in comparison with CAR T cells from WT mice, which demonstrated comparable killing potency at a 1:1 E:T ratio (Fig. 15C). Assessment of CAR T-cell polyfunctionality demonstrated comparable production of TNF α and GZMB in both CAR T^{WT} and CAR T^{IFN γ ^{-/-} cells with expected lack of IFN γ production in CAR T^{IFN γ ^{-/-} (Fig. 15D). *In vivo*, mice that received CAR T^{IFN γ ^{-/-} exhibited poor overall survival compared with mice treated with CAR T^{WT}, indicating that IFN γ deficiency in CAR T cells dampens their antitumor activity *in vivo* (Figs. 15E and 15F). Gene expression analysis of tumor and the associated TME 3 days post-CAR T-cell therapy revealed enhanced expression in genes involved in activation and proinflammatory cytokines in mice that received CAR T^{WT} cells and, conversely, reduced expression of genes involved in suppressive phenotype and function of intratumoral immune infiltrates (Fig. 15G), indicating that lack of IFN γ secretion by CAR T cells changes the glioma TME. IFN γ is a pleiotropic cytokine that induces activation of CD8 T cells, promotes polarization of Th1 CD4 cells, and reprograms or activates macrophage/microglia cells. Therefore, we then assessed whether lack of IFN γ secreted by CAR T cells affected the host immune cells. Flow cytometry analysis of TME 3 days post-CAR T-cell therapy revealed a significant decrease in T-cell number, in both endogenous and CAR T cells, which correlated with a reduction in activated (CD69⁺) T cells (Fig. 15H and 15I). Furthermore, a significant increase in frequency of MHCII⁺/MHCII⁺ and CD86⁺ macrophage/microglia cell activation in tumor-bearing mice that received CAR T^{WT} compared with CAR T^{IFN γ ^{-/-} cells (Fig. 15J) was observed. Importantly, lack of IFN γ secretion by the CAR T cells resulted in higher M2-type intratumoral macrophages in mice that received CAR T^{IFN γ ^{-/-} cells compared with CAR T^{WT} cells. Thus, IFN γ production as a consequence of CAR T-cell antitumor activity resulted in activation and reinvigoration of T cells and reprogramming of macrophage/microglia cells to enhance their activation and antigen-presenting potential.}}}}}}}

Example 6: Human CAR T cell therapy modulates patient host immune cells

We next investigated the impact of CAR T cell antitumor activity on human endogenous immune cells in GBM patients. To clinically assess if CAR T cells are able to promote activation of GBM patient monocytes or macrophages, we developed an *in vitro* assay to phenotypically characterize patient myeloid populations in the presence of CAR T cell antitumor activity. Supernatants from co-culture of human CAR T cells against patient-derived glioma tumors were collected and subsequently incubated with glioma patient derived-monocytes (Fig. 16A), *ex vivo* differentiated patient macrophages or total CD3+ patient T cells (Fig. 17A). Phenotypic and morphological assessments revealed that conditioned media (CM) from CAR T-tumor co-culture promoted differentiation and activation of patient derived-monocytes or macrophages, as demonstrated by increase in expression of activation markers (CD14+CD86+/CD80+ and CD14+HLADr+), which is correlated with enhanced expression of genes associated with classical M1 macrophages (Figs. 16B-16D and Figs. 17B-17D). Accordingly, exposure to CM from CAR T-tumor co-culture resulted in induced activation of isolated T cells from GBM patient blood, as evidenced by increased expression of CD69 (Fig. 17E and Fig. 17F). Importantly, blockade of IFN γ signaling in macrophages and T cells resulted in reduced activation (Fig. 17B-17F), highlighting the impact of IFN γ in the CAR T-mediated activation of host immune cell in GBM patients. Taken together these findings suggest that IFN γ secreted by CAR T cell antitumor function promotes polarization of monocytes into activated macrophages, further induce activation of differentiated macrophages and promote T cell activation.

Lastly, we aimed to assess if CAR T cells have the potential to induce generation of tumor-specific T cells in clinical setting, as we demonstrated in our immune competent mouse models of GBM. In order to precisely investigate this phenomenon, we evaluated samples from a case report that exhibited a complete response and was a unique responder to CAR T therapy (4). T cells were isolated from blood before CAR T cell treatment (Pre-CAR T) and during response to CAR T therapy (Post-CAR T) (Fig. 17G). Isolated T cells were stimulated and expanded in the presence of irradiated autologous tumor cells. Flow cytometry assessment of T cell populations revealed increased tumor reactivity as indicated by increased intracellular IFN γ and proliferation for T cells isolated during response versus prior to the initiation of CAR T cell therapy (Fig. 17H)

and Fig. 17I). Importantly, in a patient derived co-culture of *ex vivo* expanded T cells with autologous patient glioma tumor or irrelevant tumor cell line, T cells isolated during response to CAR T therapy exhibited tumor-specific killing against autologous versus irrelevant tumor cells (Fig. 17J). These results were in light of the tumor cells being IL13R α 2 negative (Fig. 17K).

5 These findings confirm our preclinical studies that an effective CAR T therapy has the potential to stimulate the host immune cells, promote generation of tumor-specific T cell responses and furthermore, the host immune cells play an important role in a successful CAR T treatment.

Example 7: Co-Expression of IL-13 CAR T and interferon gamma

10 We examined the impact of co-expressing interferon gamma by creating an expression cassette in which the IL-13 CAR of Example 1 (Fig. 1A) is joined to immature murine interferon gamma via a T2A skip sequence (Fig. 20A). We designed and constructed IL13R α 2-CAR/IFN γ for murine and human platforms and demonstrate that incorporating IFN γ in the CAR cassette is
15 feasible with comparable transduction and expansion of CAR T cells (Fig. 20A). The vectors were sequenced and verified. Murine T cells were isolated transduced with either a vector expressing the IL-13 CAR and truncated CD19 (lacking a signaling domain) or the IL-13 CAR and murine interferon gamma (Fig. 20B). Culture supernatant was collected and flow cytometry was used to assess the presence of IL-13 as a marker for CAR expression. Both constructs
20 expressed the IL-13 CAR, and transduction efficiency is above 50% by FACS (Fig. 20C). Furthermore, to verify that IL13R α 2-CAR/IFN γ vectors endow T cells with the ability to express and secrete IFN γ , we collected supernatant from *ex vivo* expanded IL13R α 2- CAR/ IFN γ and IL13R α 2-CAR T cells and measured IFN γ levels with ELISA (Fig. 1D). Measurement of interferon gamma production by ELISA showed only the IL-13 CAR T-interferon gamma
25 construct expressed interferon gamma (Fig. 20D).

Next, we assessed the phenotype of the CAR T cells. Our studies demonstrated no phenotypic differences between IL13R α 2-CAR/IFN γ and IL13R α 2-CAR in murine (Fig. 21E) and human (Fig. 22C) T cells. Briefly, murine IL-13 CAR T cells and murine IL-13 CAR-interferon gamma
30 T cells were co-cultured with murine glioma tumor cells at a 1:3 effector : target ratio for 24 hours. As demonstrated in Figs. 21A-21C, the IL-13 CAR-interferon gamma T cells exhibited

both superior proliferation and tumor cell killing. T cell activation was assessed by measuring CD69 expression. As can be seen in **Fig. 21D**, the activation of IL-13 CAR T cells and the IL-13 CAR T-interferon gamma T cells was similar.

- 5 CAR T cells expressing an human IL-13 CAR (human IL-13 with E13Y mutation, human CD8 hinge, human CD8 TM, human 4-1BB co-stimulatory domain and human CD3 zeta) with or without co-expressed human interferon gamma were produced. The human IL-13 CAR T cells and human IL-13 CAR-interferon gamma T cells were co-cultured with patient-derived glioma tumor cells at a 1:25 effector : target ratio for 24 hours. T cells and tumor cells were assessed.
- 10 As can be seen in **Fig. 22A**, the IL-13 CAR-interferon gamma T cells exhibited superior proliferation and roughly similar T cell killing. Importantly, when co-cultured with antigen positive tumors, there is increased expansion of IL13R α 2-CAR/IFN γ T cells compared to IL13R α 2-CAR T cells in murine (**Figs. 21C**) and human (**Fig. 22B**). In both murine and human, a slightly enhanced killing capacity is observed in IL13R α 2-CAR/IFN γ T cells compared to
- 15 IL13R α 2-IFN γ CAR T cells.

Importantly, to verify that the secreted IFN γ from IL13R α 2-CAR/IFN γ T cells were functional and have immune stimulatory components, supernatant from *ex vivo* expanded T cells engineered to constitutively express IFN γ were added to macrophage cultures (**Fig. 26A**).

- 20 Macrophages exposed to IL13R α 2-CAR/IFN γ culture conditions exhibited greater proinflammatory and metabolically active phenotype as compared to supernatants collected from IL13R α 2-CAR T cells (**Fig. 26B**). Collectively, these results show that IL13R α 2-CAR/IFN γ express and secrete IFN γ and have the potential to activate endogenous innate immune populations such as macrophages.

- 25 We have conducted a pilot study assessing the antitumor activity of IL13R α 2-CAR/IFN γ vs. IL13R α 2-CAR in mice bearing tumors with high (**Fig. 28B**; left) or medium/low (**Fig. 28C**; right) IL13R α 2 antigen in our syngeneic model. While the function of the two CARs are comparable in high antigen expressing tumors, there is a significant change in the antitumor activity in mice bearing medium antigen expressing tumors treated with IL13R α 2-CAR/IFN γ
- 30 compared to IL13R α 2-CAR.

As part the global impact of IFN γ , we also tested whether IL13R α 2-IFN γ CARs exhibit superior antitumor activity against metastatic diseases or tumors at distant sites. Thus, murine IL-13 CAR T cells and murine IL-13 CAR-interferon gamma T cells were assessed in a murine model of metastatic melanoma (**Fig. 23**). Briefly, tumor cells were injected into both flanks of mice. Once the tumors reached a predetermined size, Murine IL-13 CAR T cells and murine IL-13 CAR-interferon gamma T cells were injected locally to one tumor. Tumor size was measured on both sides. The graph in **Fig. 23** shows the tumor volume on the non-treated side. As can be seen, the IL-13 CAR T-interferon gamma T cells exhibited a greater abscopal effect than the IL-13 CAR T cells. Interestingly, our studies on melanoma bearing mice demonstrate that IL13R α 2-CAR/IFN γ T cells have superior capacity to target distant tumors. These findings strongly highlight IL13R α 2-CAR/IFN γ as an important therapeutic approach for cancer with multifocal lesions such as GBM.

Experiments were designed to test *in vivo* functional activity of IL13R α 2-IFN γ CAR in syngeneic immunocompetent glioma models and NSG mice implanted with IL13R α 2+ primary brain tumor lines (PBTs). We demonstrated that IL13R α 2-CAR/IFN γ T cells have superior antitumor activity in mice bearing medium/low antigen tumors and eradicate tumors at distant sites in metastatic melanoma model. To assess the importance of IFN γ as immunostimulatory agent and therapeutic importance of IL13R α 2-CAR/IFN γ T cells, we developed a 3-way coculture system using CAR T cells, macrophages, and tumor cells (**Figs. 31A-31B**). Our results suggests that in the presence of other immune cells such as macrophages, IL13R α 2-CAR/IFN γ T cells exhibit a significantly superior antitumor function and stimulates endogenous immunity.

Example 8: Optimizing Co-Expression of IL-13 CAR T and interferon gamma

We also designed and constructed different IL13R α 2-CAR/IFN γ variants to prioritize for both efficacy and safety by optimizing and regulating IFN γ expression. We successfully designed and sequence checked the constructs shown in **Fig. 29A**. T cells were transduced with CAR/IFN γ variants, and supernatant was collected to validate IFN γ production and cocultured with IL13R α 2+ tumors to confirm functionality (**Fig. 29B**). The CAR/IFN γ variants were assessed

for different levels of IFN γ expression and secretion. Relative to EF1 promoter, which is a strong promoter, the pkg100 promoter is a weaker promoter. CAR T cells having IFN γ under the pkg100 promoter showed reduced level of IFN γ (Fig. 29C). The IL13R α 2-CAR/IFN γ^{low} T cell addresses safety concerns related to excessive production of IFN γ . Next, to confirm cytotoxic function, IL13R α 2-CAR/IFN γ^{low} CAR T cells were cocultured with IL13R α 2+ tumors at 1:50 ratio effector to target for 5 days. Assessment of viable tumor count showed that IL13R α 2-CAR/IFN γ^{low} T cells exhibited comparable cytotoxic function to standard IL13R α 2-CAR T cells (Fig. 29D).

We designed an inducible construct system using a synthetic NFAT promoter to control IFN γ expression. This construct was designed to control the expression of the gene of interest, ensuring that expression of IFN γ will only occur when CAR T cells are activated. As proof of concept, we placed GFP under the control of an NFAT promoter. Our studies demonstrated that upon CAR activation in the presence of IL13R α 2 antigen positive tumors, the NFAT promoter is functional and can induce GFP expression (Figs. 27A-27C).

Next, we replaced the GFP gene with IFN γ (Fig. 30A). T cells transduced with the constructs were cocultured with IL13R α 2+ tumors at 1:50 effector to target ratio for 5 days (Fig. 30B). Our results confirms that NFAT CARs are functional and exhibit comparable killing capacity to standard IL13R α 2-CAR T cells (Fig. 30C). These results indicated the incorporation of synthetic NFAT promoter in the CAR construct did not impair the function of the CAR T cells.

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20

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed
5 description thereof, the foregoing description is intended to illustrate and not limit the scope of
the invention.

WHAT IS CLAIMED IS:

1. A nucleic acid molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR), wherein the chimeric antigen receptor comprises: a targeting domain, a
5 spacer, a transmembrane domain, a co-stimulatory domain, and a CD3 ζ signaling domain; and a nucleotide sequence encoding a polypeptide comprising a human interferon gamma or a variant thereof.
2. The nucleic acid molecule of claim 1, wherein the nucleic acid molecule comprises a promoter that controls expression of both the CAR and human interferon gamma.
- 10 3. The nucleic acid molecule of claim 1, wherein a first promoter controls expression of the CAR and a second promoter controls expression of the human interferon gamma or variant thereof.
4. The nucleic acid molecule of claim 3, wherein the first promoter is a constitutive promoter and the second promoter is a constitutive promoter or is an inducible promoter.
- 15 5. The nucleic acid molecule of claim 1, wherein a nucleotide sequence encoding a 2A skip sequence is located between the nucleotide sequence encoding a CAR and the nucleotide sequence encoding a human interferon gamma or a variant thereof.
6. The nucleic acid molecule of claim 5, wherein the 2A skip sequence is selected from the group consisting of T2A, P2A, E2A and F2A.
- 20 7. The nucleic acid molecule of claim 1, wherein the human interferon gamma or variant thereof comprises the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: B.
8. The nucleic acid molecule of claim 1, wherein the CAR comprises the amino acid sequence of SEQ ID NO: C.
9. The nucleic acid molecule of claim 1, wherein the transmembrane domain is
25 selected from: a CD4 transmembrane domain, a CD8 transmembrane domain, a CD28 transmembrane domain, and a NKG2D transmembrane domain.

10. The nucleic acid molecule of claim 1, wherein the transmembrane domain is a CD28 transmembrane domain or a CD8 transmembrane domain.

11. The nucleic acid molecule of claim 1, wherein the costimulatory domain is a CD28, a 4-1BB, or a 2B4 costimulatory domain.

12. The nucleic acid molecule of claim 1, wherein the costimulatory domain comprises the amino acid sequence of any of SEQ ID NOs:22-25 and 66.

13. The nucleic acid molecule of claim 1, wherein the CD3 ζ signaling domain comprises the amino acid sequence of SEQ ID NO:21 or a variant thereof comprising any of SEQ ID NOs: 50-56.

14. The nucleic acid molecule of claim 1, wherein a linker of 3 to 15 amino acids is located between the costimulatory domain and the CD3 ζ signaling domain or variant thereof.

15. The nucleic acid molecule of claim 1, wherein the spacer comprises any one of SEQ ID NOs:2-12 and 44.

16. The nucleic acid molecule of claim 1, wherein the targeting domain comprises an scFv targeted to any cancer cell antigen.

17. The nucleic acid molecule of claim 16, wherein the scFv is target to any one or more of CD19, MUC16, MUC1 (or tMUC1), CAIX, CEA, CD20, CD22, CD30, HER-2, ERBB2, MAGEA3, p53, PSCA BCMA, CD123, CD44V6, Integrin B7, ICAM-1, CD70, CEA, GD2, PSMA, B7H3, CD33, Flt3, CLL1, folate receptor, EGFR, CD7, EGFRvIII, glypican3, CD5, ROR1, CS1, AFP, CD133, and TAG-72.

18. The nucleic acid molecule of claim 1, wherein the targeting domain comprises a ligand.

19. The nucleic acid of claim 18, wherein the ligand is selected from an IL-13 or a variant thereof, a chlorotoxin or variant thereof.

20. The nucleic acid molecule of claim 1, wherein the CAR comprises the amino acid sequence of any of SEQ ID NOs 70-76, or a variant thereof having 1-5 amino acid modifications.

21. The nucleic acid molecule of claim 1, wherein the polypeptide comprising human interferon gamma comprises a signal sequence for secretion of human interferon gamma.

5 22. The nucleic acid molecule of claim 1, wherein the polypeptide comprising human interferon gamma comprises a signal sequence for secretion of human interferon gamma that differs from the native human interferon gamma signal sequence.

23. A population of human T cells harboring: (a) a nucleic acid molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR), wherein the chimeric antigen
10 receptor or polypeptide comprises: a targeting domain, a spacer, a transmembrane domain, a co-stimulatory domain, and a CD3 ζ signaling domain; and a nucleotide sequence encoding a polypeptide comprising a human interferon gamma or a variant thereof

Or (b) a nucleic acid molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR), wherein the chimeric antigen receptor or polypeptide comprises: a
15 targeting domain, a spacer, a transmembrane domain, a co-stimulatory domain, and a CD3 ζ signaling domain; and a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a human interferon gamma or a variant thereof.

24. A population of human T cells harboring: (a) a nucleic acid molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR), wherein the chimeric antigen
20 receptor or polypeptide comprises: a targeting domain, a spacer, a transmembrane domain, a co-stimulatory domain, and a CD3 ζ signaling domain; and a nucleotide sequence encoding a polypeptide comprising a human interferon gamma or a variant thereof

Or (b) a nucleic acid molecule comprising a nucleotide sequence encoding a chimeric antigen receptor (CAR), wherein the chimeric antigen receptor or polypeptide comprises: a
25 targeting domain, a spacer, a transmembrane domain, a co-stimulatory domain, and a CD3 ζ signaling domain; and a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a human interferon gamma or a variant thereof.

25. The population of human T cells of claim 23 or 24, wherein the nucleic acid molecule comprises a promoter that controls expression of both the CAR and human interferon gamma.

26. The population of human T cells of claim 23 or 24, wherein the nucleic acid molecule comprises a promoter that controls expression of both the CAR and human interferon gamma.

27. The population of human T cells of claim 23 or 24, wherein a first promoter controls expression of the CAR and a second promoter controls expression of the human interferon gamma or variant thereof.

28. The population of human T cells of claim 27, wherein the first promoter is a constitutive promoter and the second promoter is a constitutive promoter or is an inducible promoter.

29. The population of human T cells of claim 23 or 24, where a nucleotide sequence encoding a 2A skip sequence is located between the nucleotide sequence encoding a CAR and the nucleotide sequence encoding a human interferon gamma or a variant thereof.

30. The population of human T cells of claim 23 or 24, wherein the 2A skip sequence is selected from the group consisting of T2A, P2A, E2A and F2A.

31. The population of human T cells of claim 23 or 24, wherein the human interferon gamma comprises the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: B.

32. The population of human T cells of claim 23 or 24, wherein the CAR comprises the amino acid sequence of SEQ ID NO: C.

33. The population of human T cells of claim 23 or 24, wherein the transmembrane domain is selected from: a CD4 transmembrane domain, a CD8 transmembrane domain, a CD28 transmembrane domain, and a NKG2D transmembrane domain.

34. The population of human T cells of claim 23 or 24, wherein the transmembrane domain is a CD28 transmembrane domain or a CD8 transmembrane domain.

35. The population of human T cells of claim 23 or 24, wherein the costimulatory domain is a CD28, a 4-1BB, or a 2B4 costimulatory domain.

36. The population of human T cells of claim 23 or 24, wherein the costimulatory domain comprises the amino acid sequence of any of SEQ ID NOs:22-25 and 66.

5 37. The population of human T cells of claim 23 or 24, wherein the CD3 ζ signaling domain comprises the amino acid sequence of SEQ ID NO:21 or a variant thereof comprising any of SEQ ID NOs: 50-56.

10 38. The population of human T cells of claim 23 or 24, wherein a linker of 3 to 15 amino acids is located between the costimulatory domain and the CD3 ζ signaling domain or variant thereof.

39. The population of human T cells of claim 23 or 24, wherein the spacer comprises any one of SEQ ID NOs:2-12 and 44.

40. The population of human T cells of claim 23 or 24, wherein the targeting domain comprises an scFv targeted to any cancer cell antigen.

15 41. The population of human T cells of claim 40, wherein the scFv is target to any one or more of CD19, MUC16, MUC1 (or tMUC1), CAIX, CEA, CD20, CD22, CD30, HER-2, MAGEA3, p53, PSCA BCMA, CD123, CD44V6, Integrin B7, ICAM-1, CD70, CEA, GD2, PSMA, B7H3, CD33, Flt3, CLL1, folate receptor, EGFR, CD7, EGFRvIII, glypican3, CD5, ROR1, CS1, AFP, CD133, and TAG-72.

20 42. The population of human T cells of claim 23 or 24, wherein the targeting domain comprises a ligand.

43. The population of human T cells of claim 42, wherein the ligand is selected from an IL-13 or a variant thereof, a chlorotoxin or variant thereof.

25 44. The population of human T cells of claim 23 or 24, wherein the CAR comprises the amino acid sequence of any of SEQ ID NOs 70-76, or a variant thereof having 1-5 amino acid modifications.

45. The population of human T cells of claim 23 or 24, wherein the polypeptide comprising human interferon gamma comprises a signal sequence for secretion of human interferon gamma.

5 46. The population of human T cells of claim 23 or 24, wherein the polypeptide comprising human interferon gamma comprises a signal sequence for secretion of human interferon gamma that differs from the native human interferon gamma signal sequence.

47. A method of treating a cancer in a patient comprising administering a population of autologous or allogeneic human T cells transduced by a vector comprising the nucleic acid molecule of any one of claims 1-22, wherein the cancer is targeted by the targeting domain of the
10 CAR.

48. A method of treating a cancer in a patient comprising administering the population of human T cells of claims 23-46, wherein the human T cells are autologous or allogeneic, and wherein the cancer is targeted by the targeting domain of the CAR.

49. The population of human T cells of claim 28, wherein the first promoter is a
15 strong constitutive promoter and the second promoter is a weaker constitutive promoter.

50. The nucleic acid molecule of claim 4, wherein the first promoter is a strong constitutive promoter and the second promoter is a weaker constitutive promoter.



