



(51) International Patent Classification:

C12N 5/0783 (2010.01) A61K 35/17 (2015.01)

(21) International Application Number:

PCT/US2021/040765

(22) International Filing Date:

07 July 2021 (07.07.2021)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/048,881 07 July 2020 (07.07.2020) US

(71) Applicant: **THE NEMOURS FOUNDATION** [US/US];
4600 Touchton Road East, Bldg. 200, Suite 500, Jack-
sonville, Florida 32246 (US).

(72) Inventor (for all designated States except US): **MA, Zhengyu**; 3108 William Road, Garnet Valley, Pennsylvan-
ia 19060 (US).

(74) Agent: **LOEB, Bronwen M.** et al.; Faegre Drinker Biddle
& Reath LLP, 1500 K. Street NW, Suite 1100, Washington,
District of Columbia 20005 (US).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN,
KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD,

ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO,
NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW,
SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

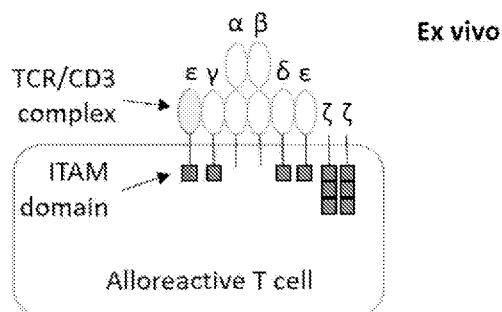
(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU,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

Published:

- with international search report (Art. 21(3))
- with amended claims (Art. 19(1))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: TUMOR-ACTIVATED ALLOREACTIVE AND XENOREACTIVE T CELLS AND THEIR USE IN IMMUNOTHER-
APY AGAINST CANCER

2A



(57) Abstract: Provided are tumor-activated alloreactive or xenoreactive T cells that are active only at the tumor sites and methods for the generation of tumor-activated alloreactive or xenoreactive T cells. Also provided are methods for using these tumor-activated alloreactive or xenoreactive T cells to treat tumors and cancers. The alloreactivity or xenoreactivity of the T cells at the tumor sites leads to the killing of tumor cells and stromal cells that express mismatched HLA molecules. The lack of activity of these T cells at non-tumor locations prevents attack on normal tissues. Further related methods and products are provided.



**Tumor-activated alloreactive and xenoreactive T cells and
their use in immunotherapy against cancer**

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of the filing date of U.S. Provisional Patent Application No. 63/048,881, filed July 7, 2020, which is hereby incorporated in its entirety for all purposes.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is herein hereby incorporated by reference in its entirety. Said ASCII copy, created on July 7, 2021, is named 045009_0053_WO_SL.txt and is 18,396 bytes in size.

FIELD

[0003] The present disclosure relates to alloreactive and xenoreactive T cells and methods of limiting their alloreactivity or xenoreactivity to tumor or cancer sites in order to kill tumor cells and tumor stromal cells without significant normal tissue damage for the purpose of immunotherapy against cancer.

BACKGROUND

[0004] T cell-based Immunotherapy against cancer has made major strides in recent years. T cells engrafted with chimeric antigen receptors (CARs) demonstrated remarkable efficacy in treating B cell malignancies (1-5). FDA approval of the first CAR T cell drug for B cell lymphoma in 2017 marked a major milestone in the fight against cancer. In this approach, autologous T cells from the patient are isolated and genetically modified to express CARs that recognizes the pan-B cell marker CD19. CAR T cells infused back to the patient are able to recognize cancer cells through CAR-CD19 interaction. Signals from CARs lead to T cell activation and cytotoxicity towards cancer cells. Significant challenges remain, however, in developing safe and effective T cell-based immunotherapies for a wider range of cancers, especially for solid tumors and non-B-cell hematopoietic malignancies.

[0005] A major challenge for T cell therapy against solid tumors is the heterogeneity of tumor antigen expression. To date, the success of CAR T cell therapy is largely limited to treating B cell cancers. Due to the homogeneous expression of CD19 on all cancerous B cells, complete eradication of cancer cells can be achieved. In fact, normal B cells are eliminated as well but the lack of B cell function is easily compensated with immunoglobulin infusion. In contrast, the expression of most solid tumor antigens is much more heterogeneous in terms of the types of tissues they are expressed in and expression levels among tumor cells. Many tumor antigens can be highly expressed in tumors but may also be expressed in certain normal tissues at lower levels than found in tumors (6). Moreover, tumor antigen expression within tumors is rarely uniform, with only a certain percentage of tumor cells expressing any given type of tumor antigen (7-10). Progresses have been made in minimizing “on-target, off-tumor” targeting of normal tissues by designing CARs that mediate T cell responses only to cells expressing tumor antigens at high levels (11) or cells expressing multiple tumor antigens (12-14). Heterogeneity in tumor antigen expression among tumor cells, however, remains a major roadblock. The challenge was highlighted by a recent clinical study targeting EGFRvIII (epithelial growth factor receptor variant III) for glioblastoma (10). CAR T cells infiltrated the tumors, killed EGFRvIII-positive tumor cells but failed to halt glioblastoma progression, most likely due to the expansion of EGFRvIII-negative tumor cells under the selection pressure from the CAR T cells. Similar results were observed in a study targeting IL13R α 2 for glioblastoma in animal models (15).