FIG. 1A

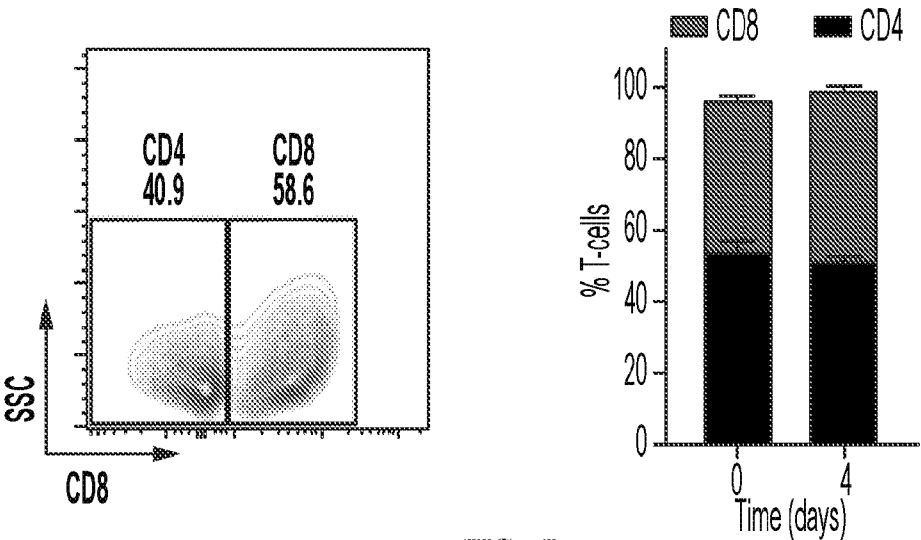


FIG. 1B

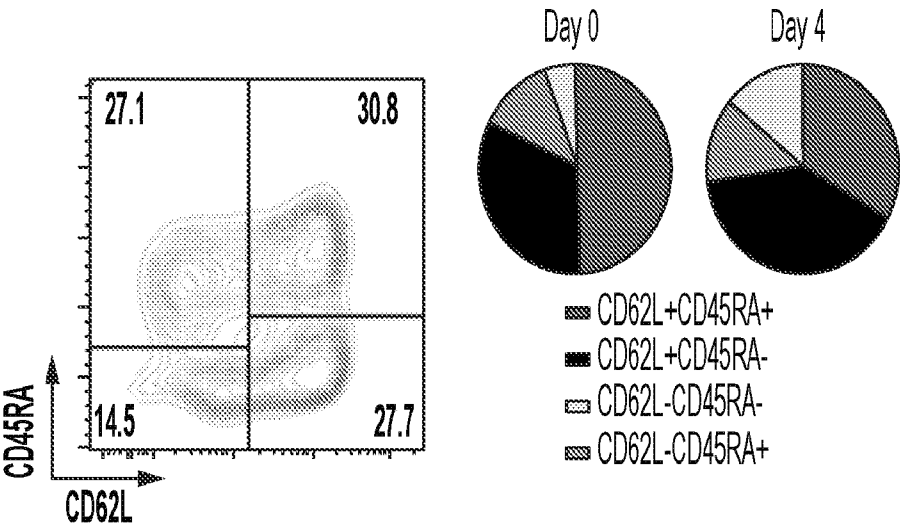


FIG. 1C

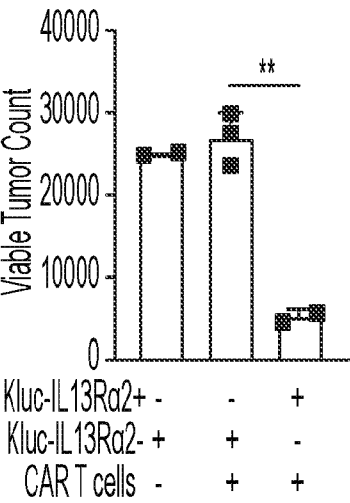


FIG. 1D

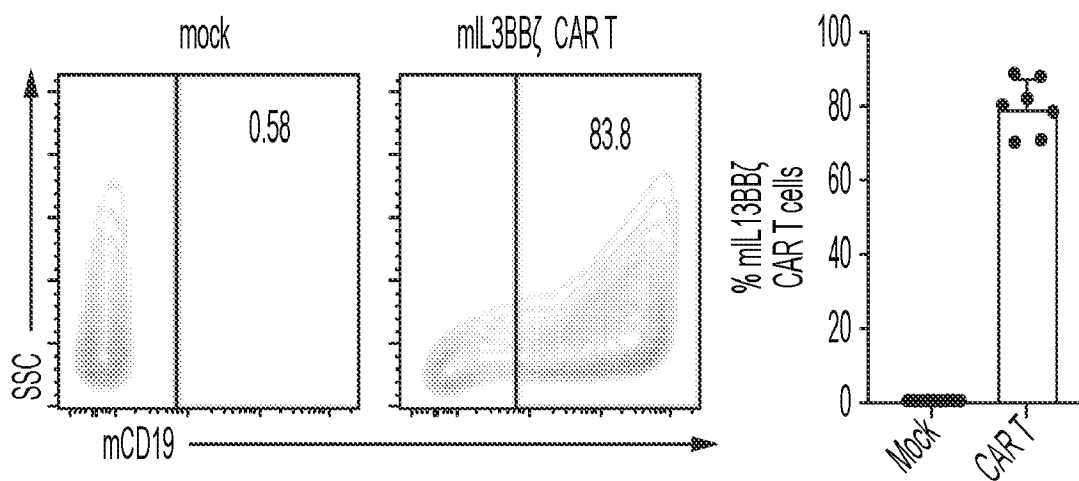


FIG. 2A

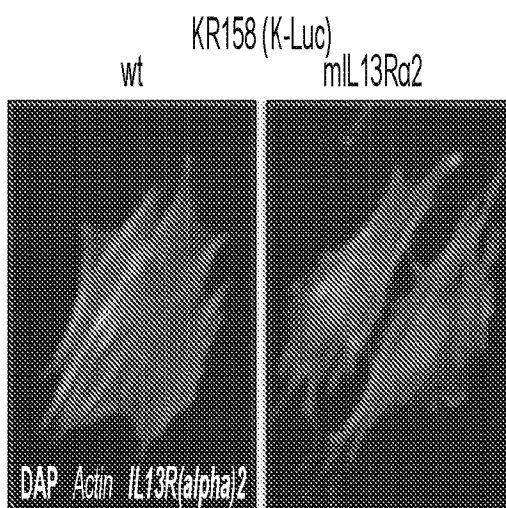


FIG. 2B

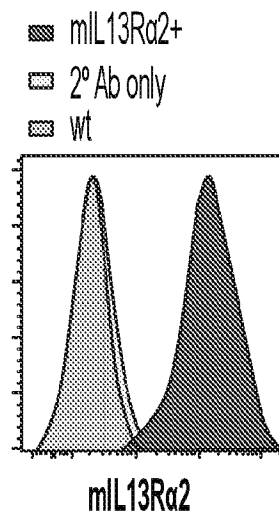


FIG. 2C

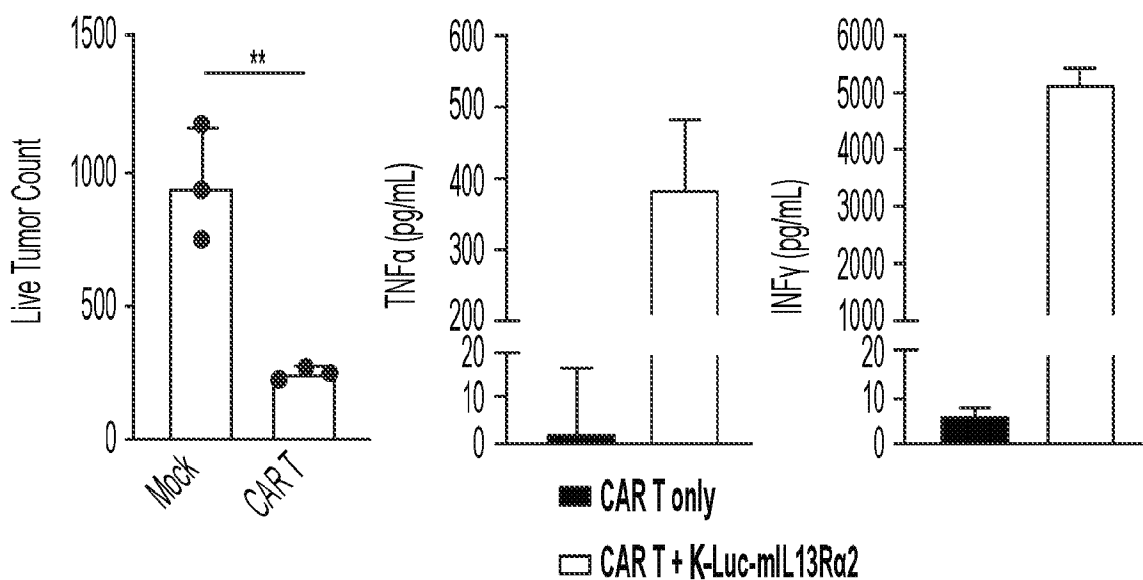


FIG. 2D

FIG. 2E

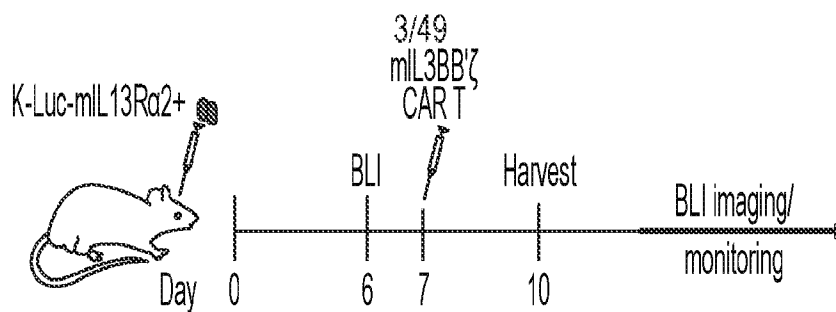


FIG. 2F

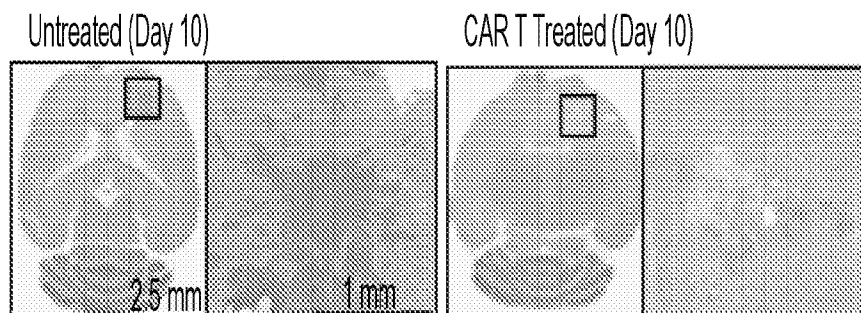


FIG. 2G

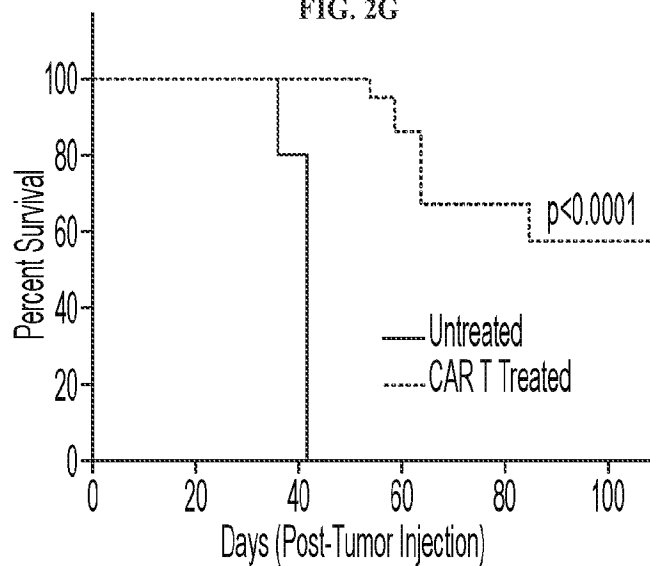


FIG. 2H

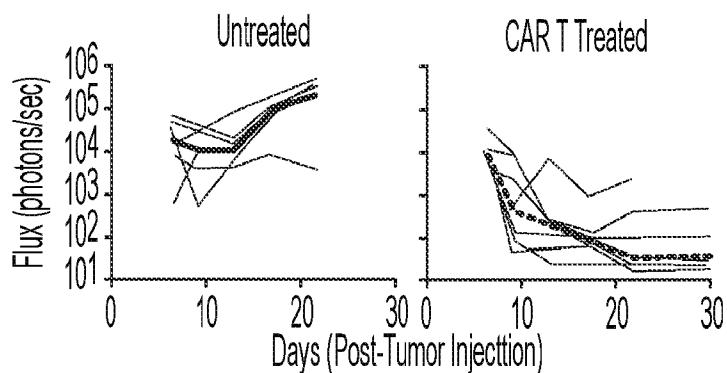
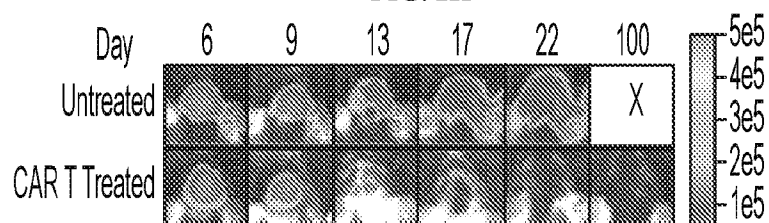


FIG. 2I

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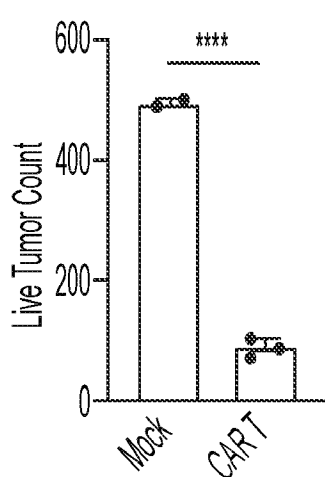
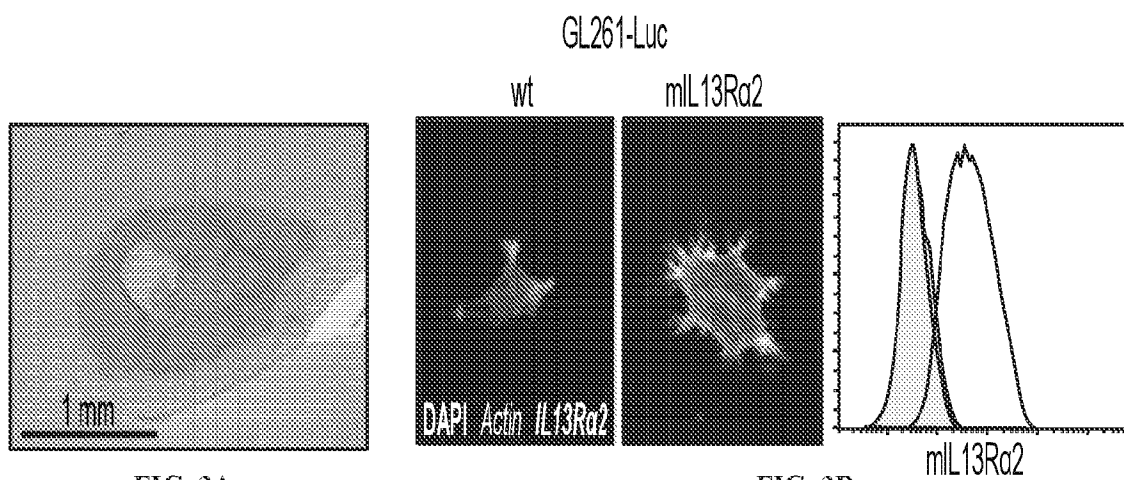


FIG. 3C

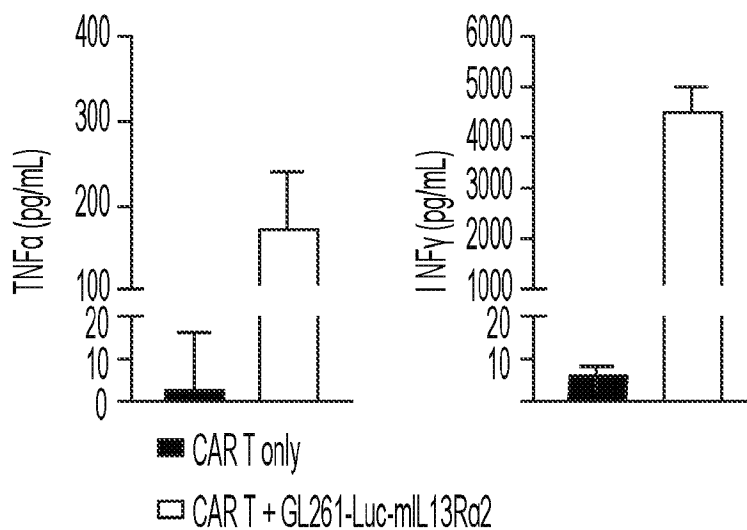


FIG. 3D

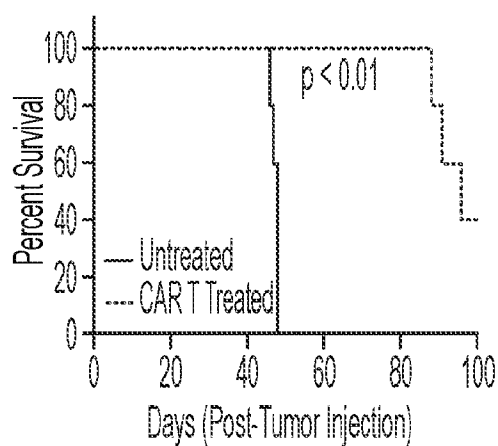


FIG. 3E

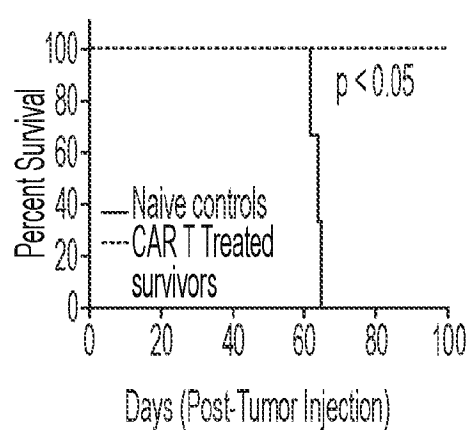


FIG. 3F

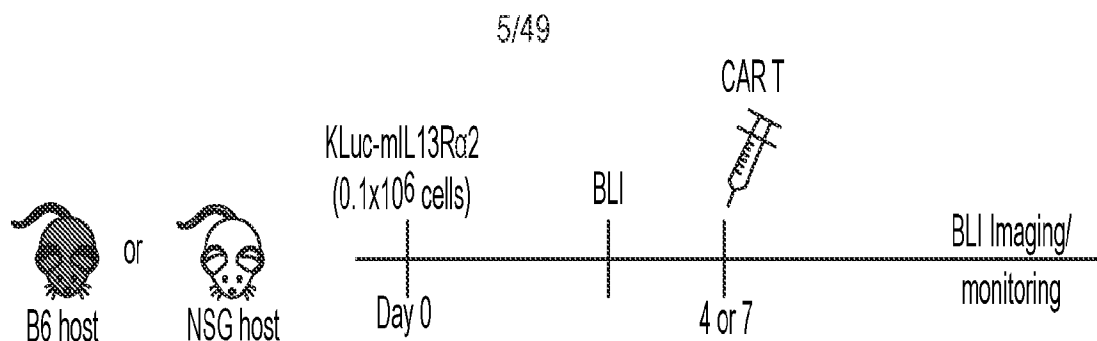
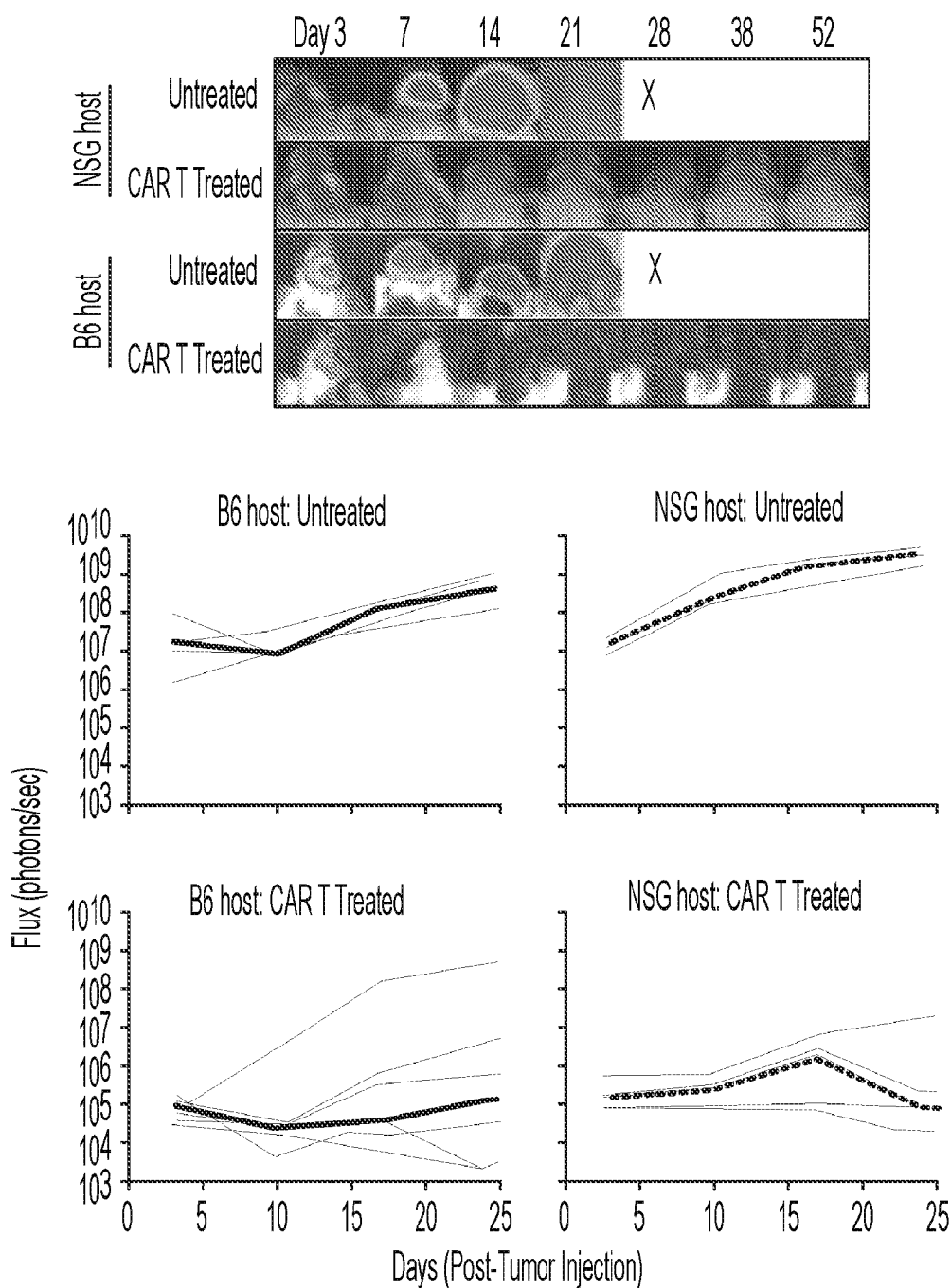


FIG. 4A



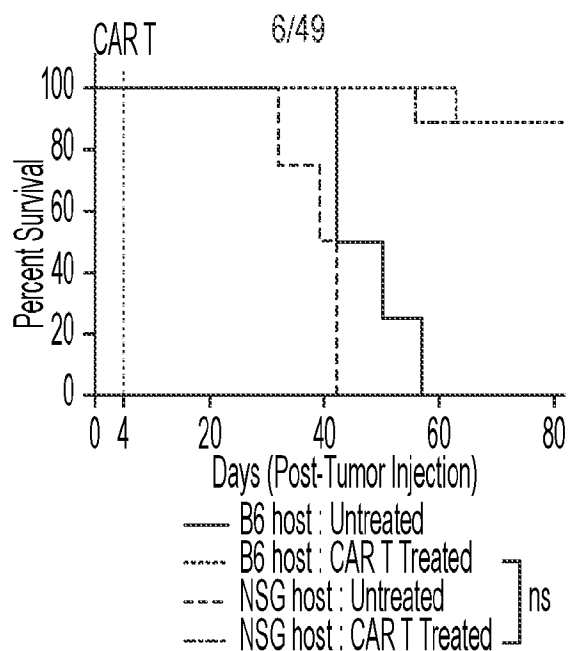


FIG. 4C

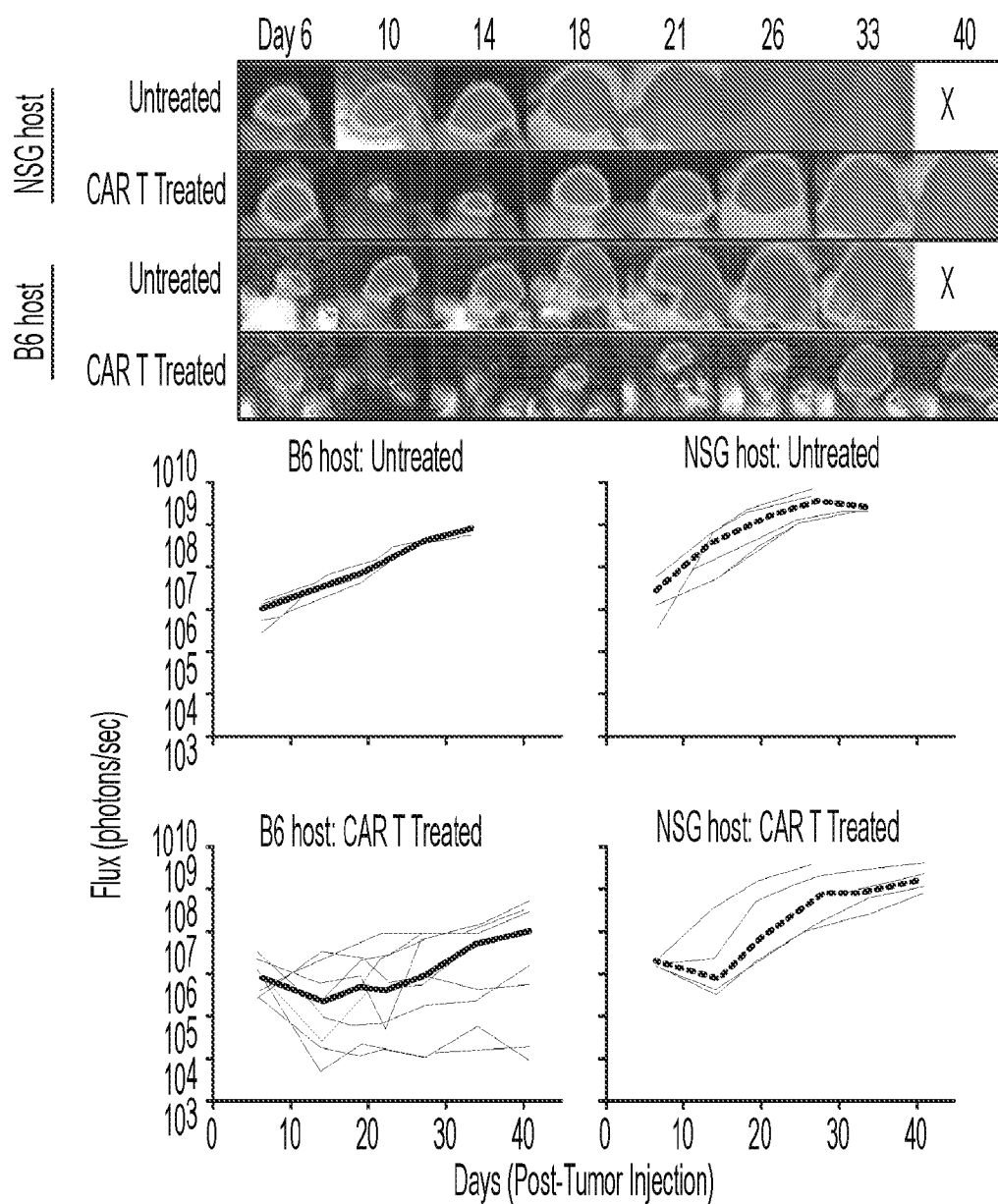


FIG. 4D

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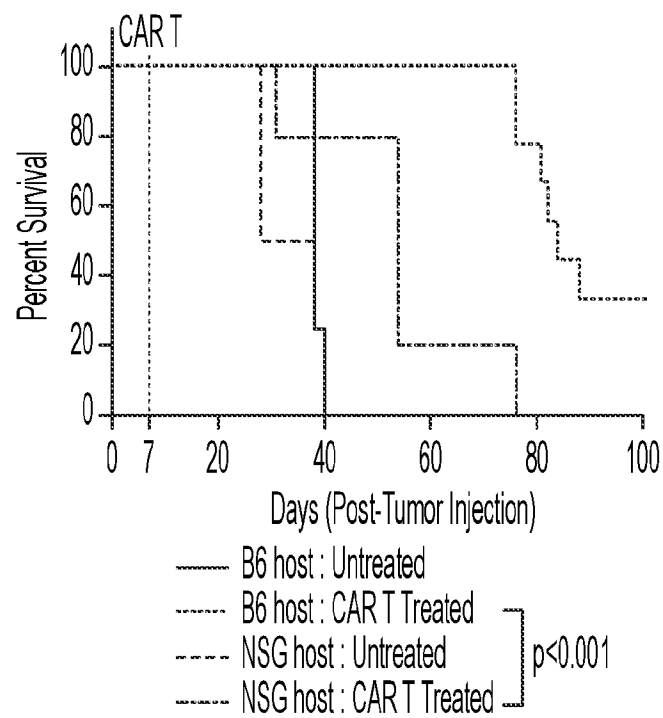


FIG. 4E

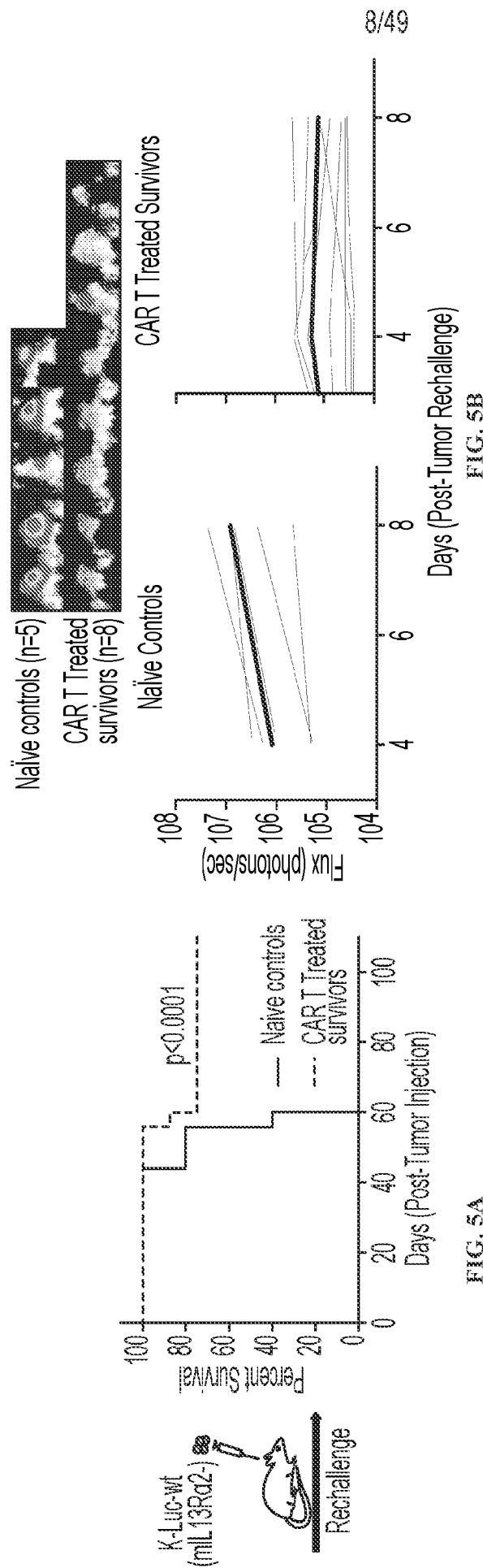


FIG. 5C

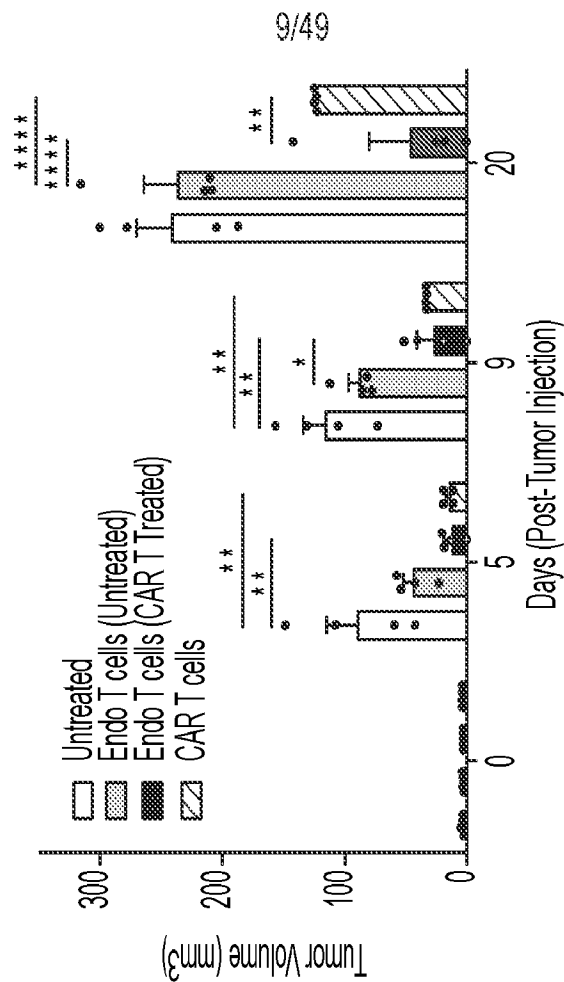


FIG. 5F

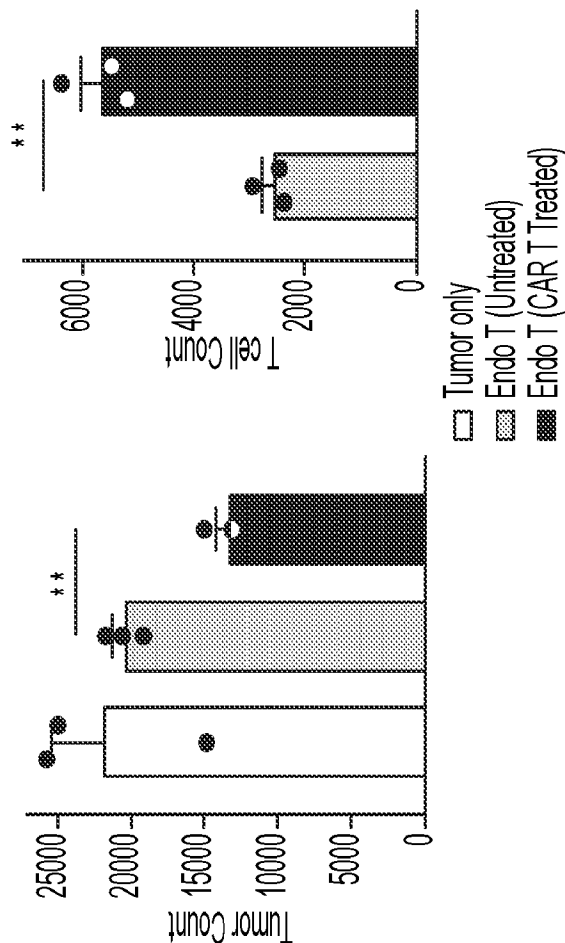
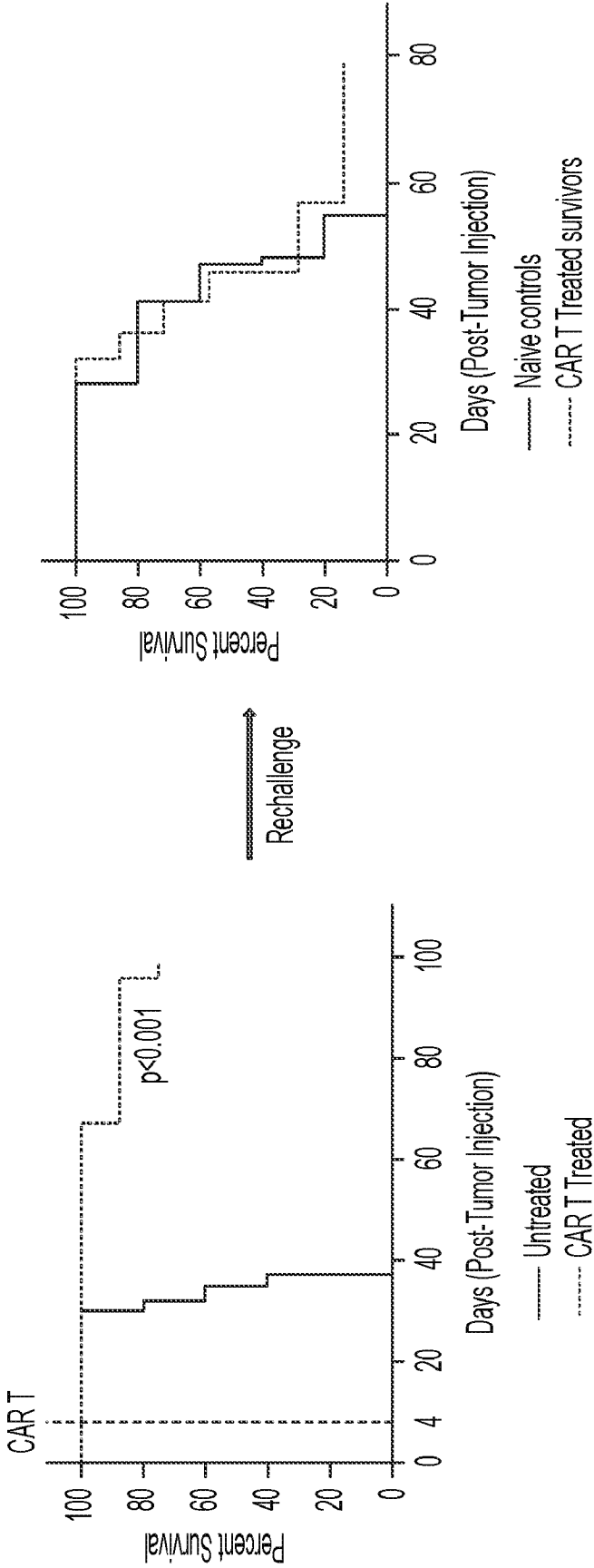


FIG. 5E

FIG. 5D

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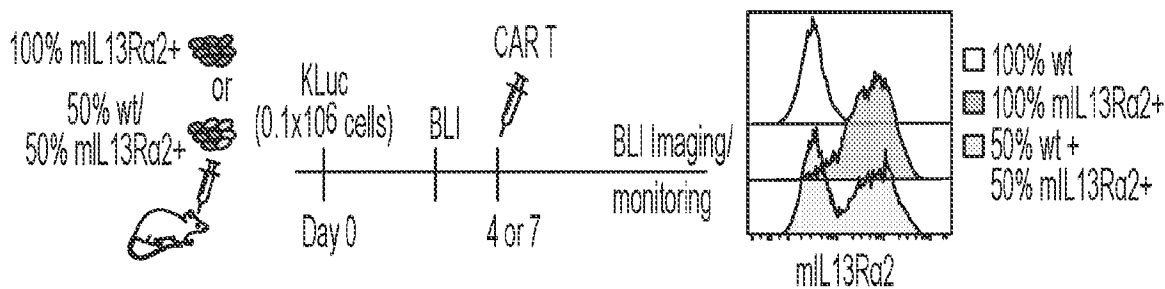


FIG. 7A

FIG. 7B

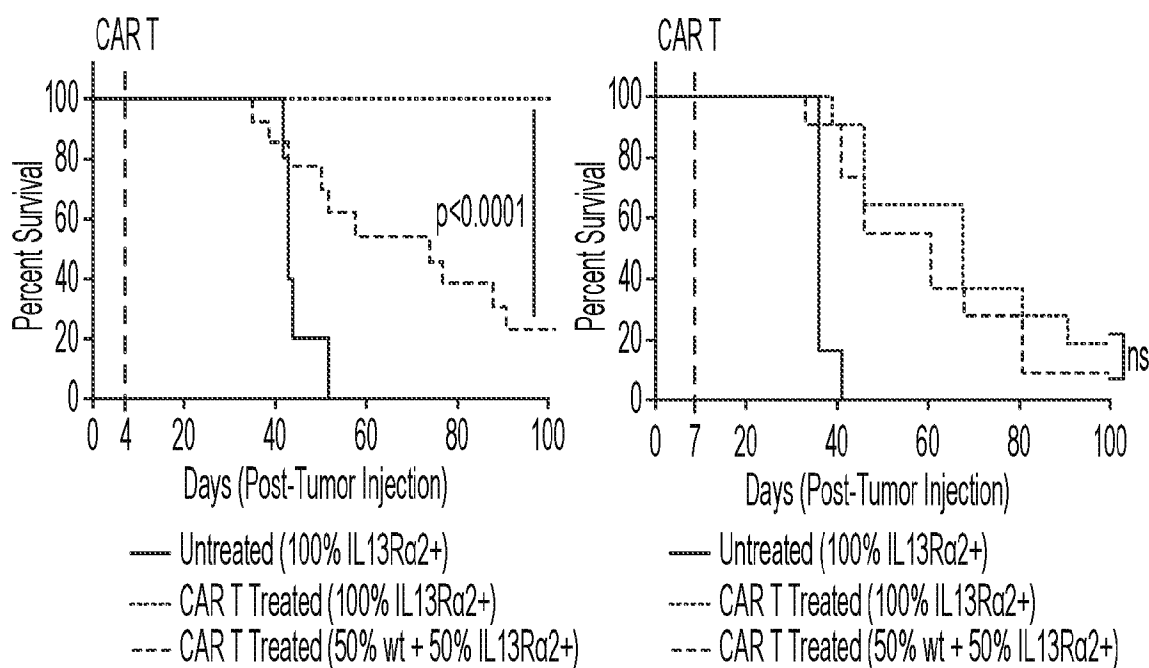


FIG. 7C

FIG. 7D

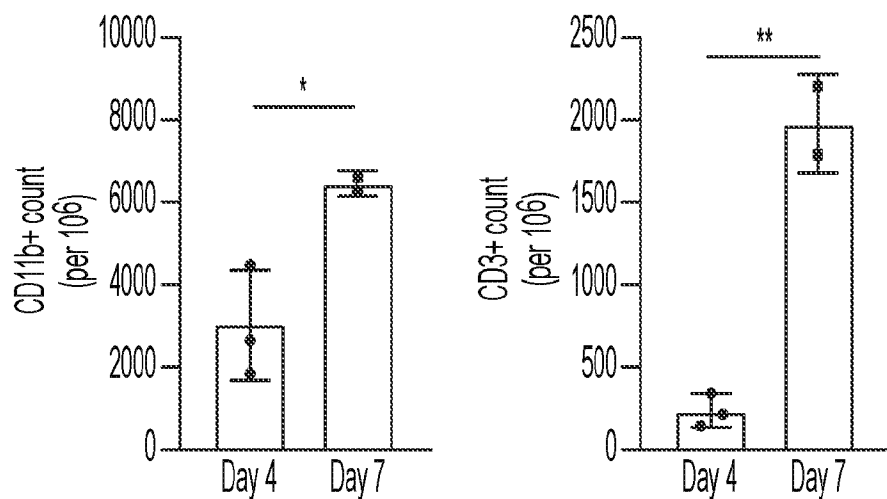


FIG. 7E

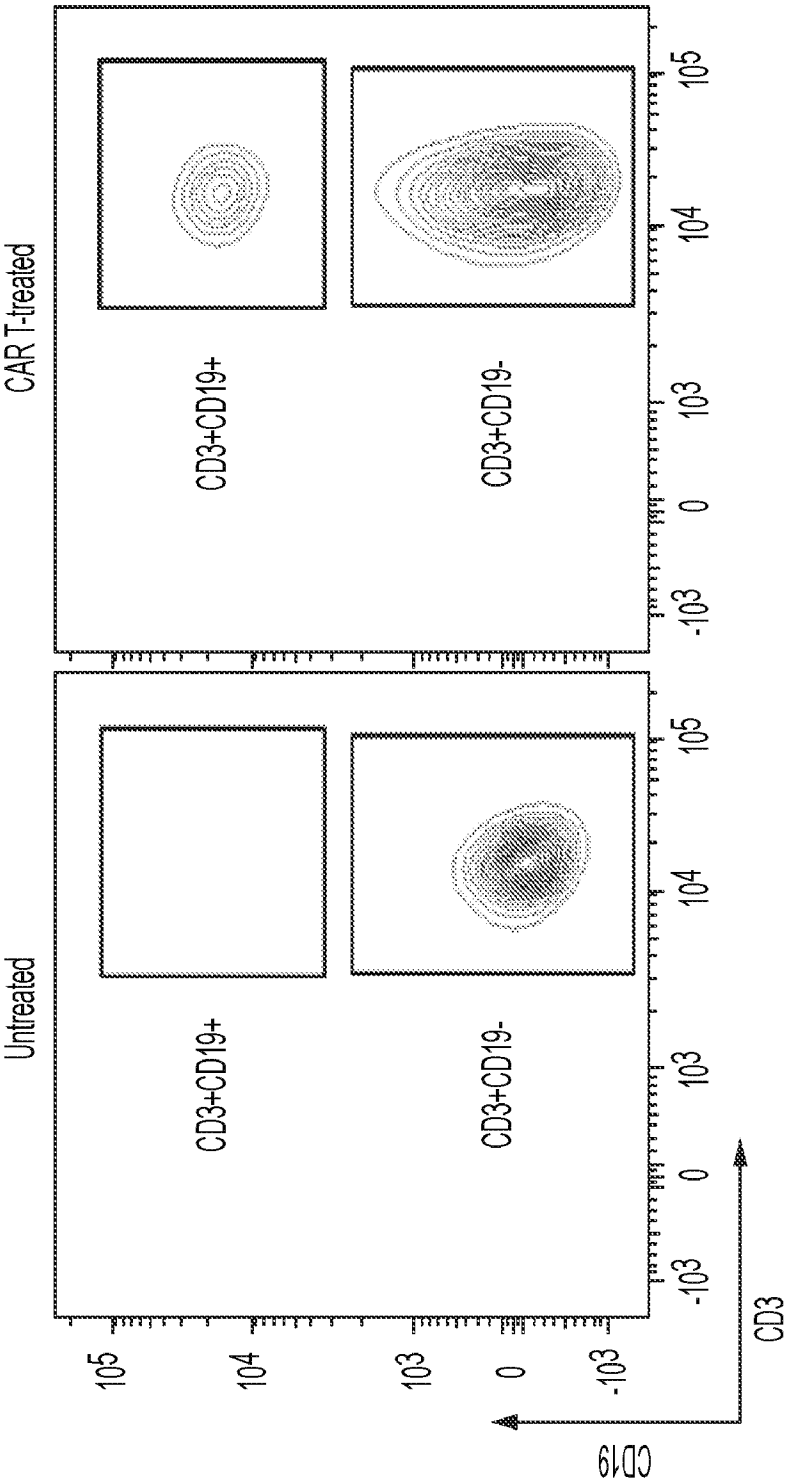


FIG. 8

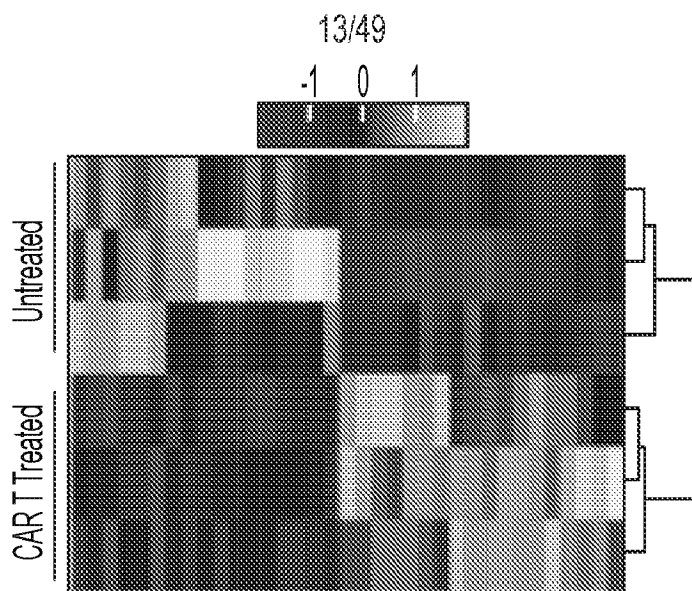


FIG. 9A

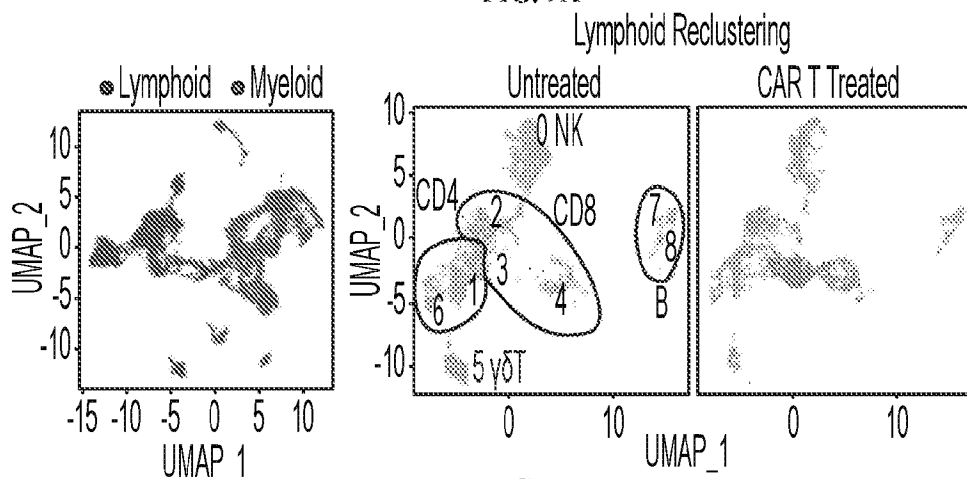


FIG. 9B

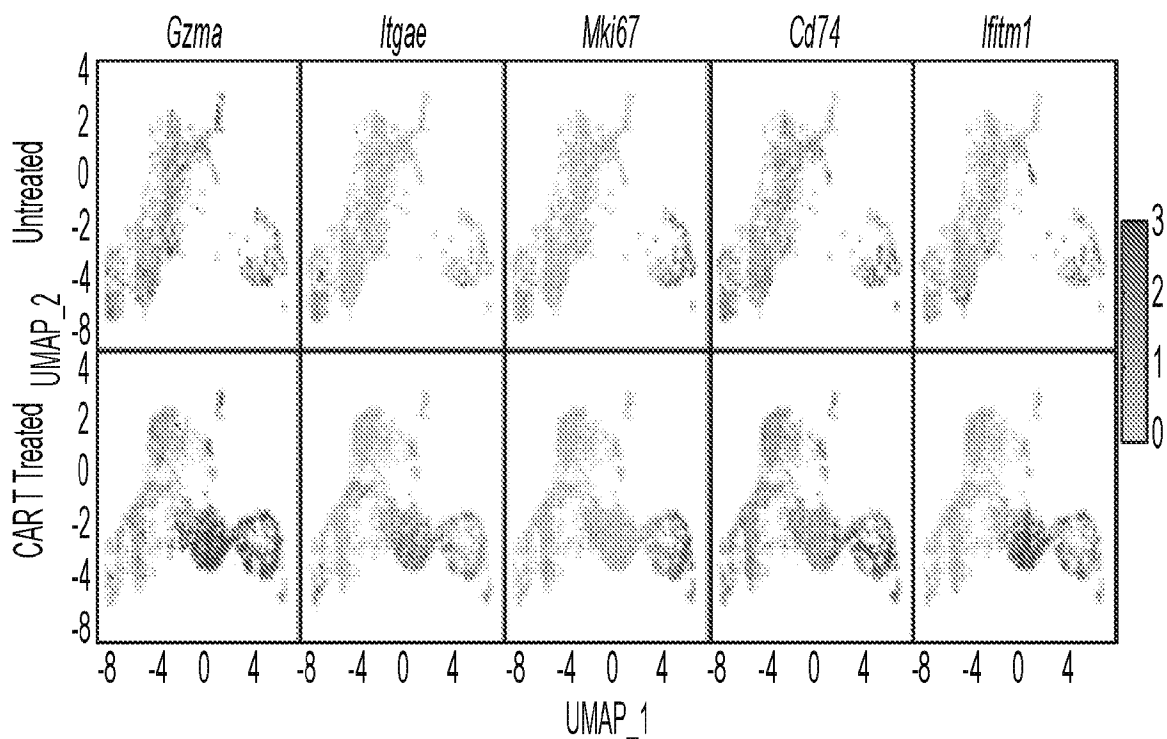


FIG. 9C

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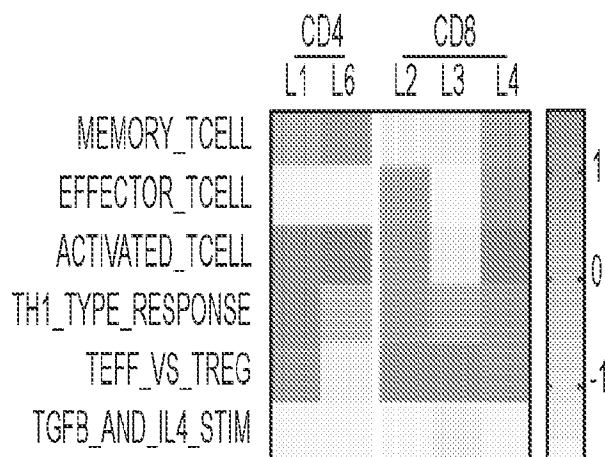


FIG. 9D

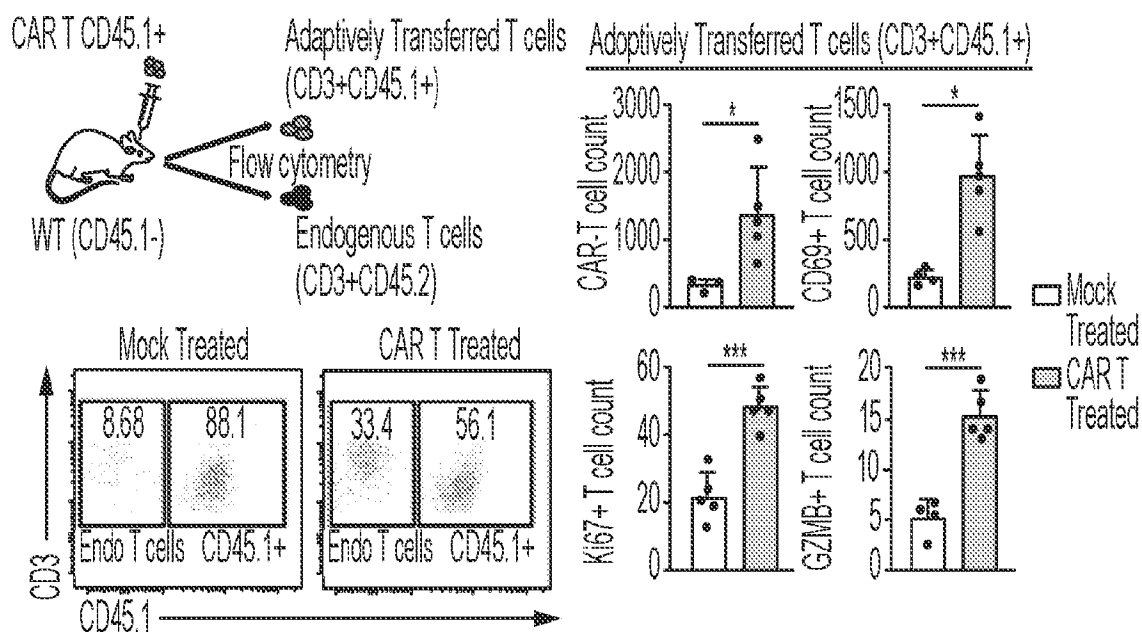


FIG. 9E

FIG. 9F

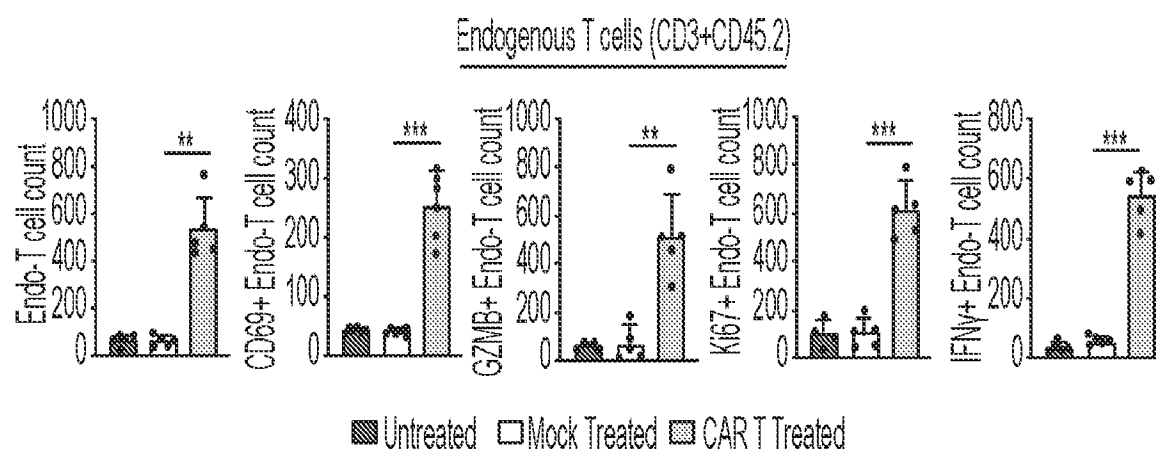


FIG. 9G

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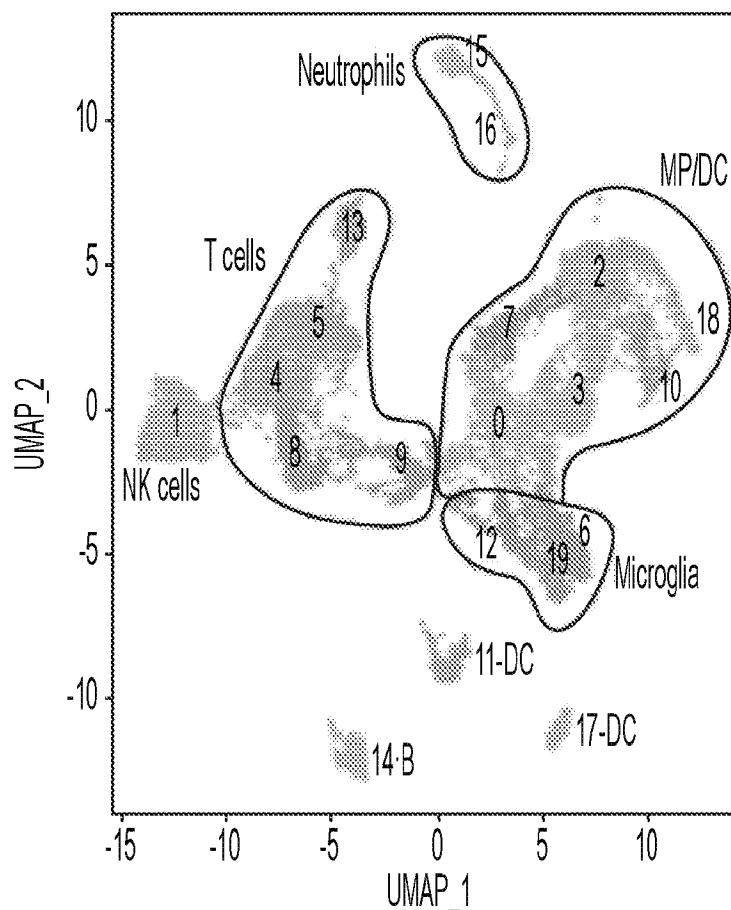