[0006] The challenge of target antigen heterogeneity also exists for non-B cell blood cancers. The lack of cancer-specific antigen has hampered the efforts to develop CAR T therapy for acute myeloid leukemia (AML) (16). Most targets identified today, such as CD33 and CD123, are also expressed on normal myeloid cells and hematopoietic stem cells (17, 18). Targeting these antigens therefore often requires rescue hematopoietic stem cell transplantation. Heterogeneous expression of target antigens among cancer cells is a main contributor to relapse post CAR T cell therapy. The expression of B-cell maturation antigen (BCMA) in myeloma biopsies, for example, varies in terms of both percentage and level of expression (19).

[0007] Therefore, an urgent need remains for effective strategies that can overcome antigen-negative immune escape by broadening the target to include tumor antigen-negative tumor cells and stromal cells that support tumor growth. Approaches that target alternative antigens are

also needed to overcome the barriers for non-B cell hematopoietic malignancies. This disclosure addresses these needs.

SUMMARY

[0008] The present disclosure relates to alloreactive and xenoreactive T cells and methods of limiting their alloreactivity or xenoreactivity to tumor sites or sites enriched in cancer cells in order to kill tumor cells, tumor stromal cells, and cancer cells without significant damage to normal tissues outside of the sites for the purpose of immunotherapy against cancer.

[0009] The present disclosure provides a tumor-activated alloreactive or xenoreactive T cell. The T cell can be used to kill tumor cells, tumor stromal cells, and cancer cells. Advantageously, the T cell should not cause significant normal tissue damage. Thus, a method of treating a patient having a malignancy, in particular, a solid tumor and a non-B cell hematopoietic malignancy, is also provided. A method of preparing the tumor-activated T cell and related kits are also provided.

[0010] The therapeutic methods and compositions used in these methods as described herein can be alternatively considered as a use of genetically-modified tumor-activated alloreactive or xenoreactive T cells for use in treating cancer in a patient in therapeutic need thereof, or for use in the preparation of a medicament for treating cancer. The use of the disclosed genetically-modified tumor-activated T cell can be applied to any of the methods and combinations described above and *infra*.

[0011] Provided is a genetically modified T cell comprising: (i) genetic disruption of expression of at least one endogenous gene encoding a molecule necessary for TCR signaling and T cell activation, (ii) an exogenous nucleotide sequence encoding a tumor-sensing receptor that releases or activates a transcription activator in response to direct or indirect binding to molecules enriched on tumor cells, in the tumor microenvironment or in tissues with blood cancer cell accumulation, and (iii) an exogenous nucleotide sequence comprising an expression cassette that expresses a copy of the disrupted endogenous gene of (i) in response to the released or activated transcription activator of (ii).

[0012] In some aspects, the at least one disrupted endogenous gene encoding a molecule necessary for TCR signaling and T cell activation encodes a transmembrane protein selected

from, for example, one or more of CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58. In certain embodiments, the endogenous gene disrupted is selected from CD3 ϵ , CD3 ζ , CD3 γ , and CD3 δ . In an embodiment, the endogenous gene disrupted is CD3 ϵ . In some aspects, the at least one
 5 disrupted endogenous gene encoding a molecule necessary for TCR signaling and T cell activation encodes an intracellular signaling molecule chosen from Lck, Zap70, calcineurin, PI3K, Fyn, PLC γ , SLP76, PKC θ , AKT, and PDK1.

[0013] In aspects of the genetically modified T cell, the tumor-sensing receptor comprises (i) an extracellular domain that binds directly or indirectly to a target molecule enriched on or
 10 in at least one of tumor cells, tumor microenvironment, and tissues with blood cancer cell accumulation; and (ii) an intracellular domain that activates or releases a transcription activator in response to extracellular domain binding to the target molecule. The tumor-sensing receptor can be a Synthetic Notch (SynNotch) receptor, a Modular Extracellular Sensor Architecture (MESA) receptor, a Tango receptor, or a chimeric antigen receptor (CAR).