FIG. 10A

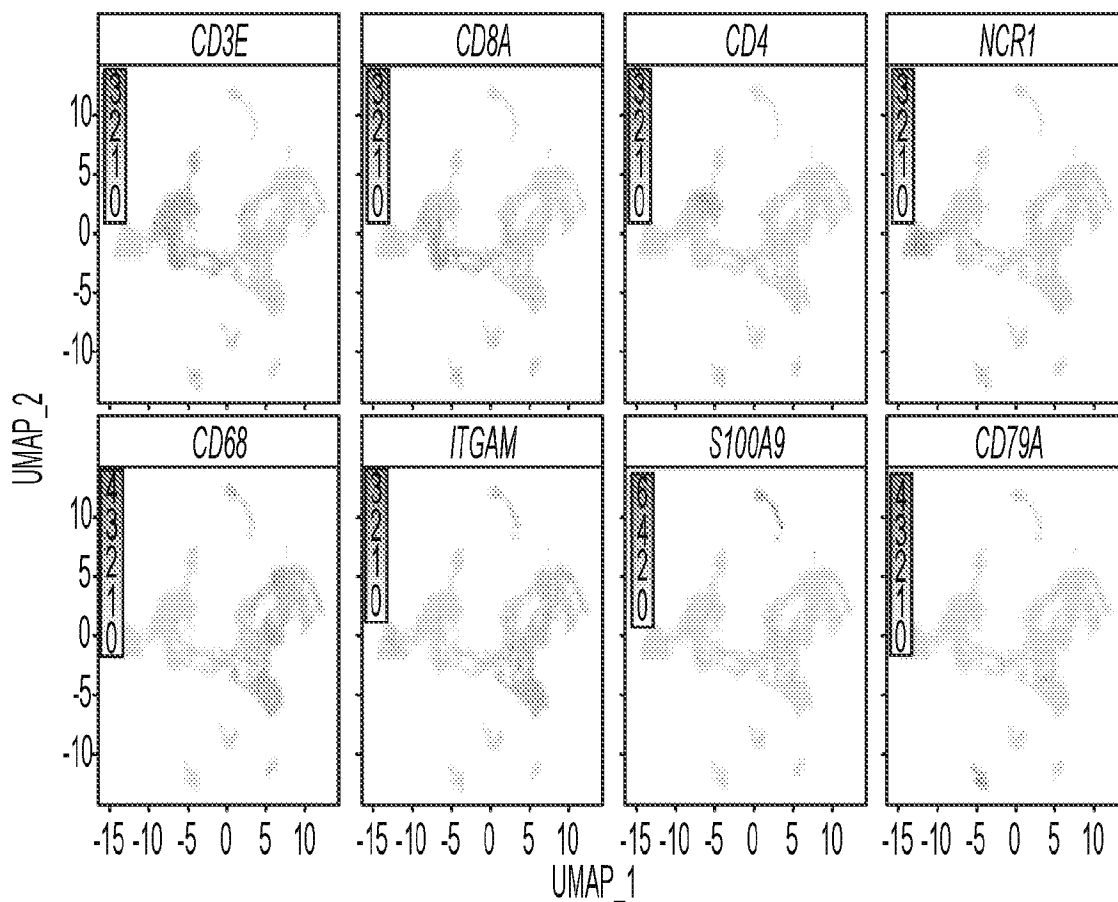


FIG. 10B

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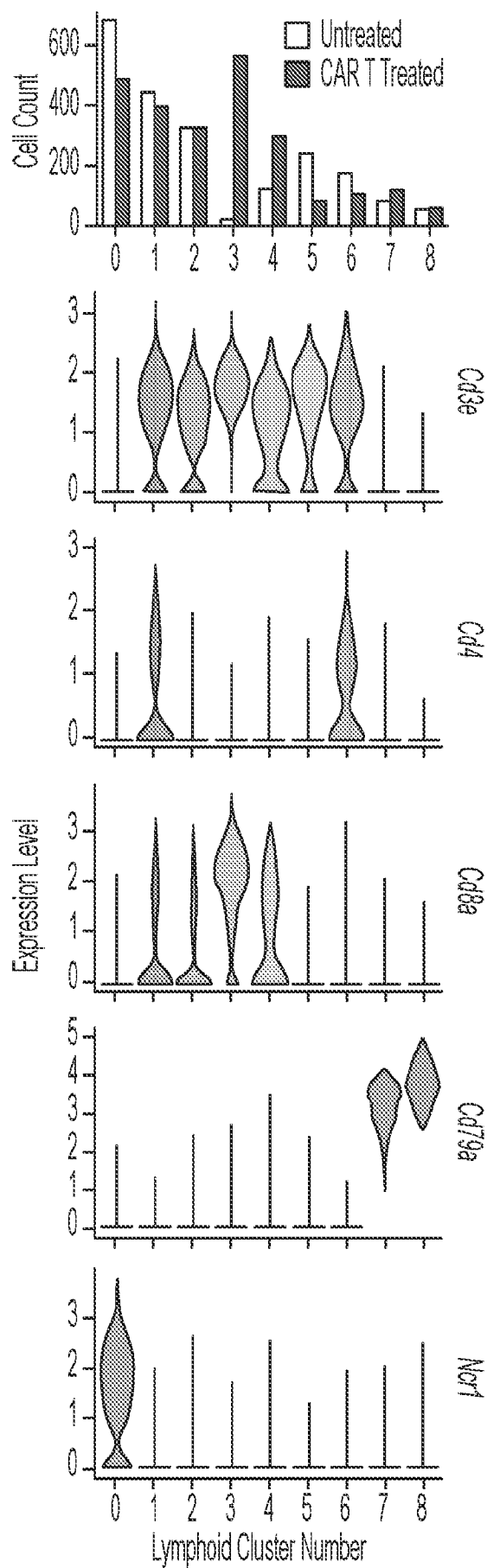


FIG. 10C

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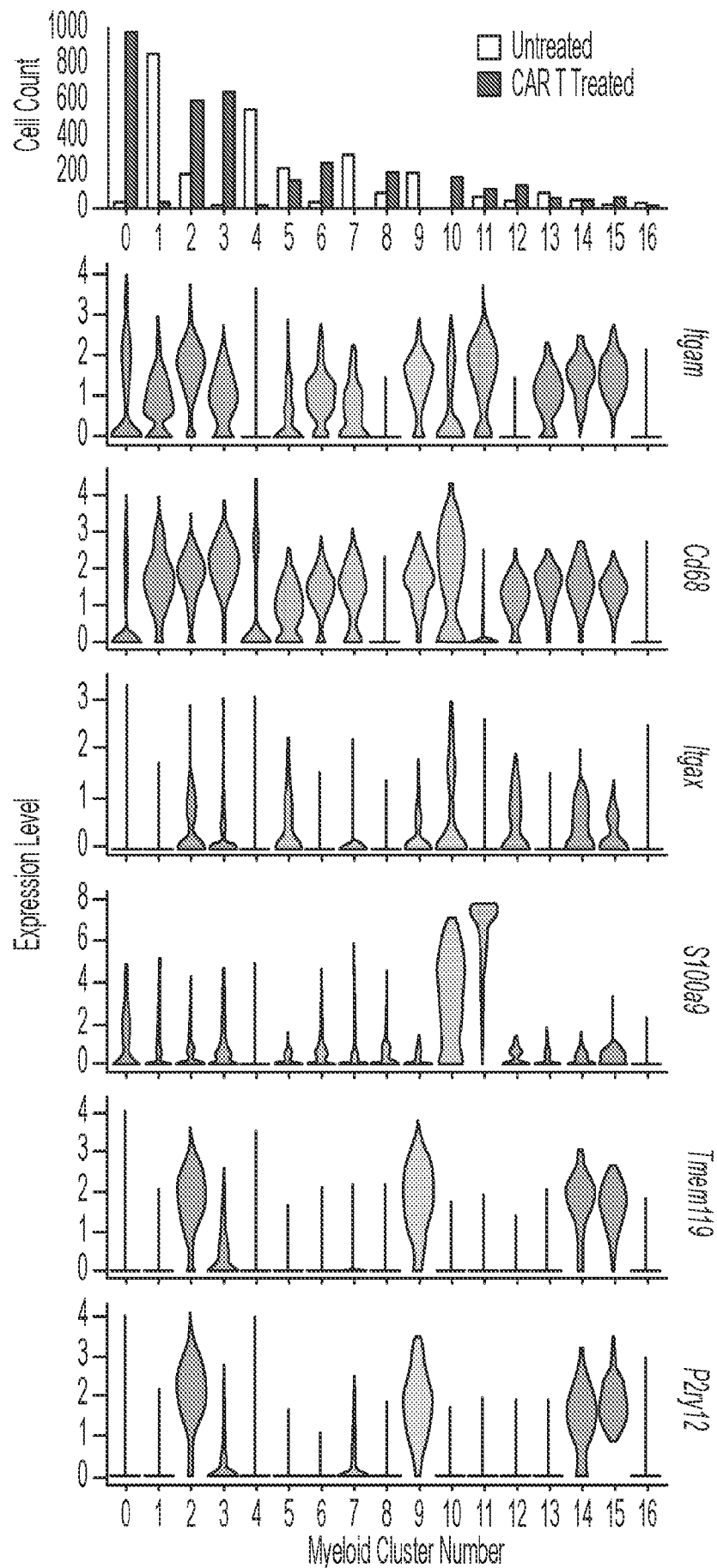


FIG. 10D

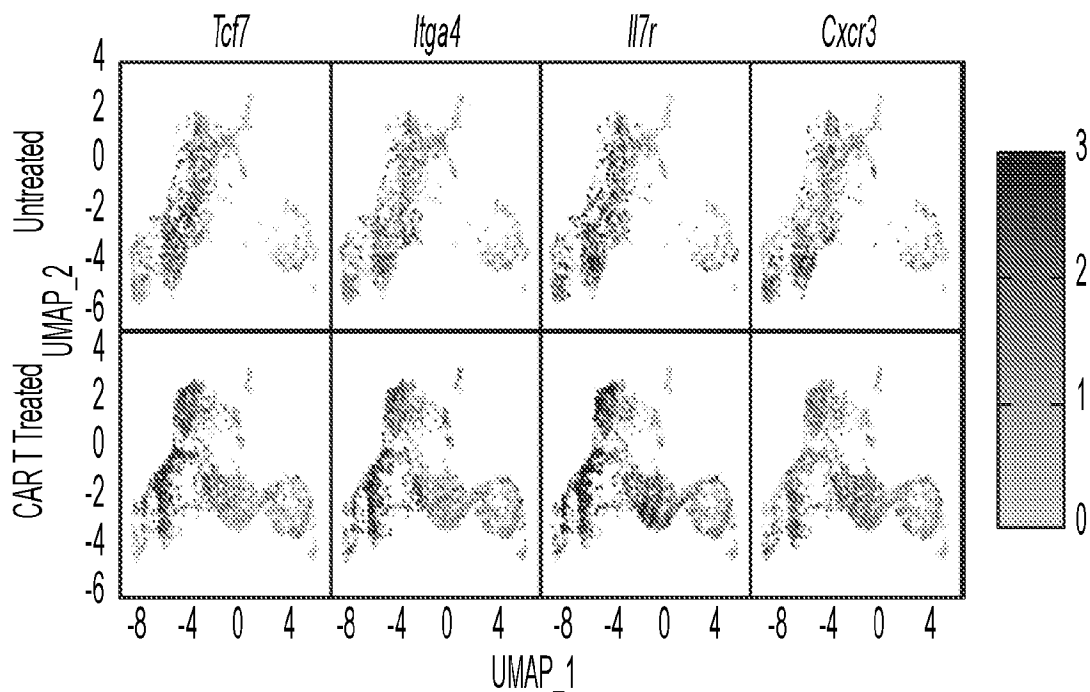


FIG. 11A

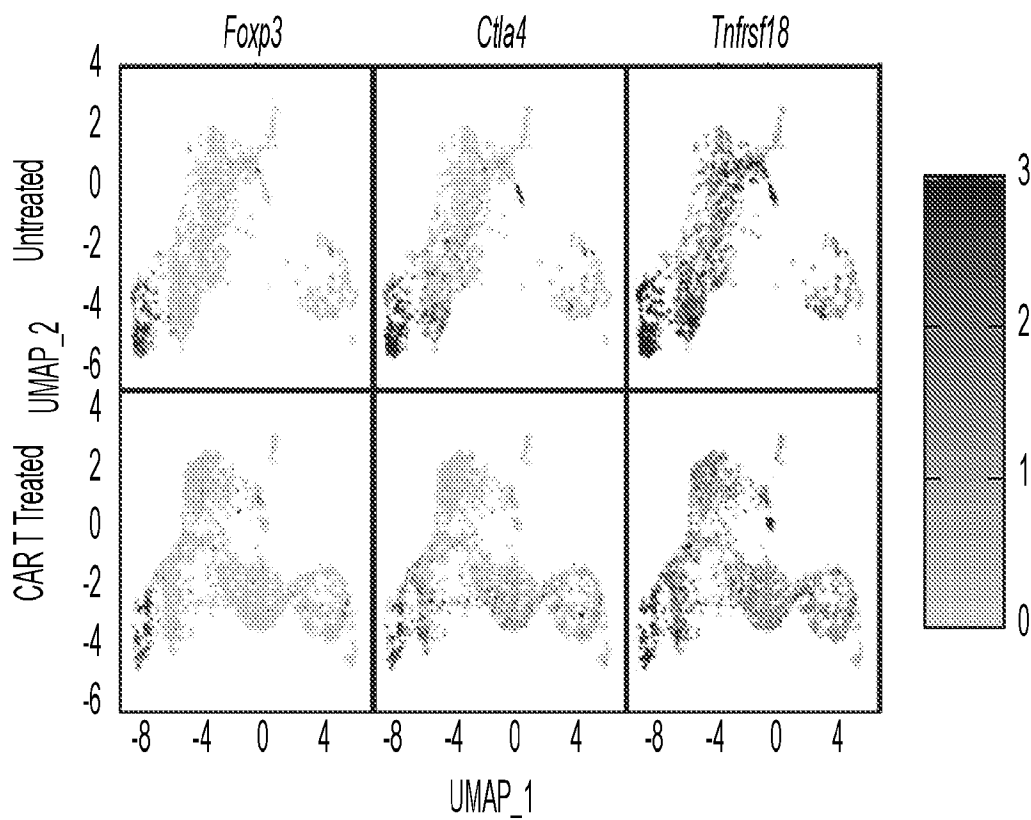


FIG. 11B

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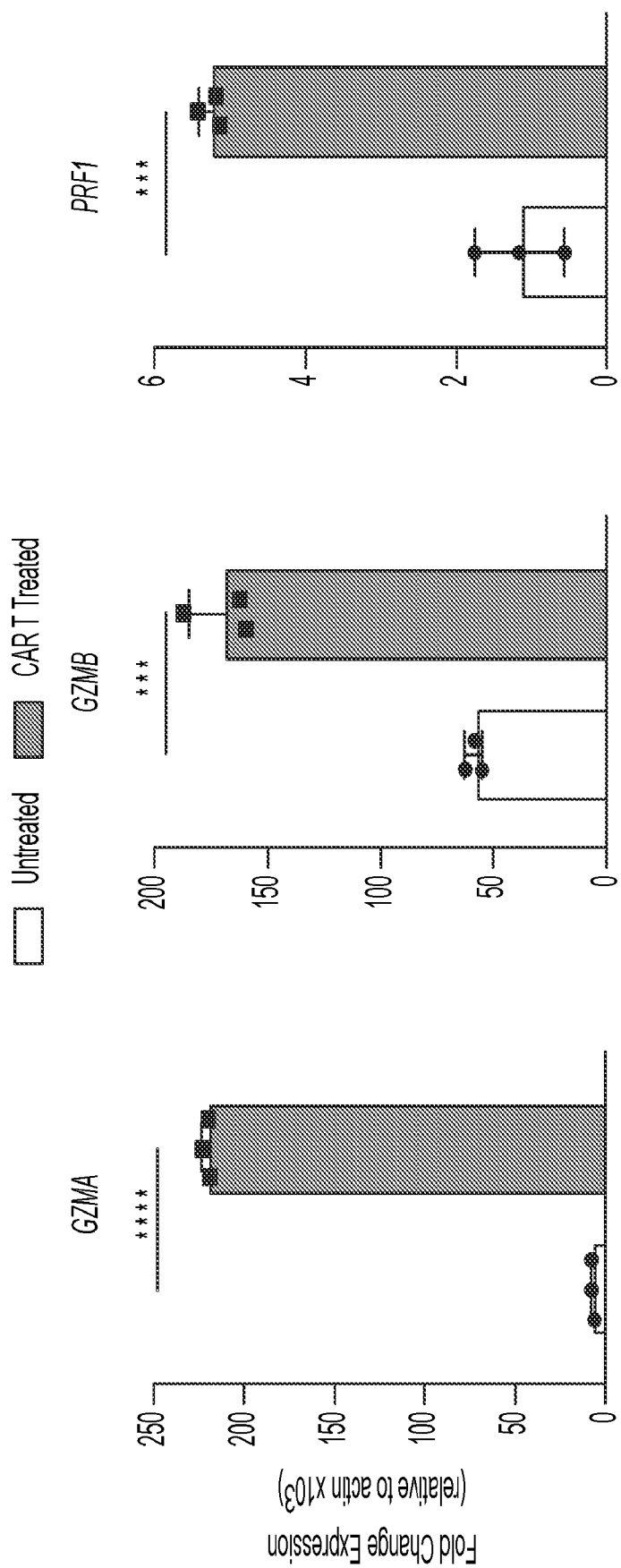


FIG. 12

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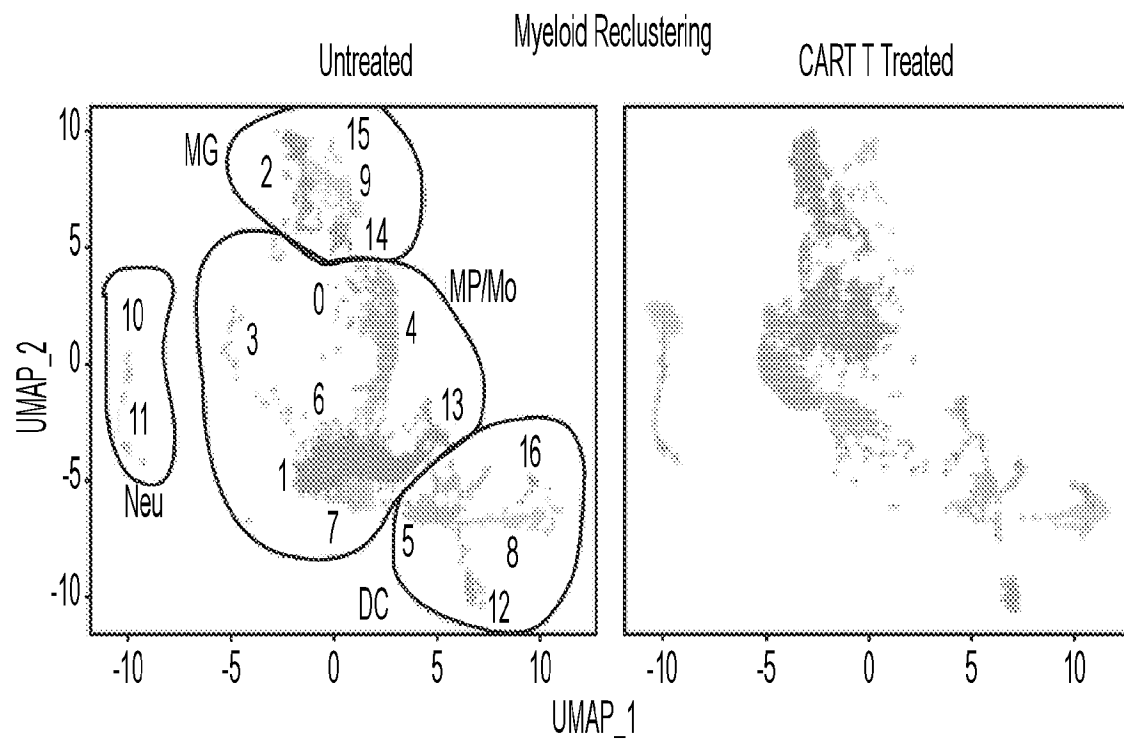


FIG. 13A

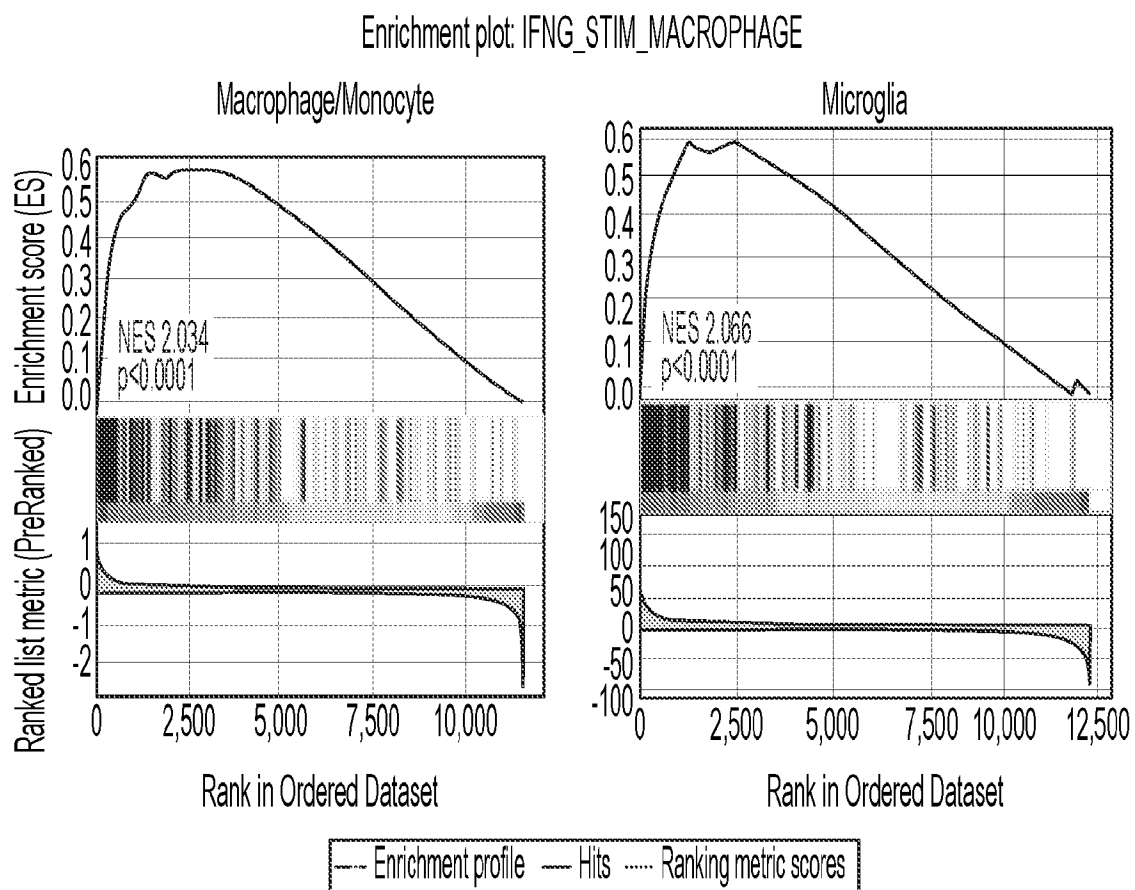


FIG. 13B

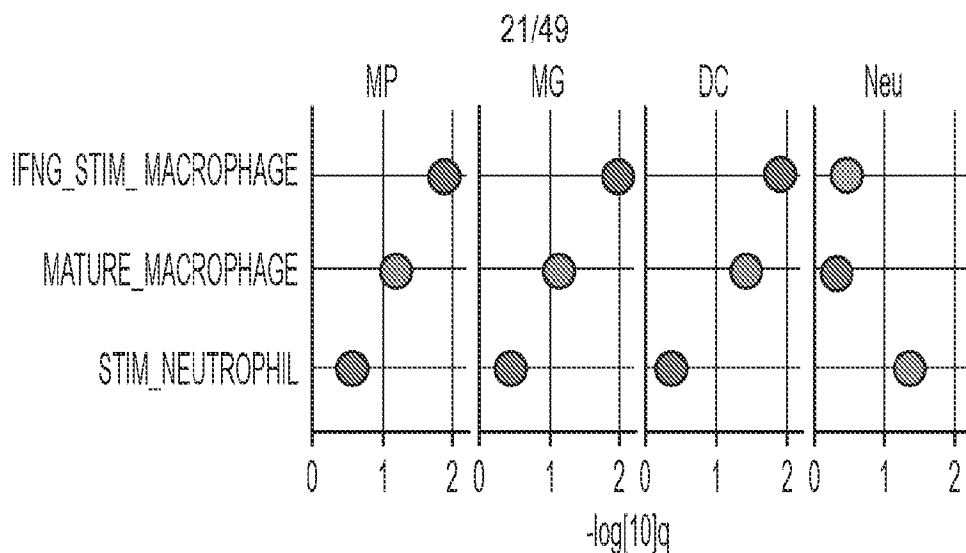


FIG. 13C

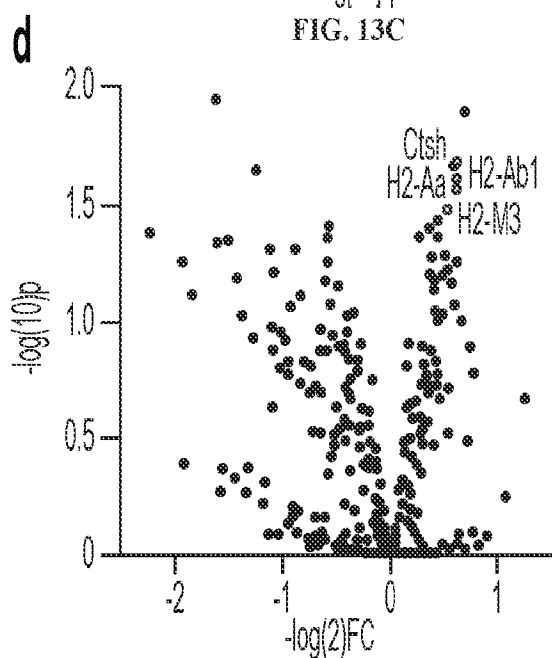


FIG. 13D

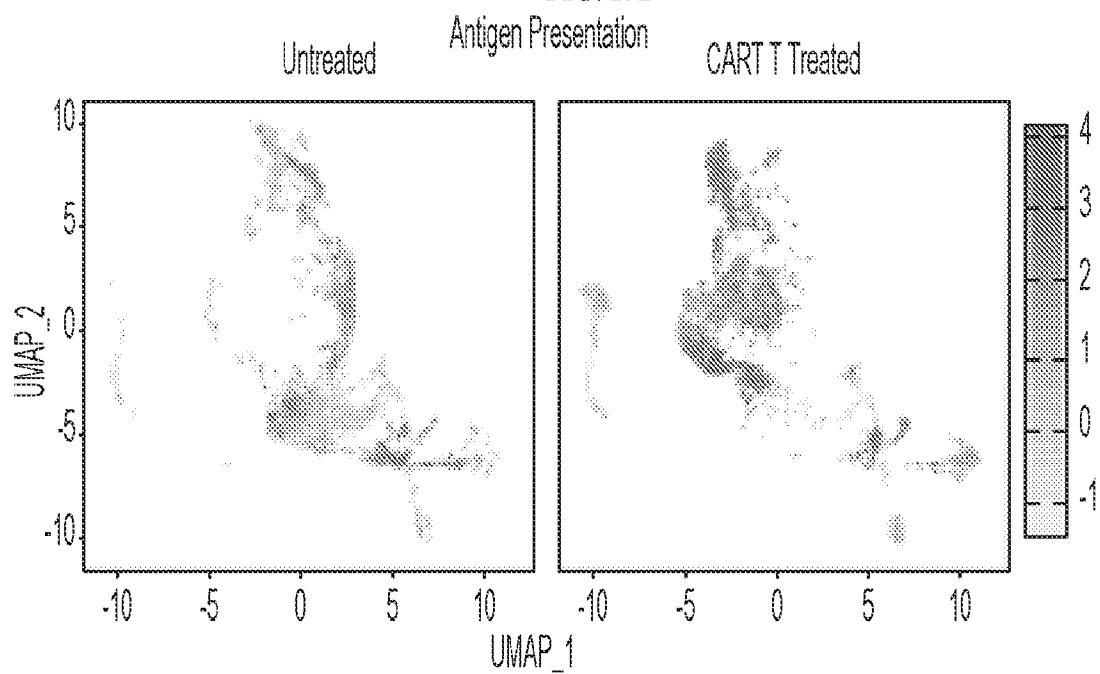


FIG. 13E

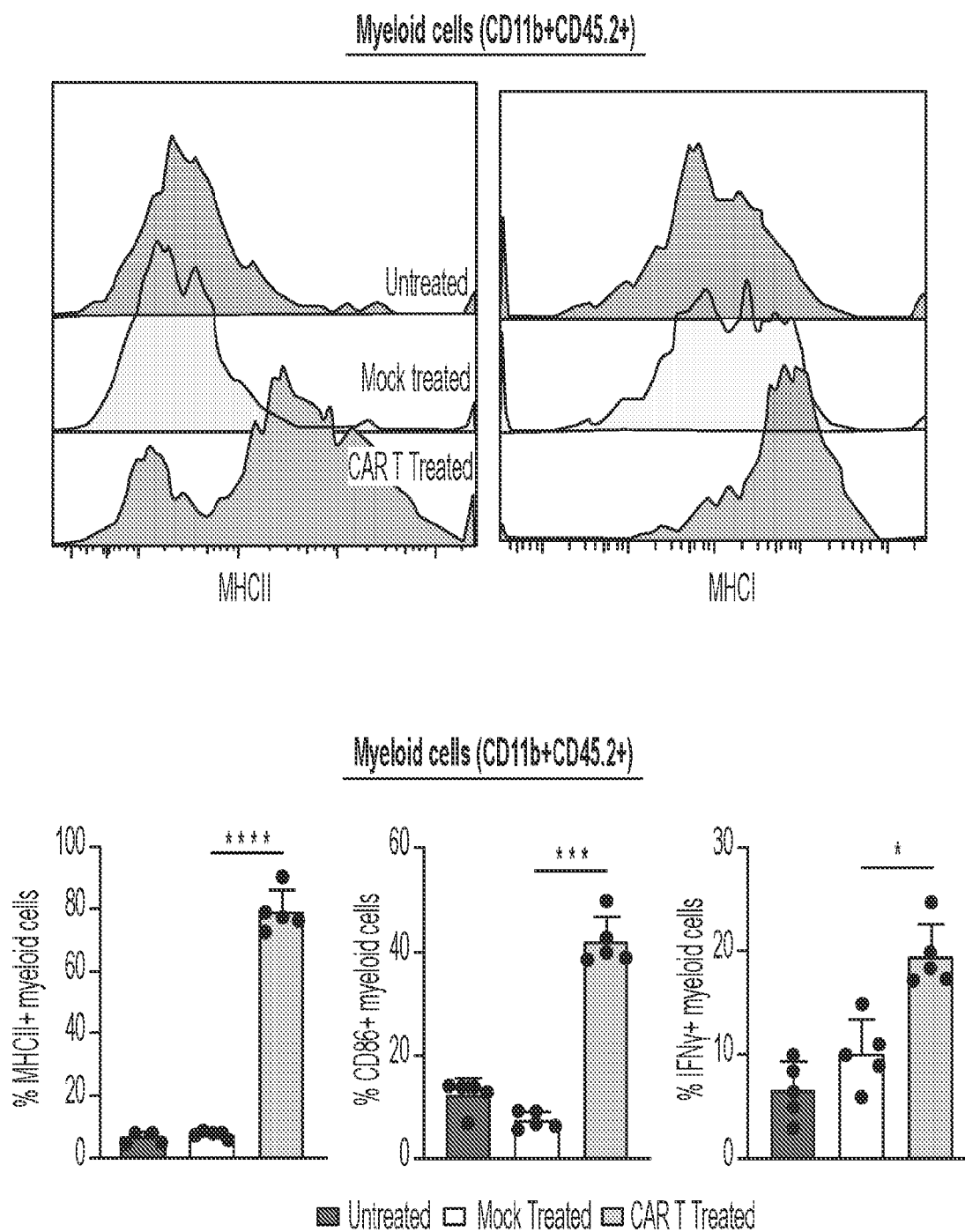


FIG. 13F

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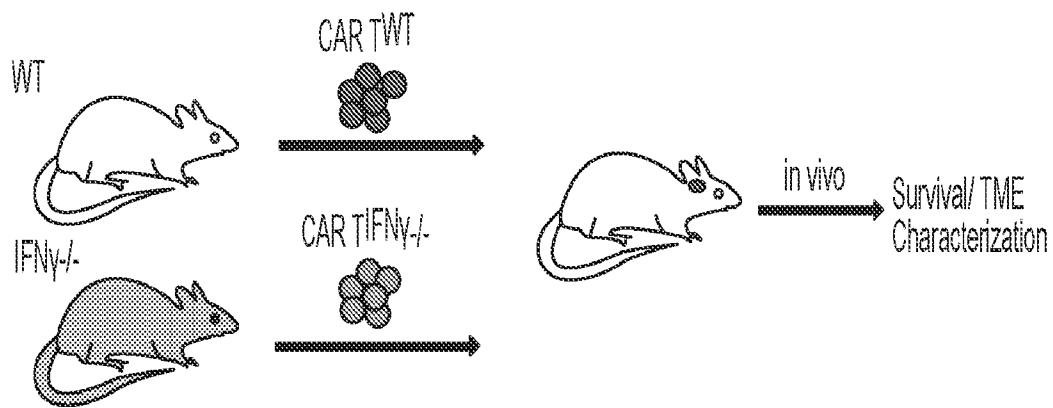


FIG. 14A

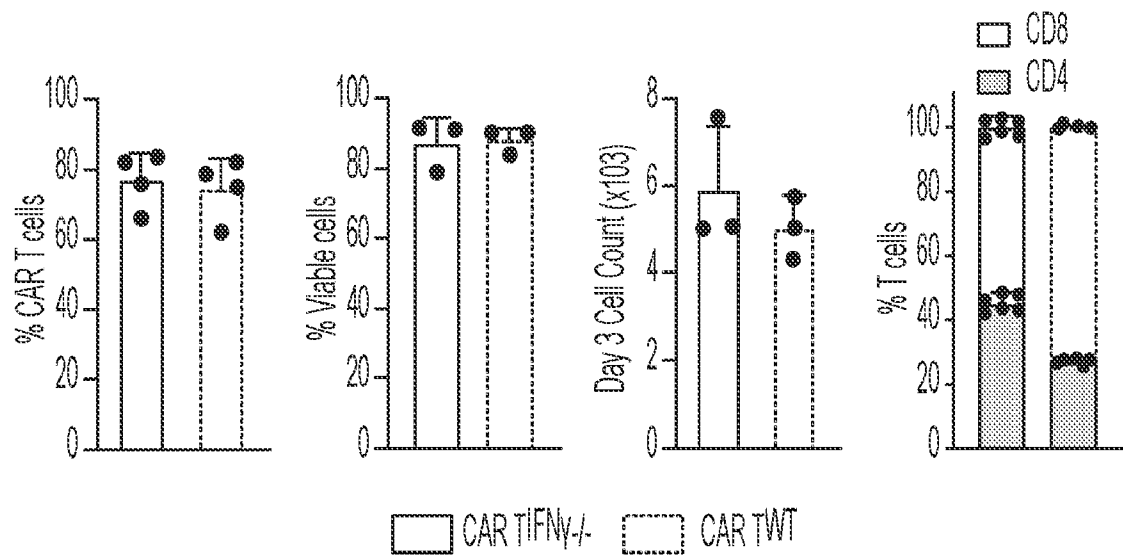


FIG. 14B

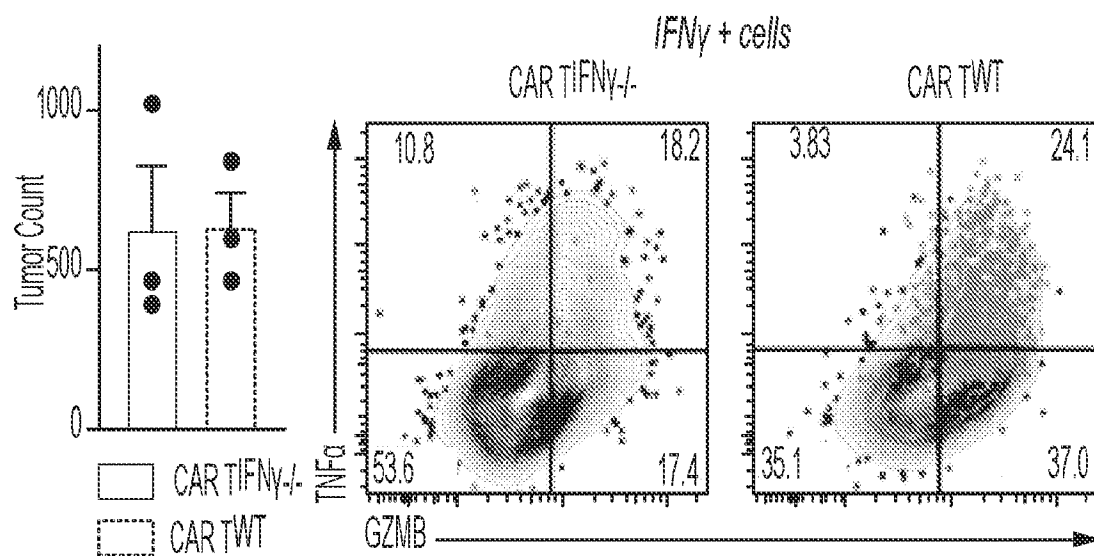
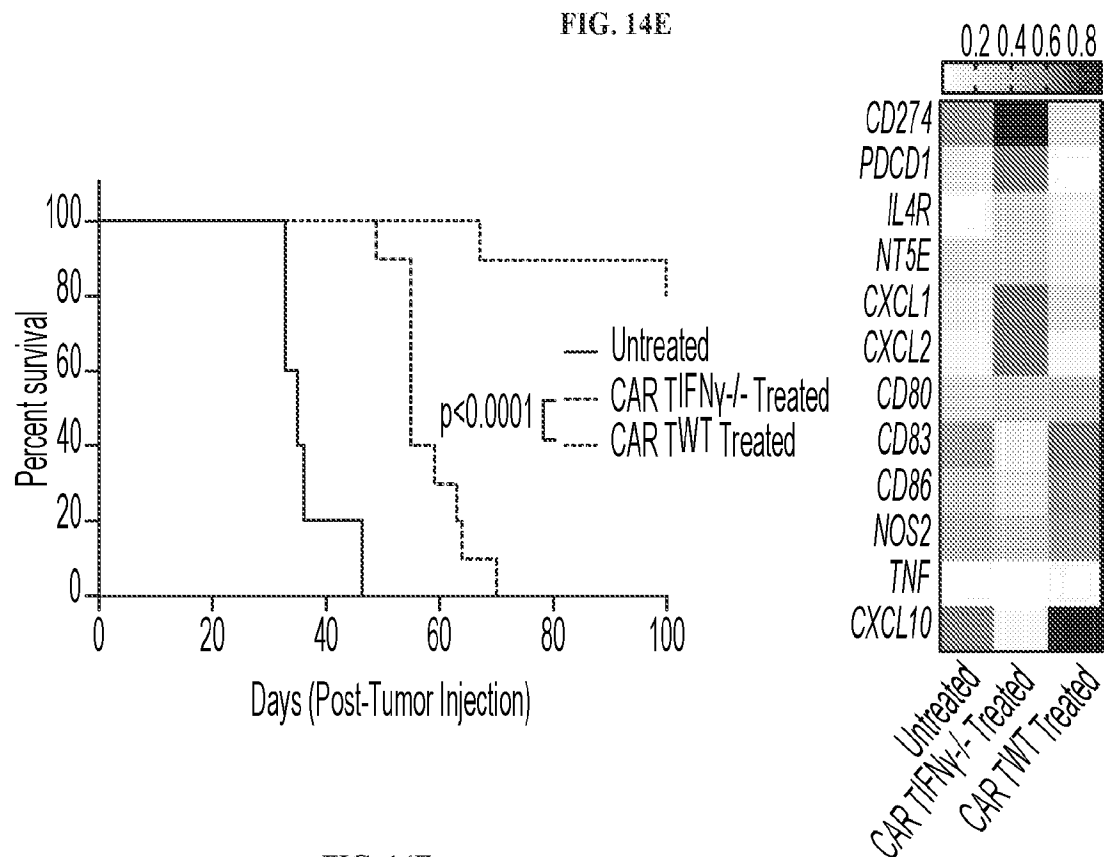
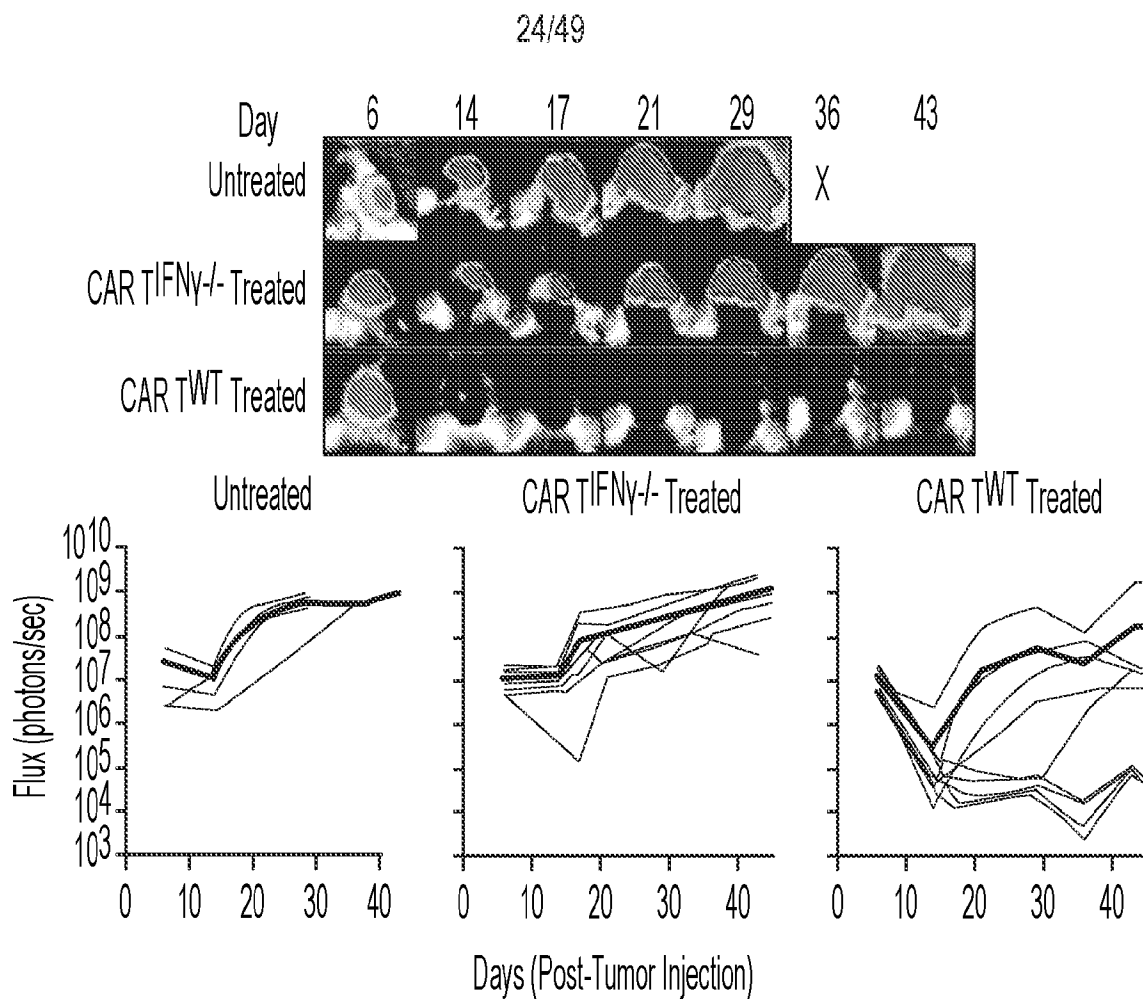


FIG. 14C

FIG. 14D



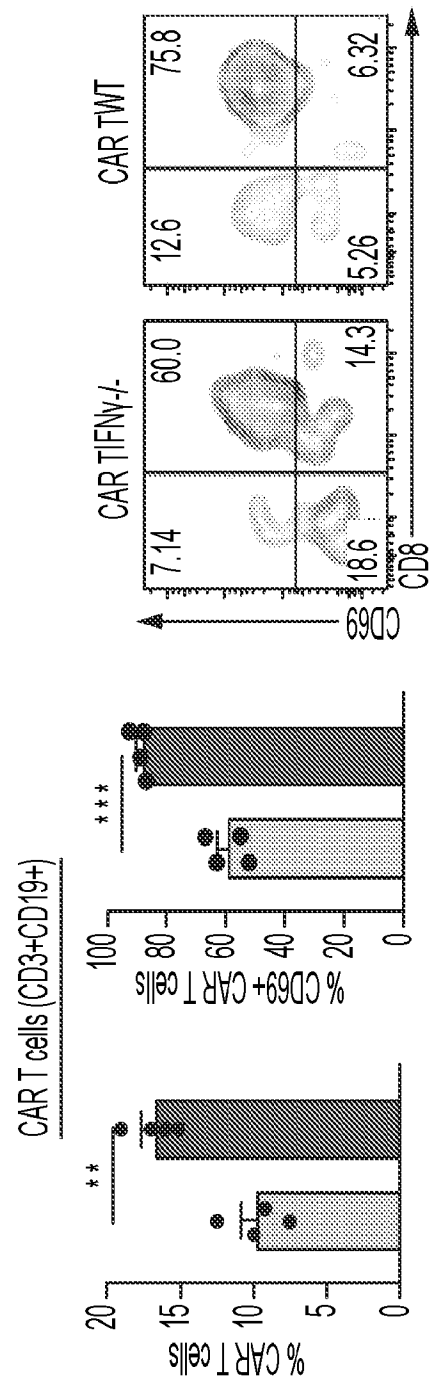


FIG. 14H

Endogenous T cells (CD3+CD19-)

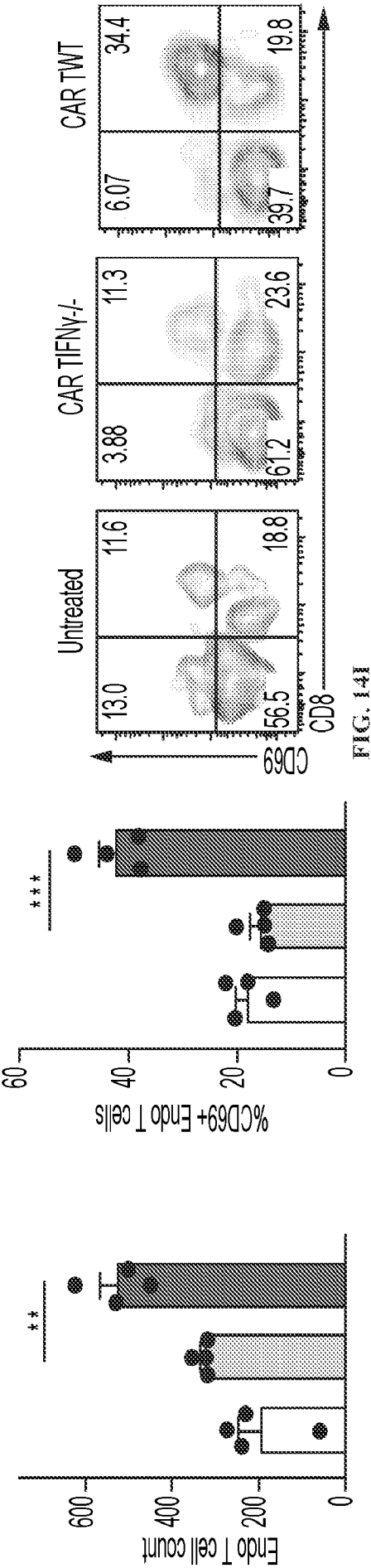


FIG. 14I

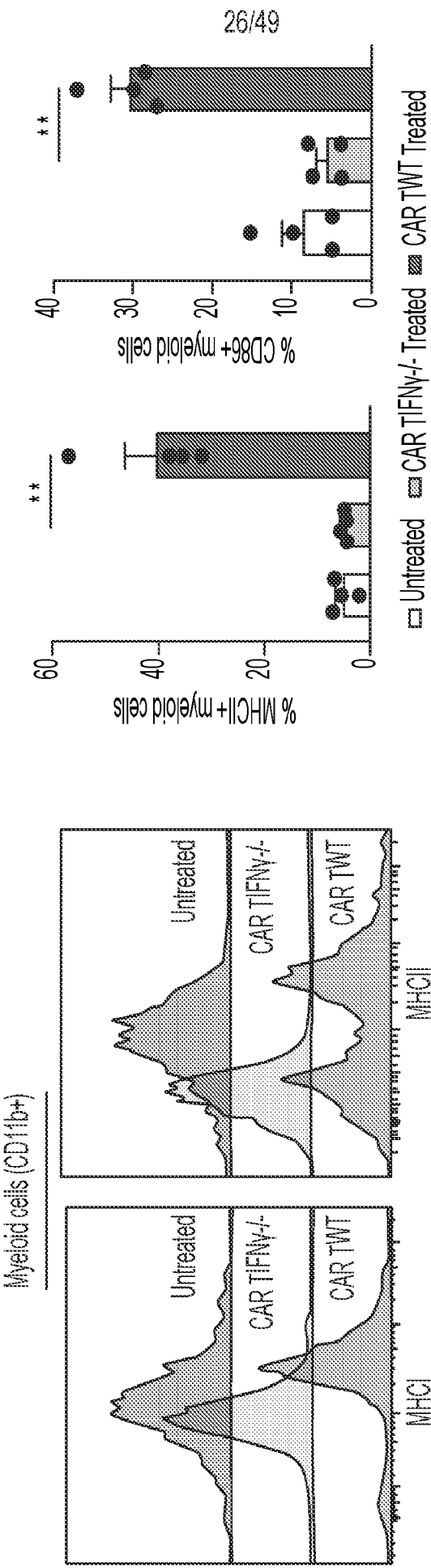


FIG. 14J

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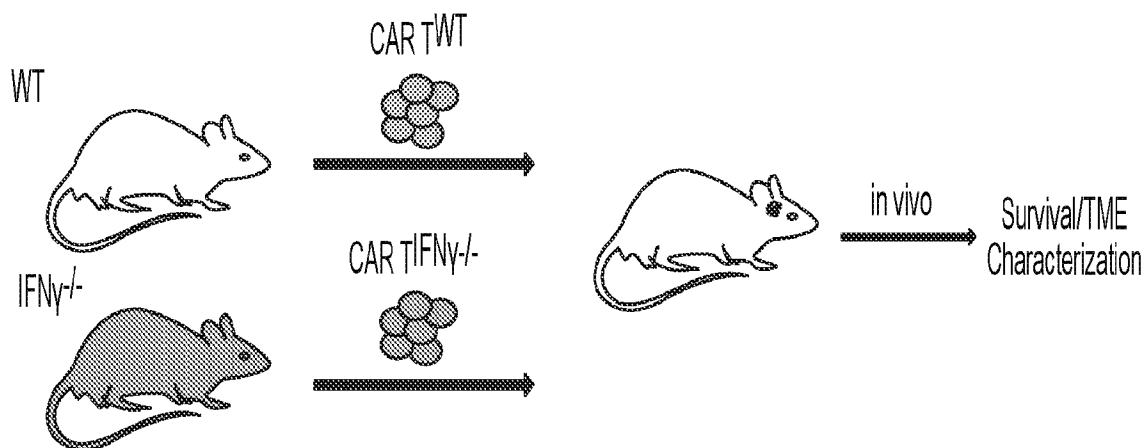


FIG. 15A

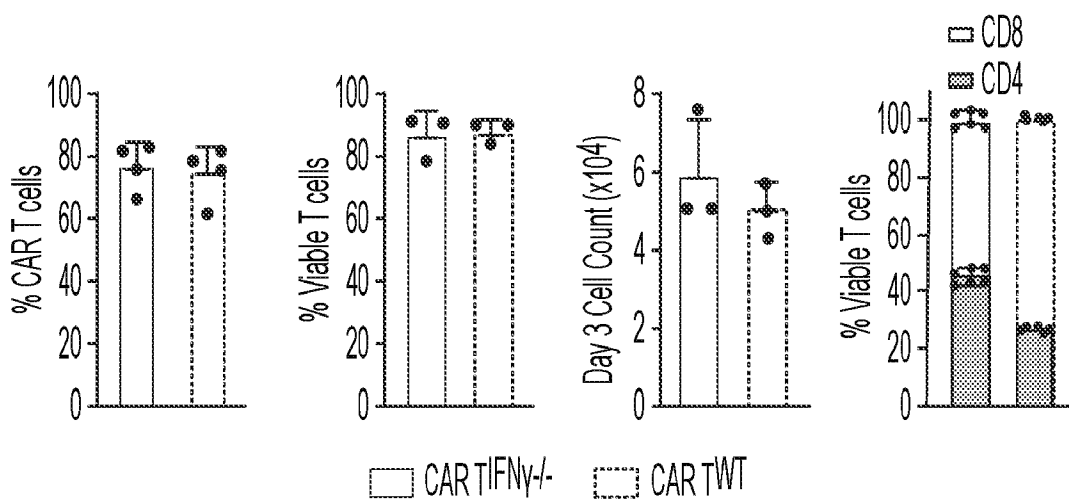


FIG. 15B

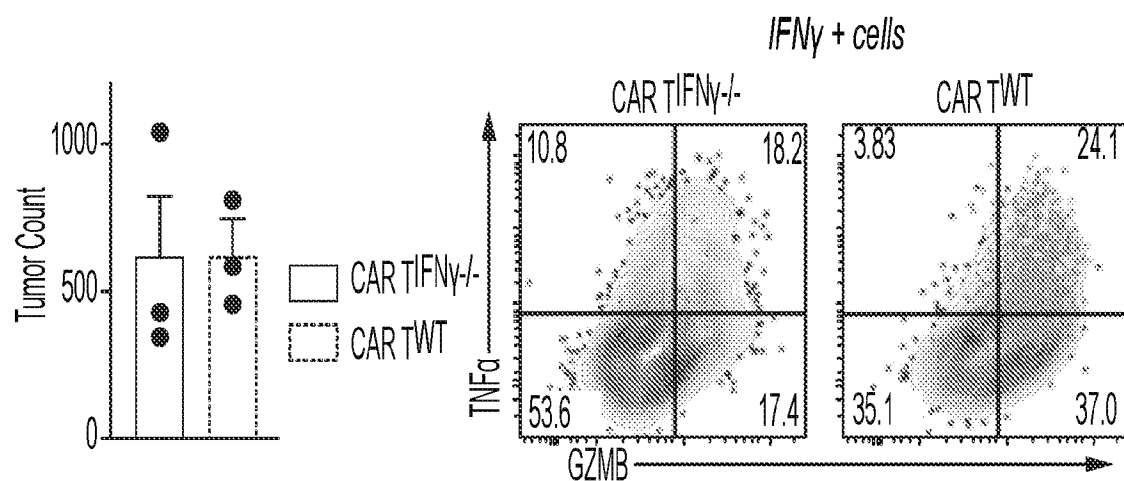
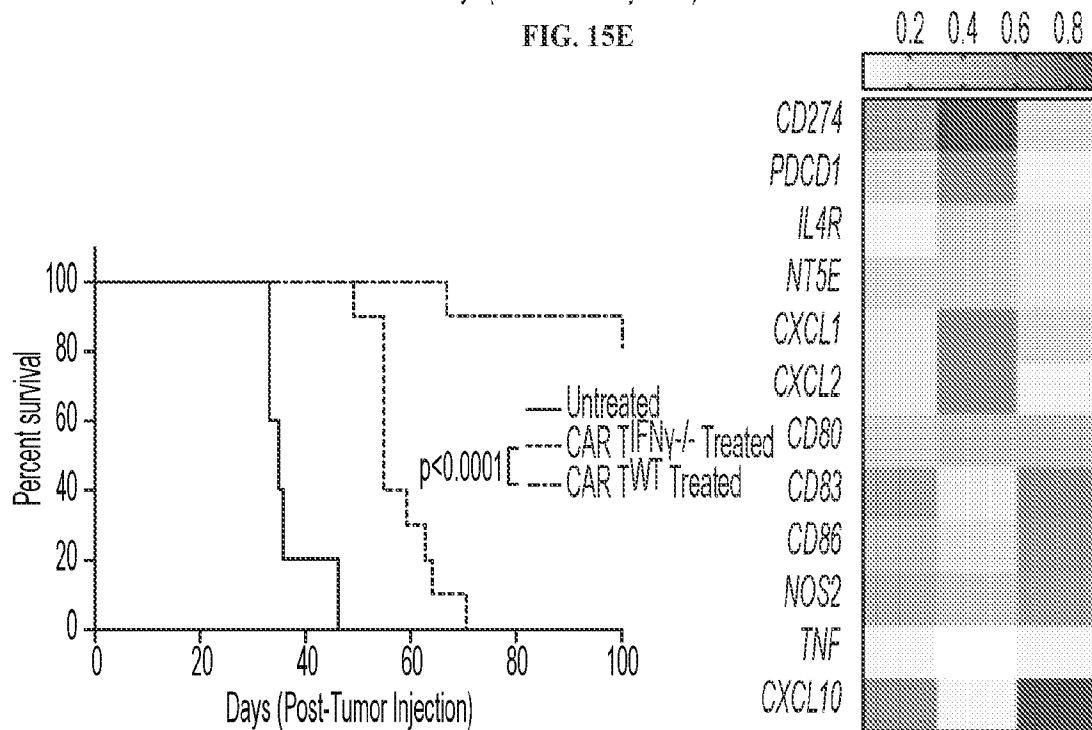
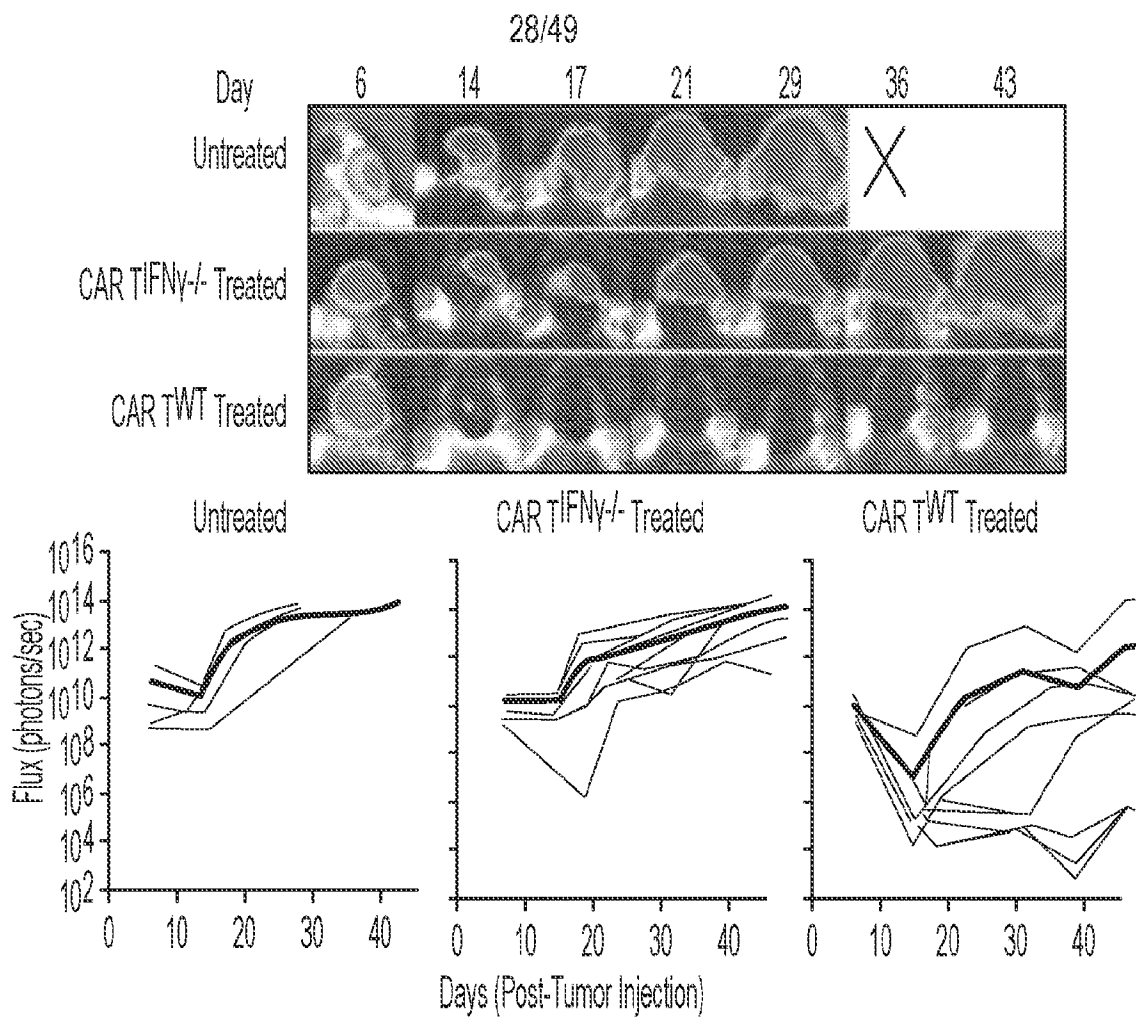


FIG. 15C

FIG. 15D



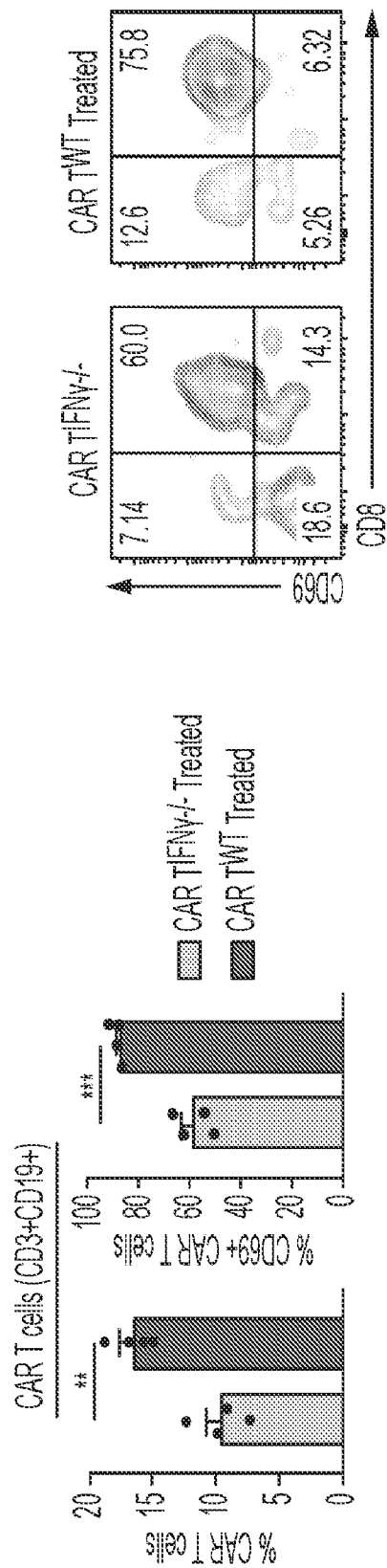


FIG. 15H

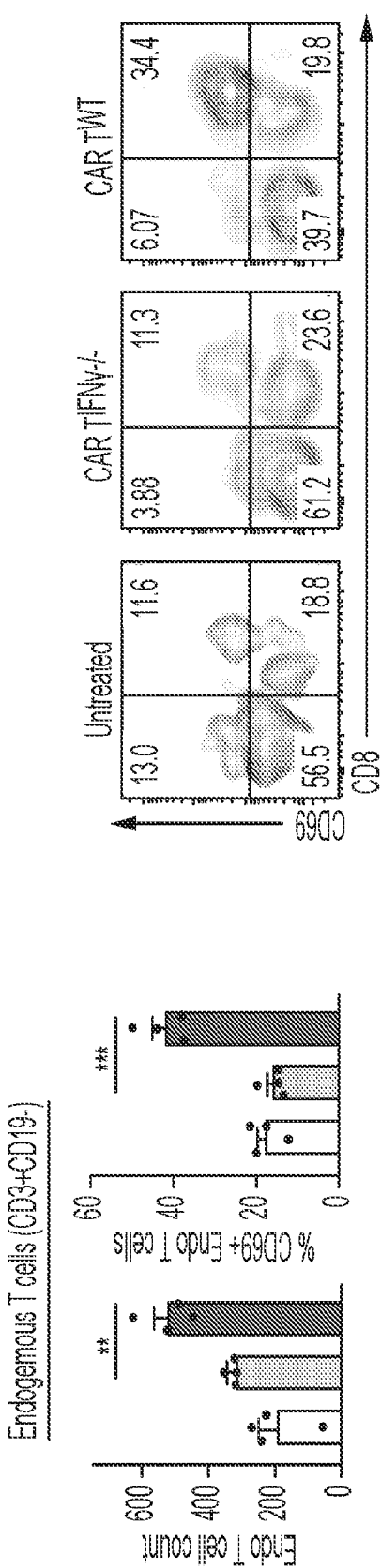


FIG. 15I

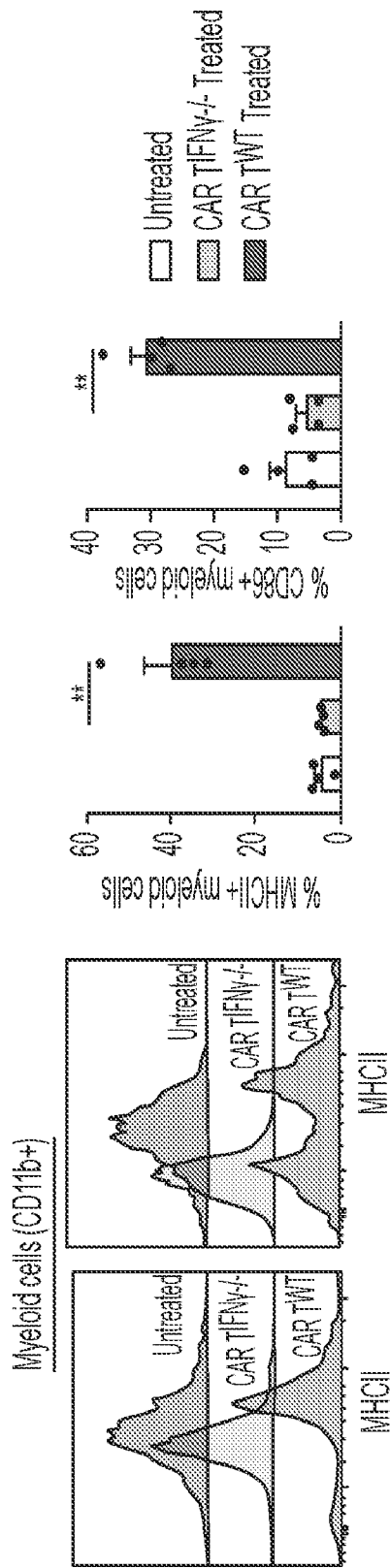


FIG. 15J

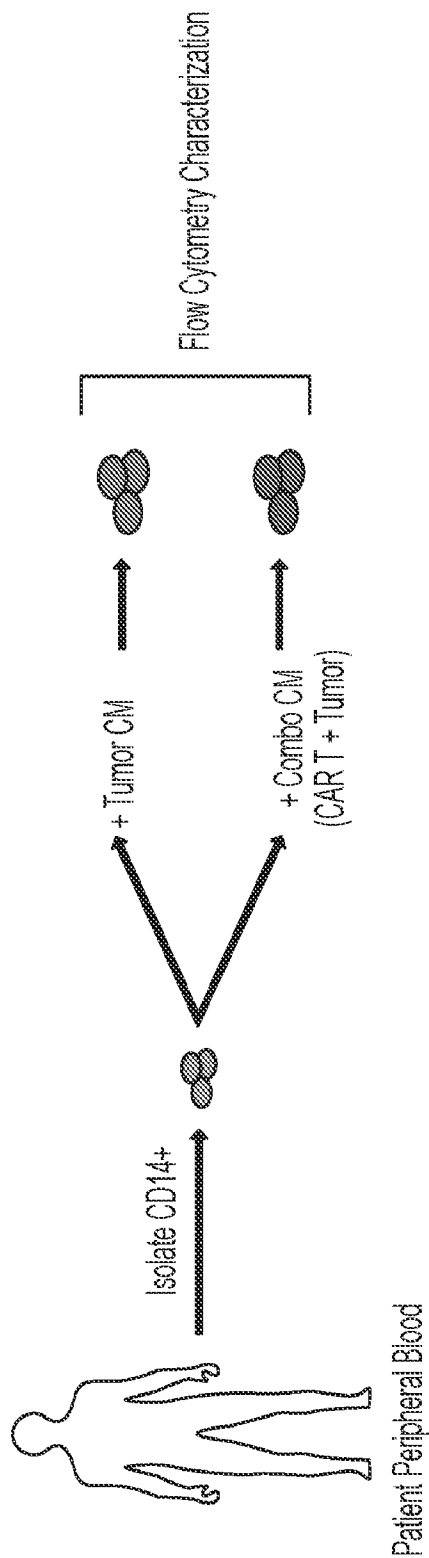


FIG. 16A

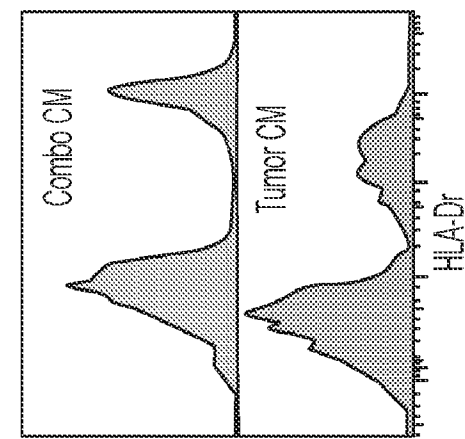
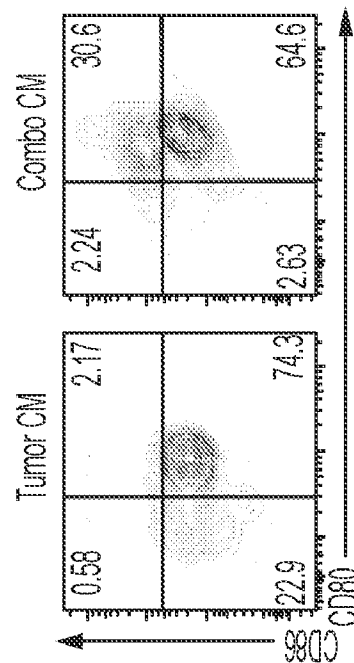
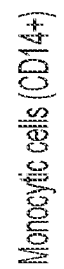
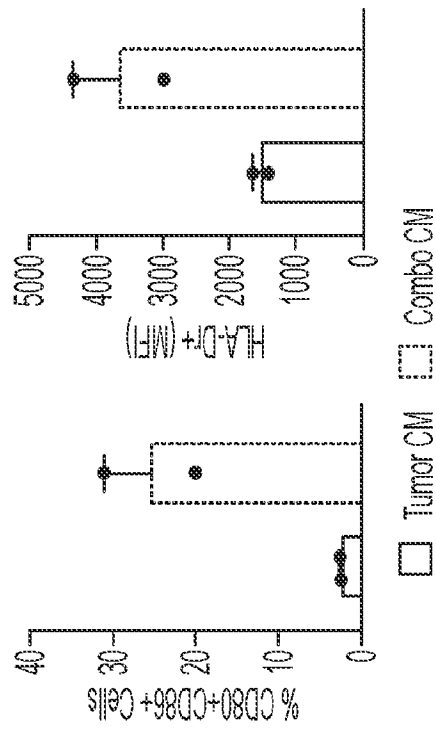


FIG. 16B



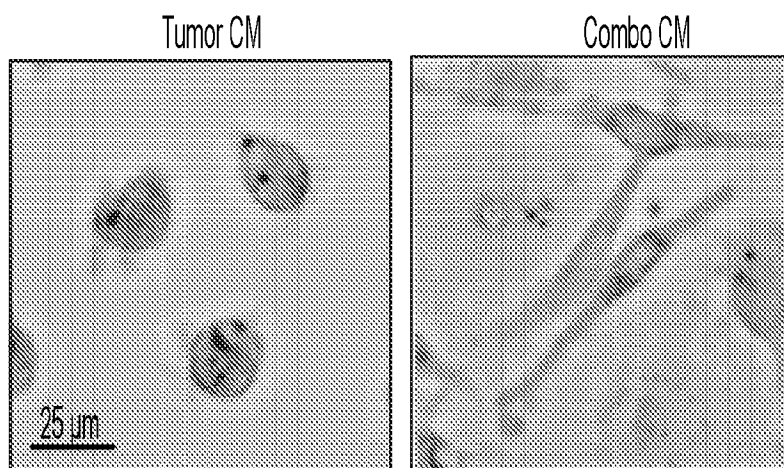


FIG. 16C

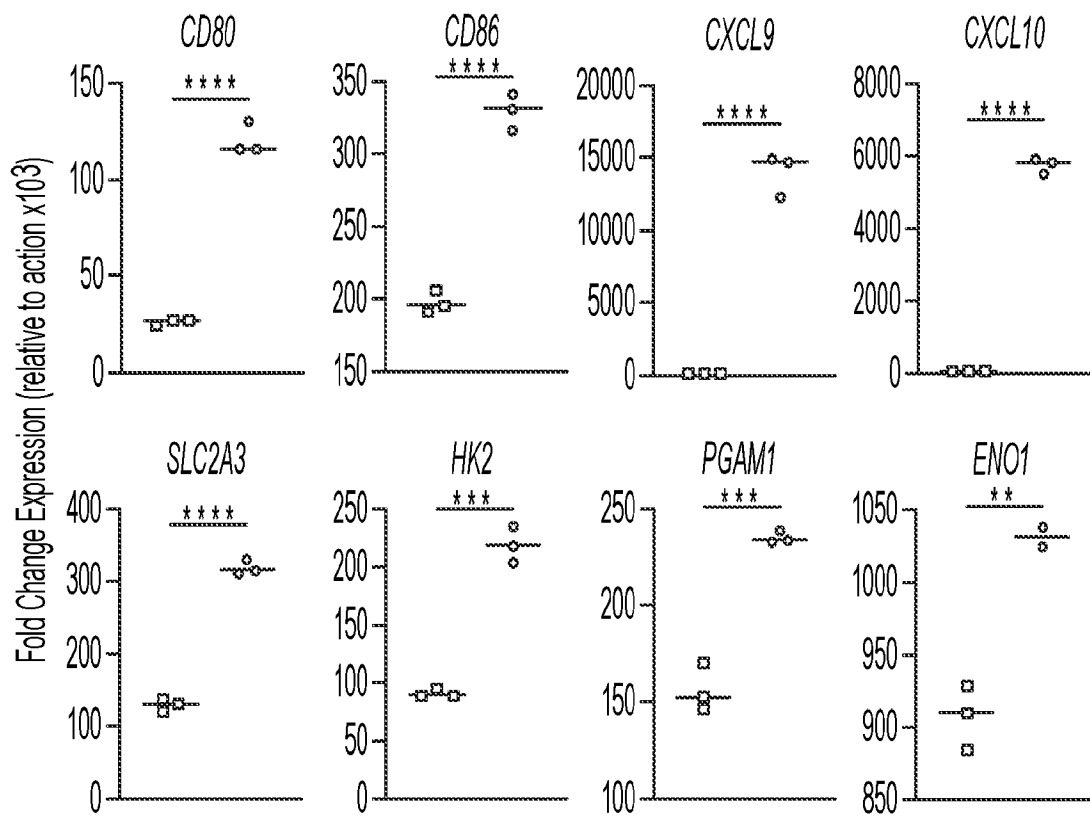


FIG. 16D

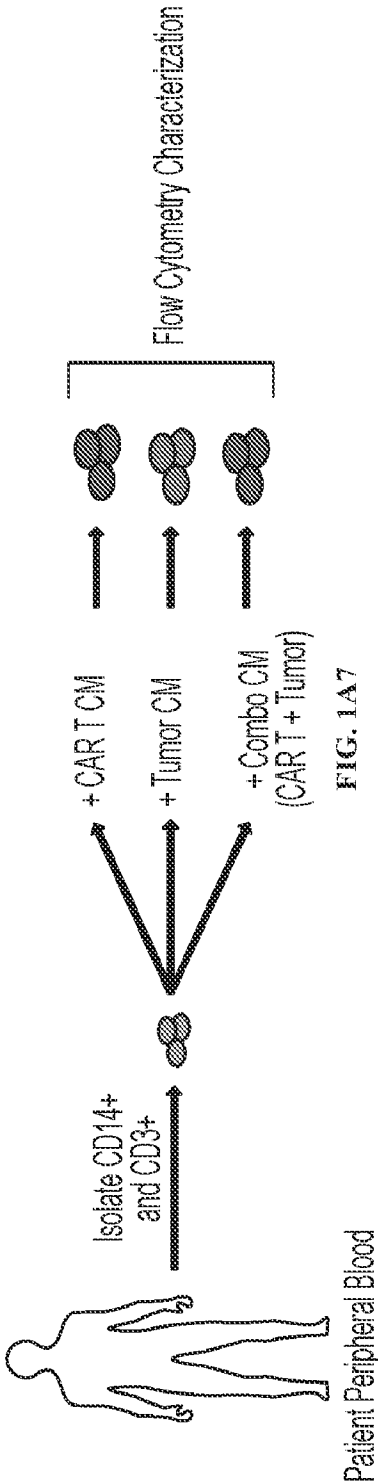


FIG. 1A7

ex vivo differentiated macrophages (CD14+)

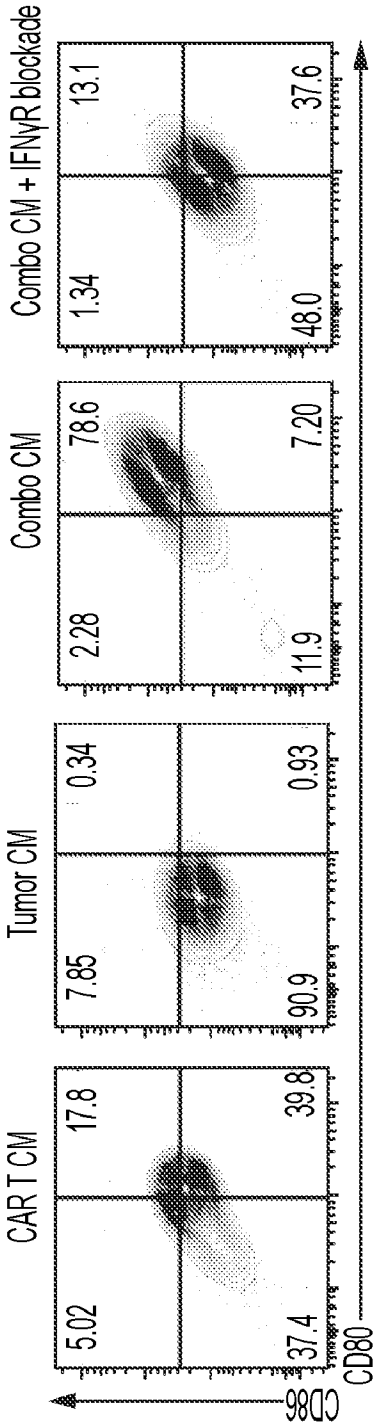


FIG. 17B

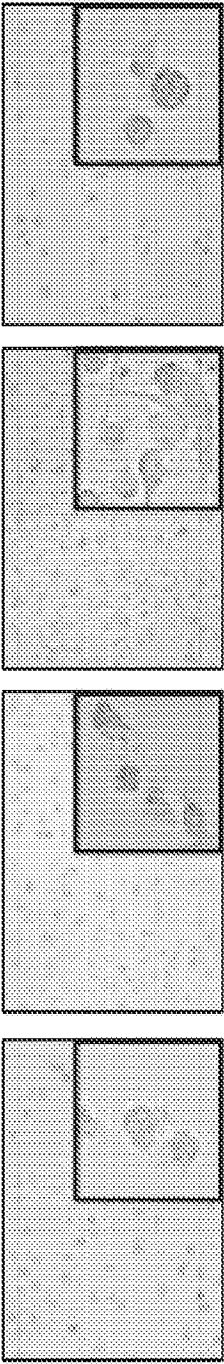


FIG. 17C

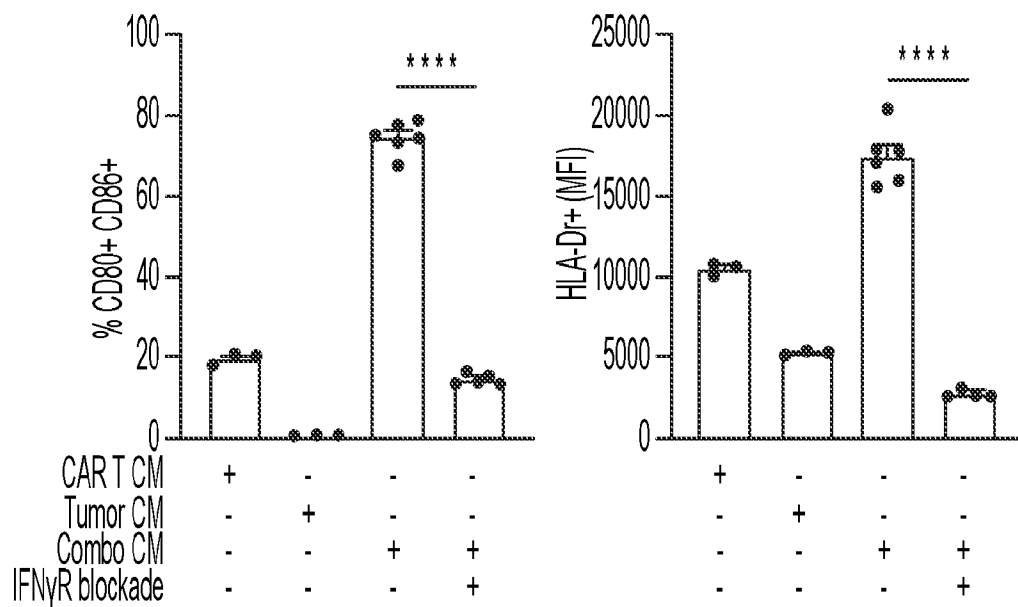


FIG. 17D

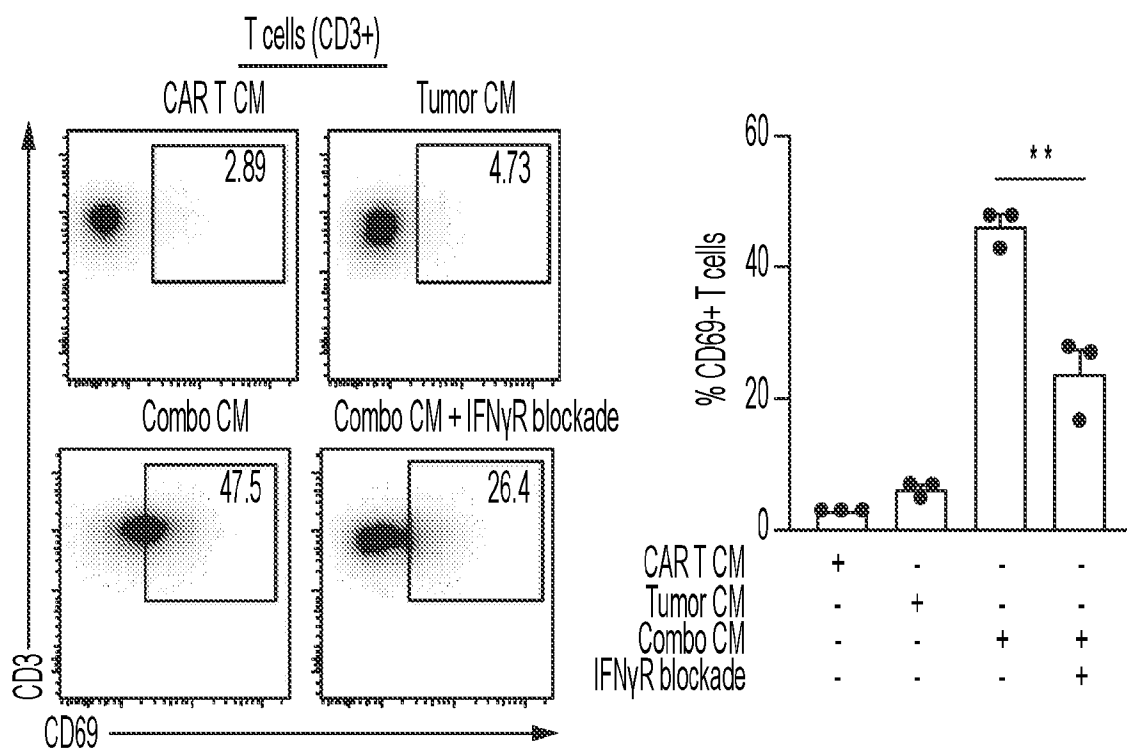


FIG. 17E

FIG. 17F

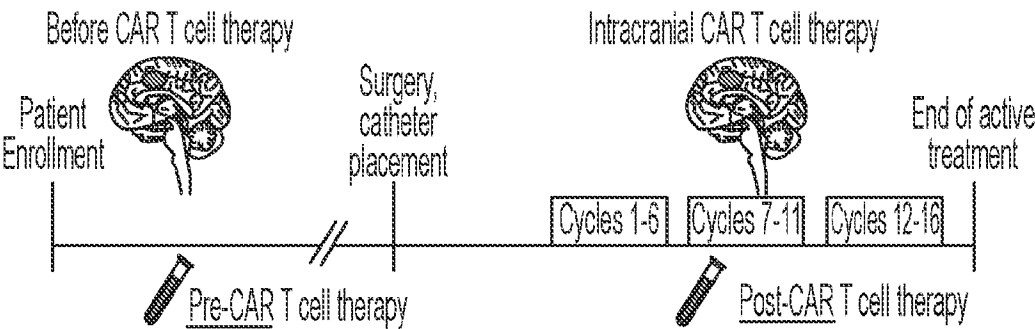


FIG. 17G

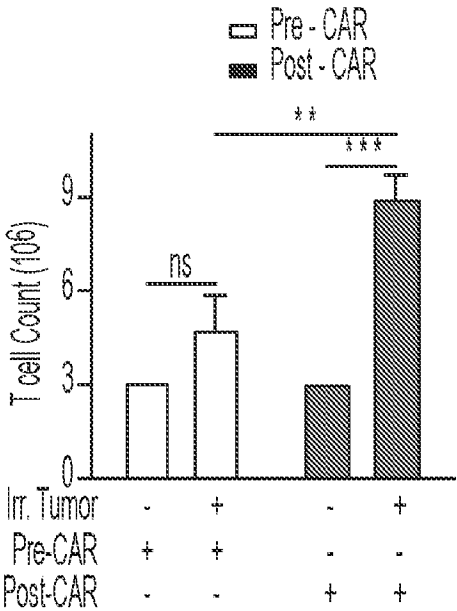
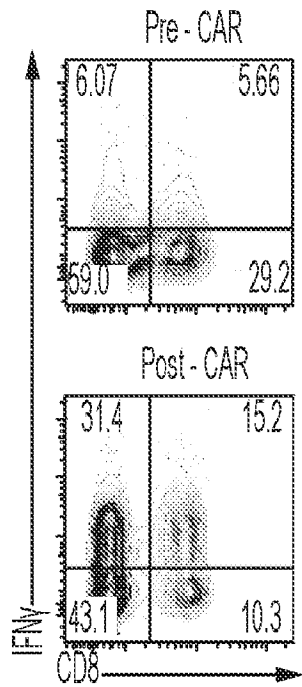


FIG. 17I

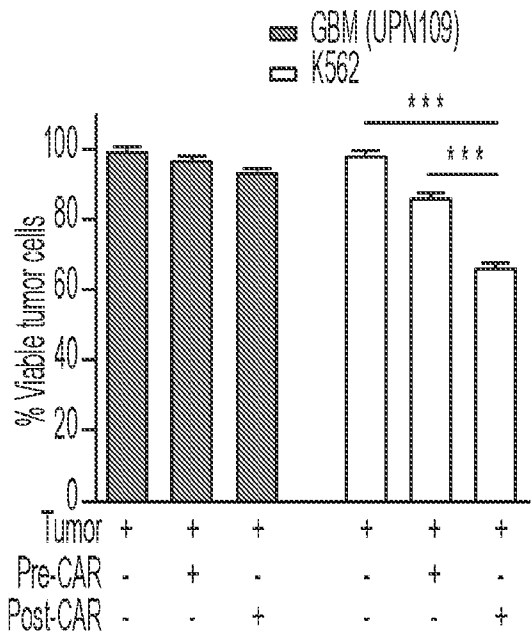


FIG. 17J

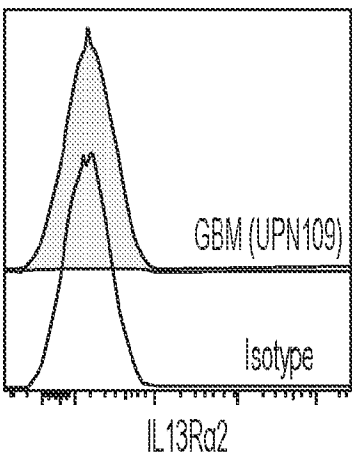


FIG. 17K

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Gene	Forward Primer	Reverse Primer
h-Actin	CCTCGCCTTTGCCGATCC	GGATCTTCATGAGGTAGTCAGTC
h-CD80	AAACTCGCATCTACTGGCAA	GGTTCTTGTA CT CGGGCCATA
h-CD86	CTGCTCATCTATACACGGTTACC	GGAAACGTCGTACAGTTCTGTG
h-CXCL10	GTGGCATTCAAGGAGTACCTC	TGATGGCCTTCGATTCTGGATT
h-CXCL9	CCAGTAGTGAGAAAGGTCGC	AGGGCTTGGGGCAAATTGTT
h-ENO1	GGTTGTGGTAAACCTCTGCTC	AAAGCTGGTGCCGTGAGAA
h-GAPDH	CTCATGACCACAGTCCATGCC	AGCCCATGCCAGTGAGCTTC
h-HK2	GAGCCACCACTCACCTACT	CCAGGCATTGCGCAATGTG
h-PGAM1	GTGCAGAAGAGAGCGATCCG	CGGTTAGACCCCATAGTGC
h-SLC2A3	GCTGGGCATCGTTGTTGGA	GCACTTTGTAGGATAGCAGGAAG
m-Actin	AGCTCATTGTAGAAGGTGTGG	GTGGGAATGGGTCAGAAGG
m-CCL5	GGGTACCATGAAGATCTCTGC	TCTAGGGAGAGGTAGGCCAAG
m-CXCL1	AACCGAAGTCATAGCCACAC	CAGACGGTGCCATCAGAG
m-CXCL10	TCAGCACCATGAACCCAA	CTATGGCCCTCATTTCTACTG
m-CXCL2	GAAGTCATAGCCACTCTCAAGG	CTTCCGTTGAGGGACAGC
m-GAPDH	CTCATGACCACAGTCCATGCC	AGCCCATGCCAGTGAGCTTC
m-NOS2	GTTCTCAGCCCAACAATAACAAGA	GTGGACGGGTCGATGTCAC

Gene	Taqman Assay ID
m-Actin	Mm02619580_g1
m-ADORA2	Mm00802075_m1
m-CD206	Mm01329362_m1
m-CD274	Mm03048248_m1
m-CD40	Mm00441891_m1
m-CD74	Mm00658576_m1
m-CD80	Mm00711660_m1
m-CD83	Mm00486868_m1
m-CD86	Mm00444540_m1
m-GZMA	Mm01304452_m1
m-GZMB	Mm00442837_m1
m-FNG	Mm01168134_m1
m-IL4R	Mm01275139_m1
m-NOS2	Mm00440502_m1
m-NT5E	Mm00501910_m1
m-PDCD1	Mm01285676_m1
m-PRF1	Mm00812512_m1
m-TGFB1	Mm01178820_m1
m-TGFBR1	Mm00436964_m1
m-TNF	Mm00443258_m1

FIG. 18

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Idiotype	Clone	Company
h-CD14	MφP9	BD Biosciences
h-CD3	UCHT1	BD Biosciences
h-CD69	L78	BD Biosciences
h-CD8	SK1	BD Biosciences
h-CD80	L307.4	BD Biosciences
h-CD86	2331 (FUN-1)	BD Biosciences
h-HLADr	LN3	eBioscience
H-IFN	B27	BD Biosciences
h-IL13Ra2	SHM38	BioLegend
M-CD11b	M1/70	eBioscience
m-CD11c	N418	eBioscience
m-CD19	1D3	BD Biosciences
m-CD3	17A2	BioLegend
m-CD4	RM4-5	eBioscience
m-CD45	30-F11	BD Biosciences
m-CD45.1	A20	BioLegend
m-CD45Ra	14.8	BD Biosciences
m-CD62L	MEL-14	eBioscience
m-CD69	H1.2F3	eBioscience
m-CD8	53-6.7	BioLegend
m-CD86	PO3	BioLegend
Donkey anti-Goat IgG	polyclonal	R&D Systems
m-GZMB	NGZB	eBioscience
m-IFNg	XMG1.2	BD Biosciences
m-IL13RA2	polyclonal	R&D Systems
m-KI67	16A8	BioLegend
m-MHCI	AF6-88.5	BioLegend
m-MHCII	NIMR-4	eBioscience
m-Thy1.1	OX-7	BioLegend
m-TNFa	MP6-XT22	eBioscience

FIG. 19

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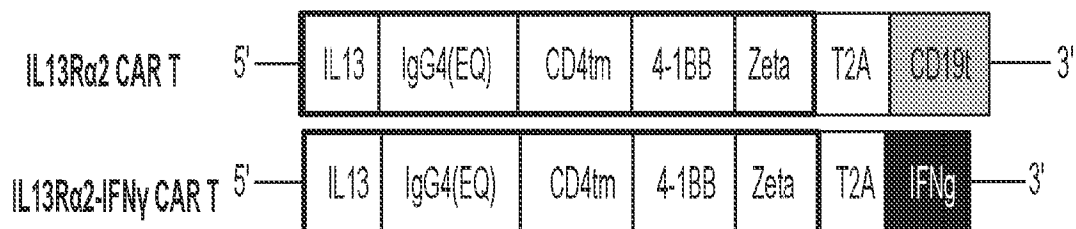


FIG. 20A

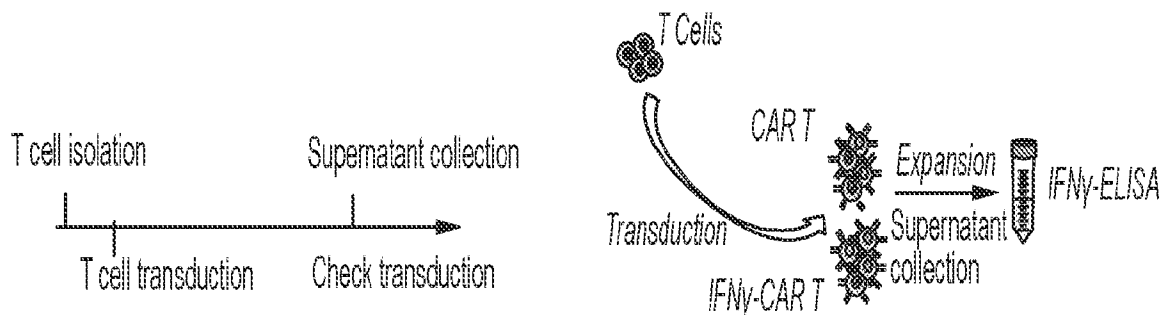


FIG. 20B

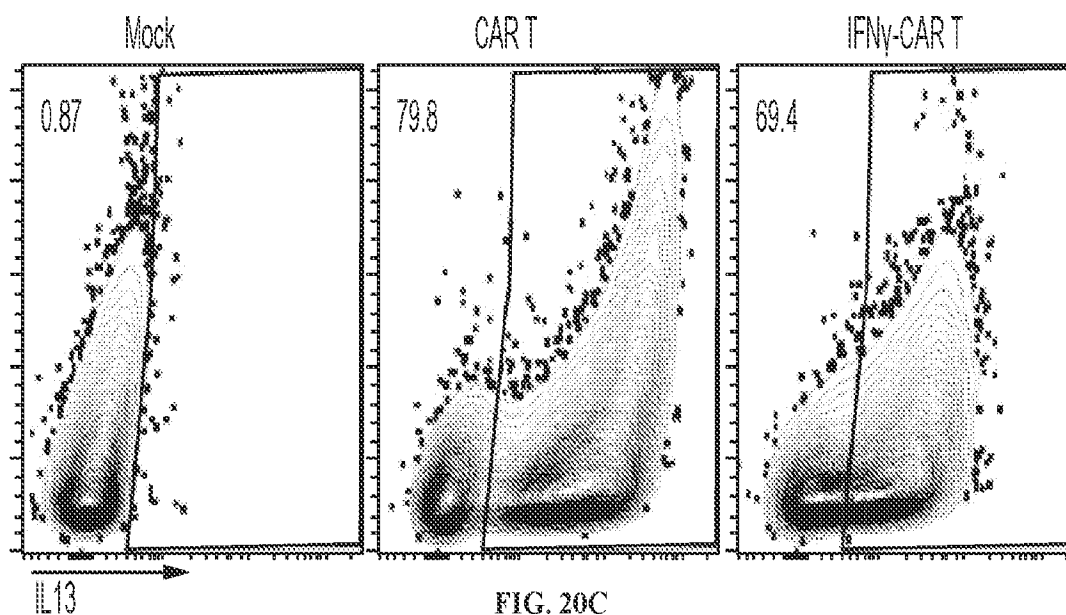


FIG. 20C

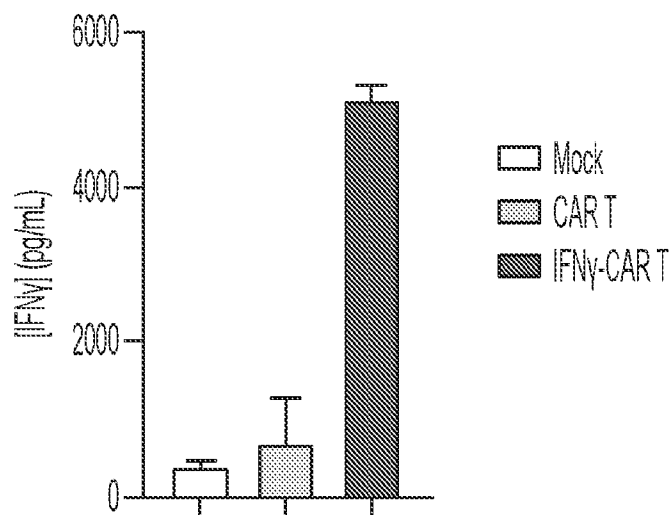


FIG. 20D

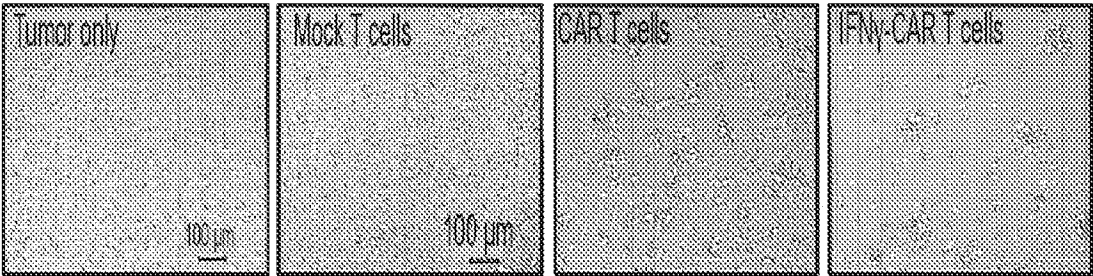


FIG. 21A

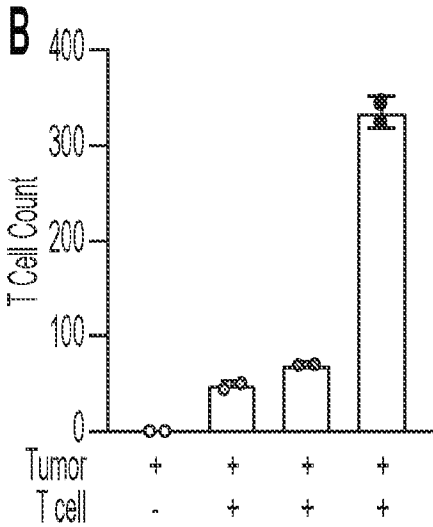


FIG. 21B

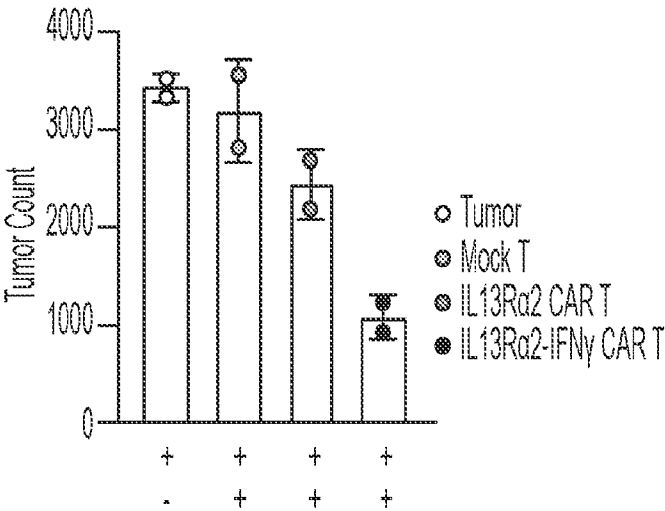


FIG. 21C

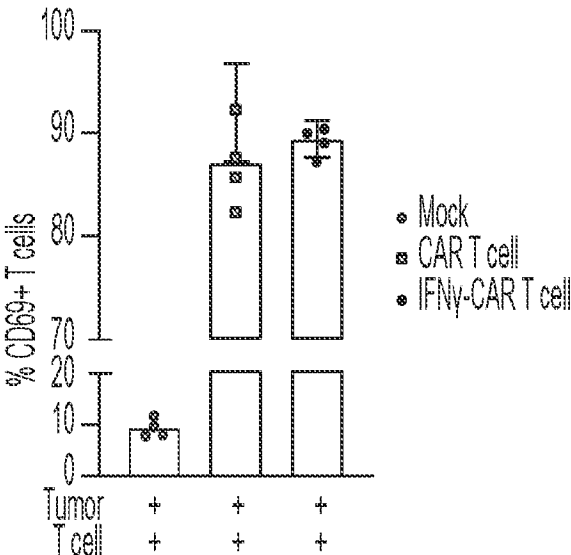


FIG. 21D

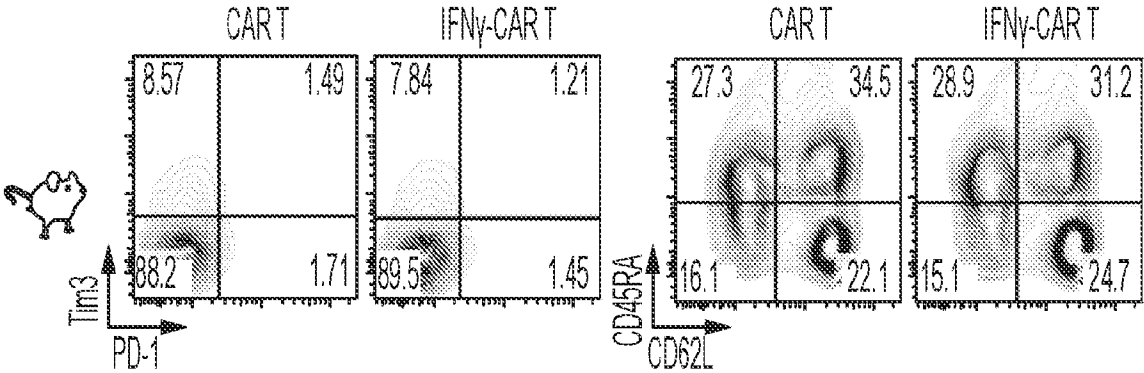


FIG. 21E

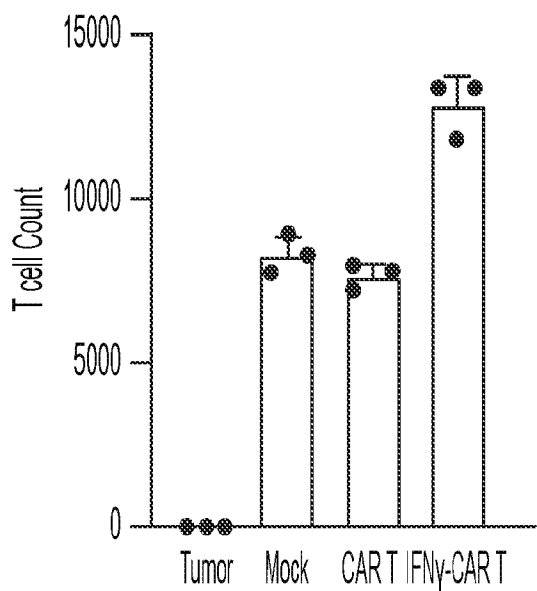


FIG. 22A

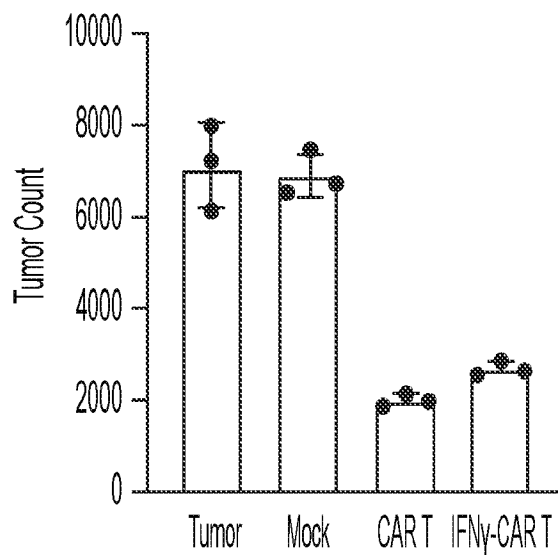


FIG. 22B

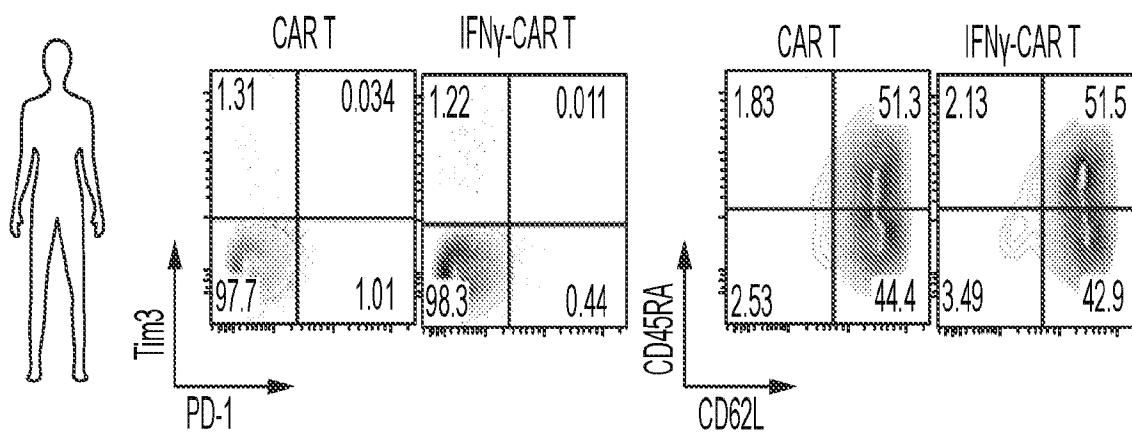


FIG. 22C

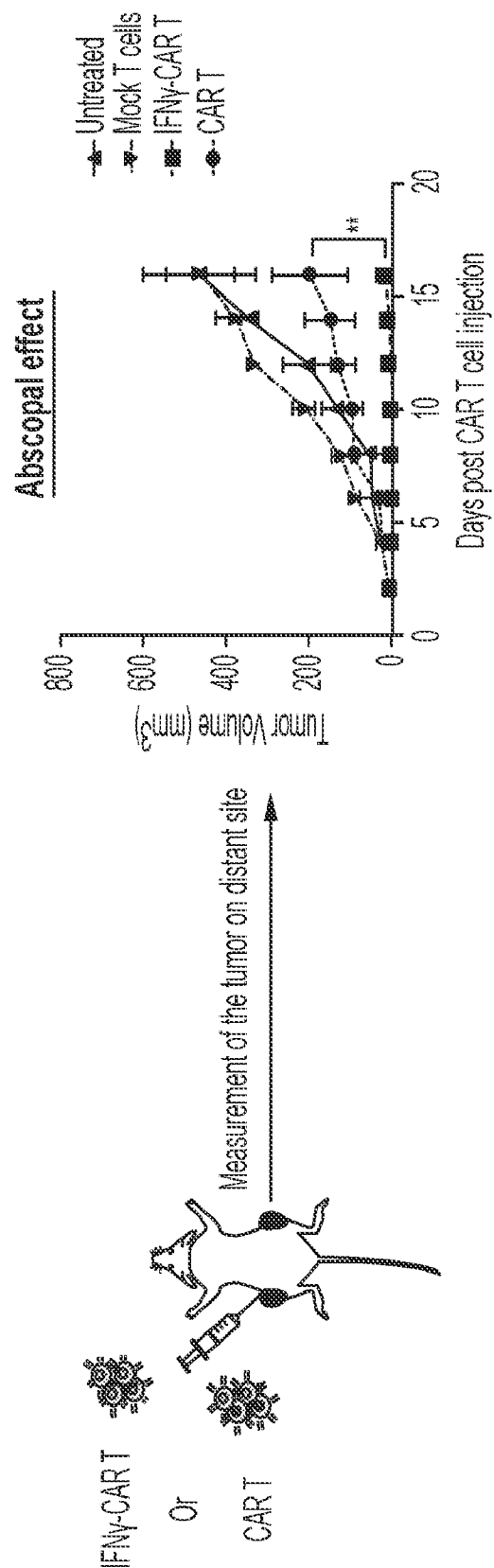


FIG. 23

IL13 variant/IgG4 (EQ) spacer/CD28TM/41BB/GGG/CD3zeta

GPVPPSTALRYLIEELVNITQNKAPLCNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTORMLSGFCPHKVSAGQF
 SSLHVRDRTKIEVAQFVKDLLLLHLKKLFREGRFNESKYGPPCPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
 QEDPEVQFNWYVDGVEVHQAKTKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTIKAKGQPRE
 POVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGOPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVE
 SCSVMHEALHNHYTQKSLSLGLKFWVLVVGGLVACYSLLTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEDDGC
 SCRFPEEEEGGCELGGGRVKFSRSADAPAYQOGONQLYNELNLRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYN
 ELQDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

IL13 variant/IgG4 (EQ) spacer/CD4TM/41BB/GGG/CD3zeta

GPVPPSTALRYLIEELVNITQNKAPLCNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTORMLSGFCPHKVSAGQF
 SSLHVRDRTKIEVAQFVKDLLLLHLKKLFREGRFNESKYGPPCPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
 QEDPEVQFNWYVDGVEVHQAKTKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTIKAKGQPRE
 POVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGOPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVE
 SCSVMHEALHNHYTQKSLSLGLKMAUUVLGGVAGLLIFGLGIFFKRGRKKLLYIFKQPFMRPVQTTQEDDGCSCRFPEE
 EGGCELGGGRVKFSRSADAPAYQOGONQLYNELNLRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQDKM
 MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

IL13 variant/IgG4 (EQ) spacer/CD8TM/41BB/GGG/CD3zeta

GPVPPSTALRYLIEELVNITQNKAPLCNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTORMLSGFCPHKVSAGQF
 SSLHVRDRTKIEVAQFVKDLLLLHLKKLFREGRFNESKYGPPCPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
 QEDPEVQFNWYVDGVEVHQAKTKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTIKAKGQPRE
 POVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGOPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVE
 SCSVMHEALHNHYTQKSLSLGLKIYWAPLAGTCGVLLLSLVITKRGGRKKLLYIFKQPFMRPVQTTQEDDGCSCRFPEE
 EGGCELGGGRVKFSRSADAPAYQOGONQLYNELNLRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQDKM
 AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

FIG. 24

IL13 variant/IgG4 ΔCH2 spacer/CD28TM/41BB/GGG/CD3zeta

GPVPPSTALRYLIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALESINVS GC SAIEKTQRM LSGFCPHKVSAGQF
 SSLHVRDTKIEVAQFVKDLLHLKLFREGRFNGQPREPQVYTLPPSQEEMTKNOVSLTCLVKGFYPSDIAVEWESNGQ
 PENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGKWWLVVVGGLACYSLLVT
 VAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGGRVKFSRSADAPAYQQGQNLNLYNELNL
 GRREEYDVLDKRRGRDPPEMGGKPRRKNPQEGLYNELOKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD
 ALHMQALPPR

IL13 variant/IgG4 ΔCH2 spacer /CD4TM/41BB/GGG/CD3zeta

GPVPPSTALRYLIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALESINVS GC SAIEKTQRM LSGFCPHKVSAGQF
 SSLHVRDTKIEVAQFVKDLLHLKLFREGRFNGQPREPQVYTLPPSQEEMTKNOVSLTCLVKGFYPSDIAVEWESNGQ
 PENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGKMAIVLGGVAGLLIFGLG
 IFFKRGKRLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGGRVKFSRSADAPAYQQGQNLNLYNELNLGRREE
 YDVLDKRRGRDPPEMGGKPRRKNPQEGLYNELOKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQ
 ALPPR

IL13 variant/IgG4 ΔCH2 spacer /CD8TM/41BB/GGG/CD3zeta

GPVPPSTALRYLIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALESINVS GC SAIEKTQRM LSGFCPHKVSAGQF
 SSLHVRDTKIEVAQFVKDLLHLKLFREGRFNGQPREPQVYTLPPSQEEMTKNOVSLTCLVKGFYPSDIAVEWESNGQ
 PENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGKIYIWAPLAGTCGVLLLSLVI
 TKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGGRVKFSRSADAPAYQQGQNLNLYNELNLGRREEYD
 VLDKRRGRDPPEMGGKPRRKNPQEGLYNELOKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQA
 LPPR

FIG. 25

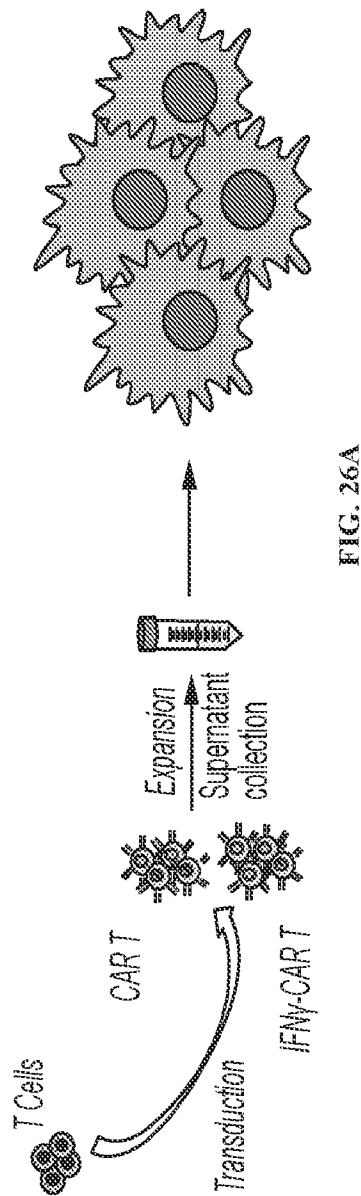


FIG. 26A

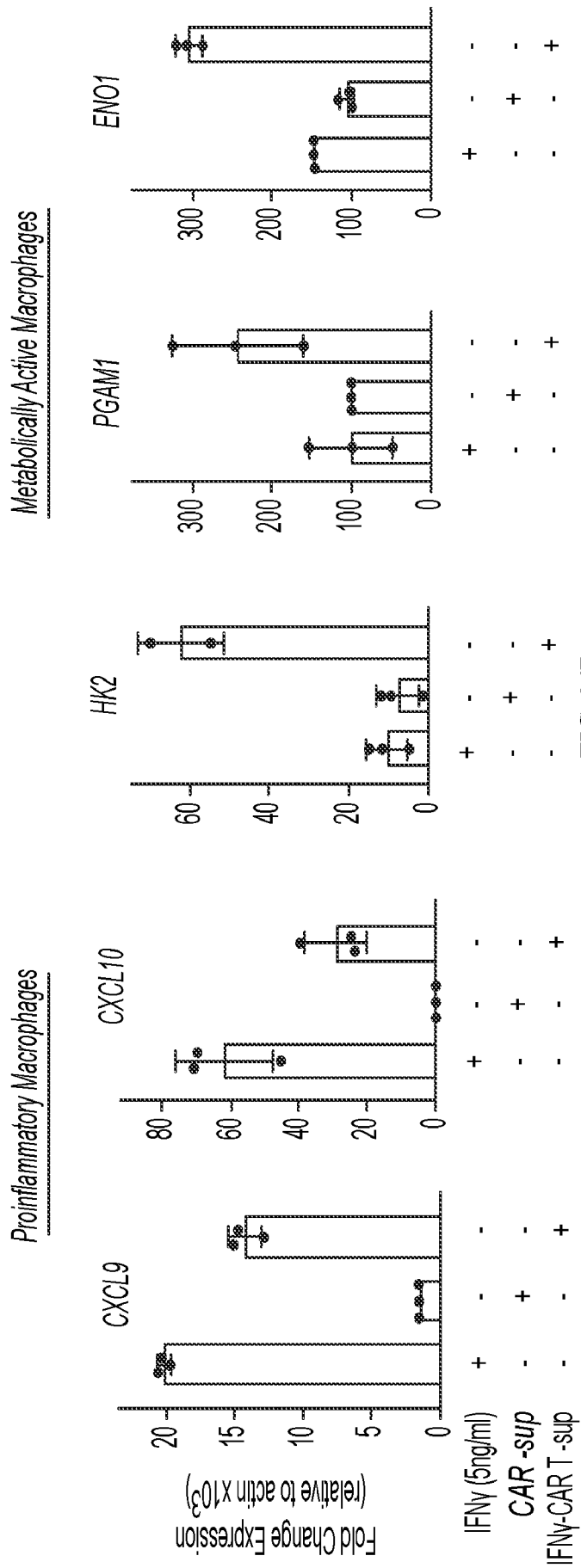


FIG. 26B

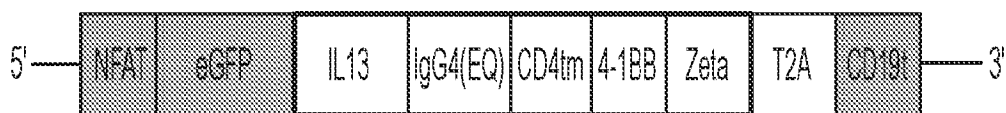


FIG. 27A

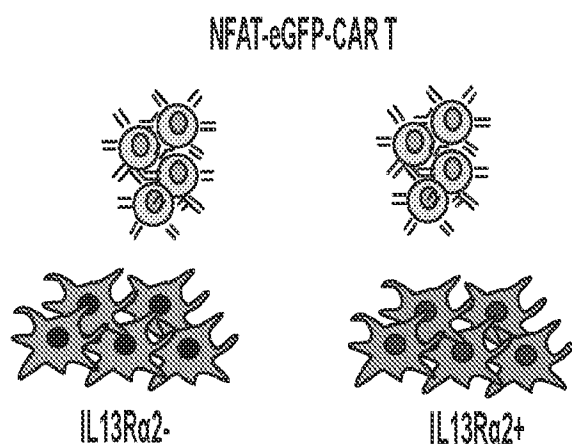


FIG. 27B

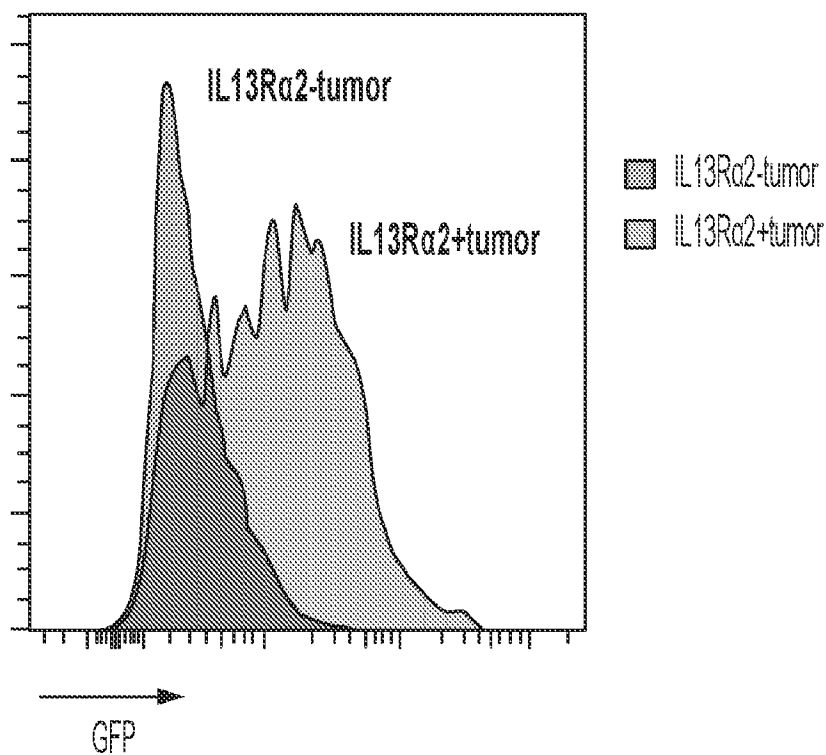


FIG. 27C

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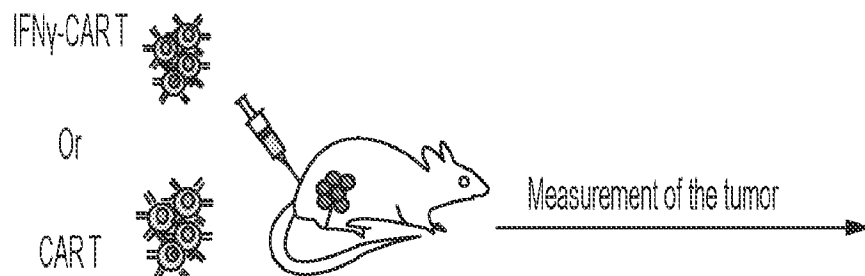


FIG. 28A

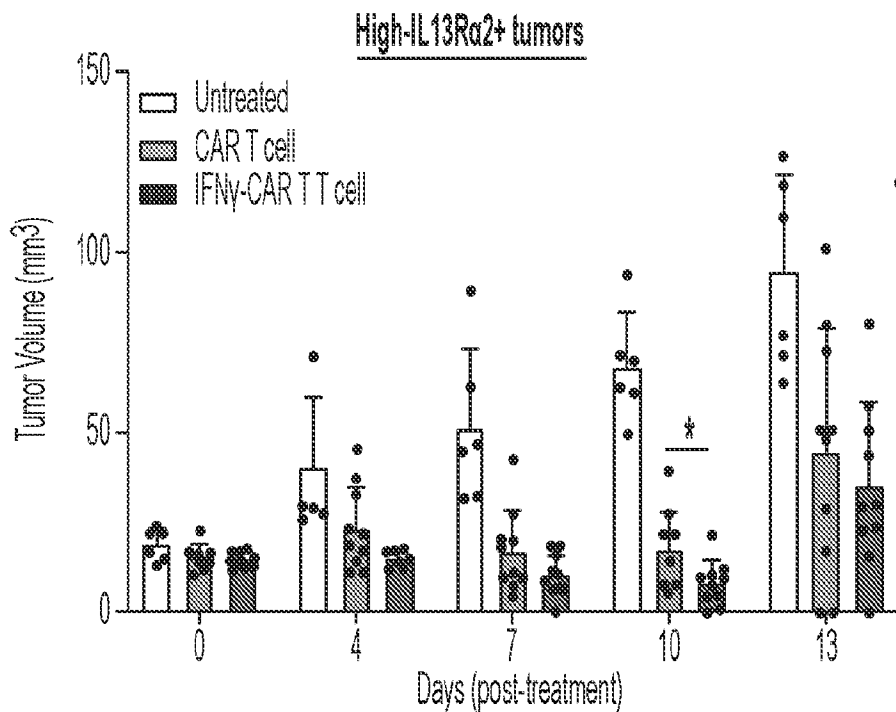


FIG. 28B

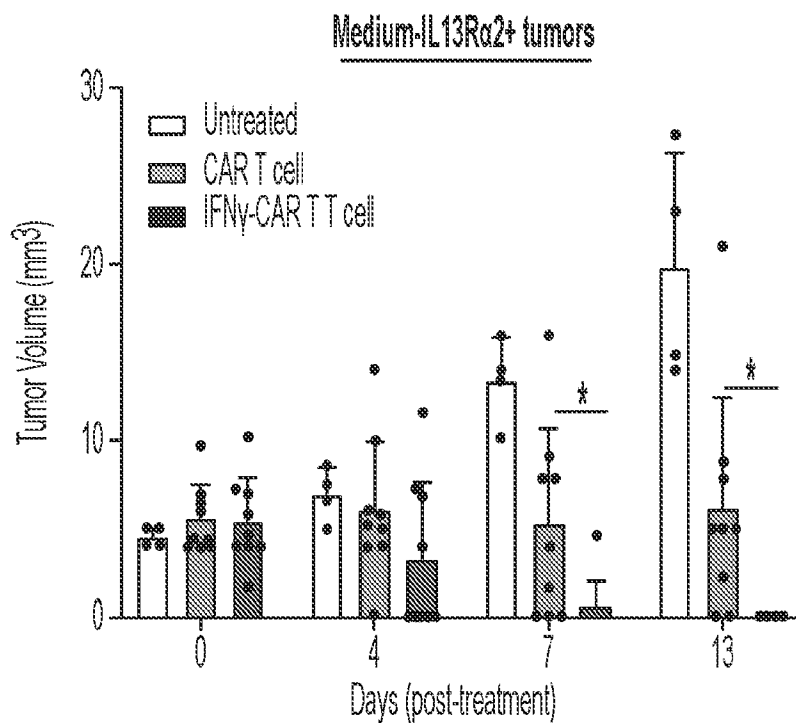


FIG. 28C

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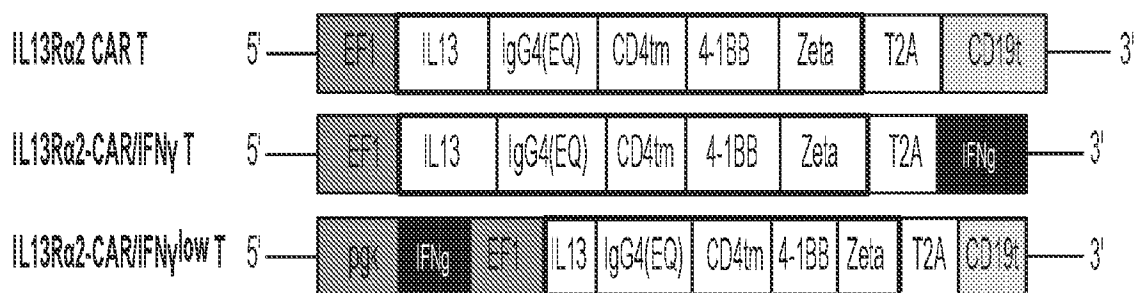


FIG. 29A

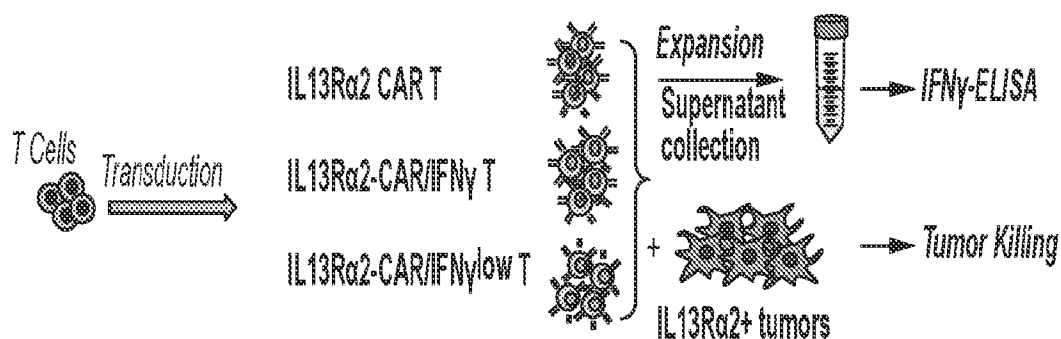


FIG. 29B

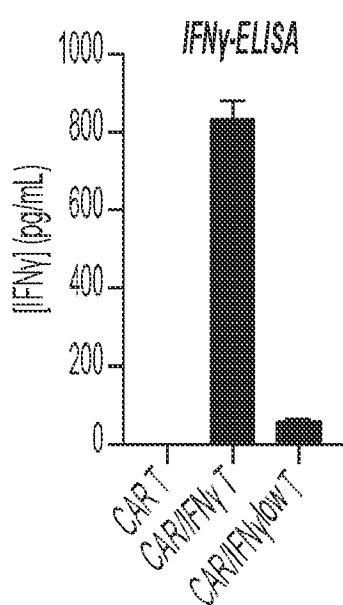


FIG. 29C

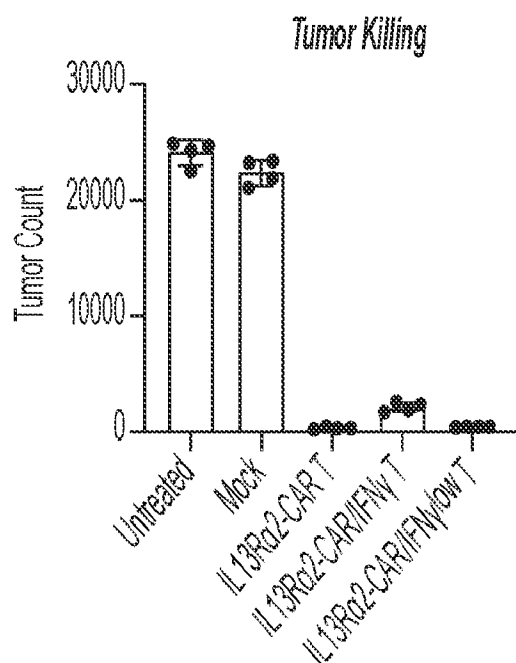


FIG. 29D

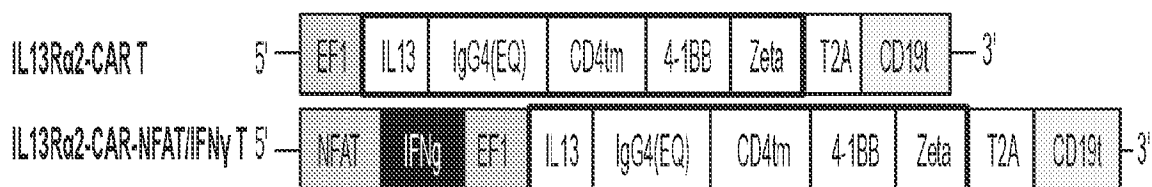


FIG. 30A

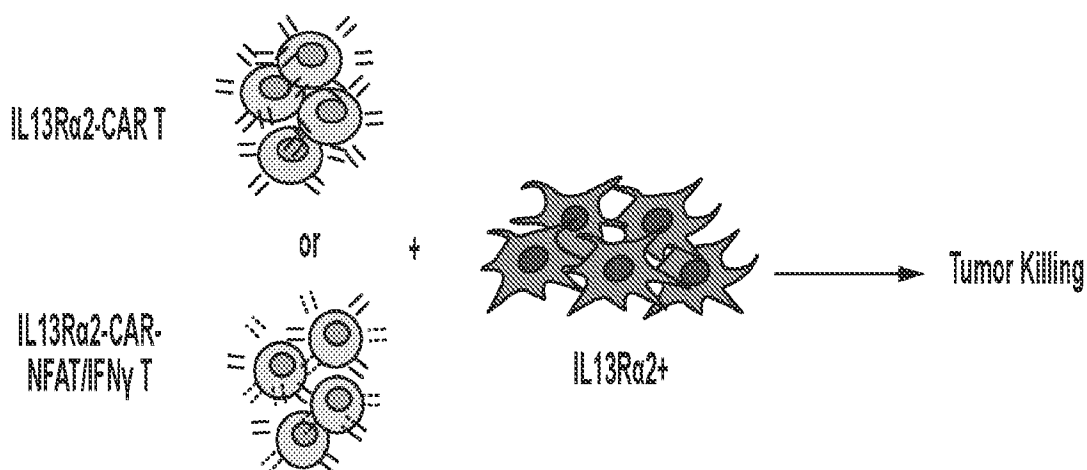


FIG. 30B

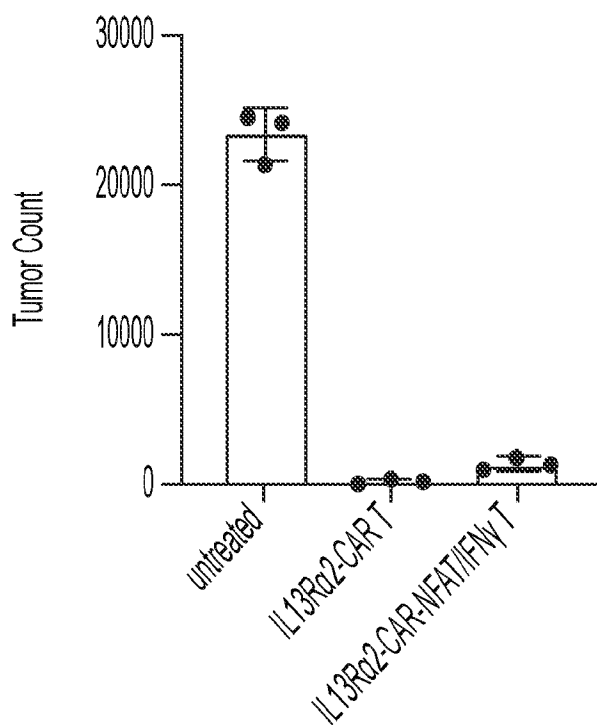


FIG. 30C

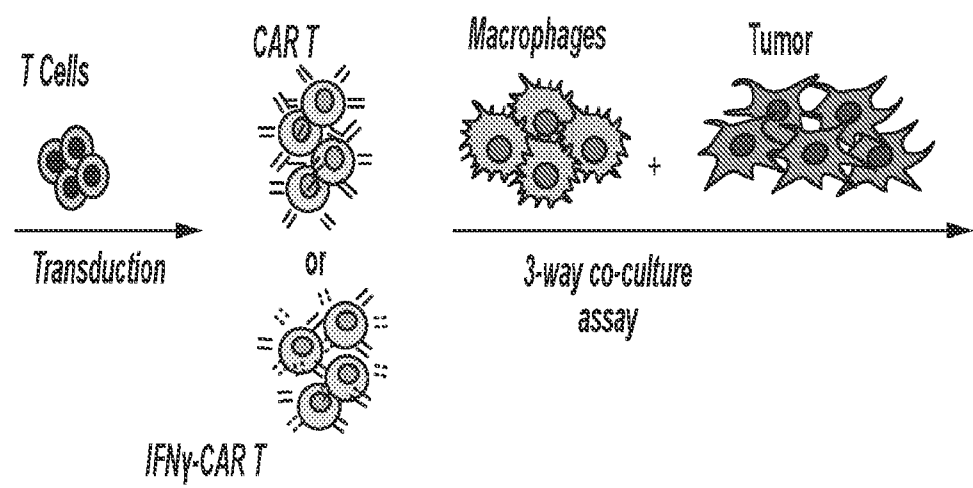


FIG. 31A

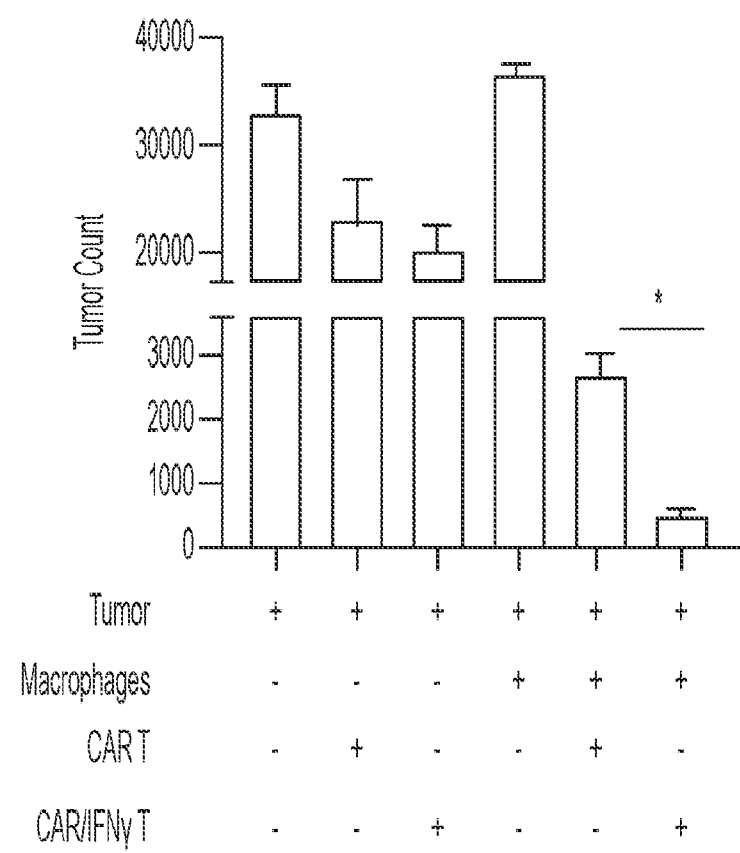


FIG. 31B