[0014] In some aspects, the target molecule is enriched on tumor cells and/or in the tumor microenvironment and is chosen from, for example. one or more of CD19, CD20, CD38, CD30, Her2/neu, ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2
 20 (VEGFR2), high molecular weight- melanoma associated antigen (HMW-MAA), MAGE-A1, IL-13R-a2, GD2, 4-1BB, 5T4, adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), C-MET, CCR4, CD152, CD19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4,
 25 DRS, EGFR, EpCAM, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgG1, LI-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin $\alpha\beta$ 3, MORAb-009, MS4A1, MUC1, mucin CanAg, N- glycolylneuraminic acid, NPC-IC, PDGF-Ra, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1,
 30 SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF- β , TRAIL-R1, TRAIL-R2, tumor antigen CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, mesothelin, NKG2D, CD147, NKR2, B7H3, and vimentin. In certain embodiments, the target molecule is selected from

Her2/neu, EGFRvIII, CD19, IL-13RR-a2, mesothelin, MUCI, EpCAM, GD2 and CEA. In certain embodiments, target molecule is selected from Her2/neu, EGFRvIII, CD19, IL-13RR-a2, mesothelin, and MUCI. In certain embodiments, the target molecule is selected from Her2/neu, EGFRvIII, and CD19. In an embodiment, the target molecule is Her2/neu.

5 [0015] In some aspects, the target molecule is enriched in a tissue with blood cancer cell accumulation, and wherein the tissue is lymphoid and/or bone marrow tissue. In certain embodiments, the target molecule can be chosen from CD45, CD19, CD20, CD4, CD8, CD2, CCR4, CD58, CD28, CD23, CD69, CD25, CD33, CD123 and CCL-1.

10 [0016] In some aspects, the extracellular domain of the tumor-sensing receptor is a single chain variable fragment (scFv), a Fab fragment, a designed ankyrin repeat protein (DARPin), a nanobody, a TCR, a Fc receptor, a growth factor receptor, a chemokine receptor, or a hormone receptor.

[0017] In some aspects, the tumor-sensing receptor is a chimeric antigen receptor, wherein a downstream transcription factor is activated through signaling pathways activated in response
15 to extracellular domain binding to the target molecule.

[0018] In certain embodiments, the expression cassette encoding the copy of the disrupted endogenous gene comprises a transcription control element driving expression of the copy of the disrupted endogenous gene is bound by a transcription activator selected from the group consisting of Gal4-VP64, Notch, tet transactivator, NFAT, AP-1, NFκB/Rel, NR4A1 (Nur77),
20 T-Bet, IRF4, STATs and eomesodermin (Eomes). In certain embodiments, the expression cassette is selected from Gal4-CD3-PGK-BFP, NR4A-CD3-PKG-BFP, and NFAT-CD3-PKG-BFP. In certain embodiments, the tumor sensing receptor and expression cassette are selected from SynNotch and Gal4-CD3-PGK-BFP, CAR and NR4A-CD3-PKG-BFP, and CAR and NFAT-CD3-PKG-BFP.

25 [0019] In certain aspects, the sample of T cells is from a donor individual, and wherein the donor individual has at least one HLA allele mismatch relative to an intended recipient and the at least one HLA allele mismatch is located in a locus selected from the group consisting of: HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DPA1 and HLA-DPB1, thereby producing tumor-activated alloreactive T cells. In certain embodiments, the at
30 least one HLA allele mismatch is located in a locus selected from the group consisting of:

HLA-A, HLA-B, HLA-C, and HLA-DRB1. In certain embodiments, the HLA allele mismatch comprises four alleles of, HLA-A, HLA-B, HLA-C, and HLA-DRB1, six alleles of, HLA-A, HLA-B, HLA-C, and HLA-DRB1 or all eight alleles of HLA-A, HLA-B, HLA-C, and HLA-DRB1. In an embodiment, the HLA allele mismatch comprises all eight alleles of HLA-A, HLA-B, HLA-C, and HLA-DRB1.

[0020] In certain embodiments, the endogenous gene disrupted is CD3 ϵ , the target molecule is selected from Her2/neu, EGFRvIII, and CD19, and the tumor sensing receptor is SynNotch. In certain embodiments, the endogenous gene disrupted is CD3 ϵ , the target molecule is selected from Her2/neu, EGFRvIII, and CD19, the tumor sensing receptor is SynNotch, and the expression cassette is Gal4-CD3-PGK-BFP. In an embodiment, the endogenous gene disrupted is CD3 ϵ , the target molecule is Her2/neu, the tumor sensing receptor is SynNotch, and the expression cassette is Gal4-CD3-PGK-BFP. In an embodiment, the endogenous gene disrupted is CD3 ϵ , the target molecule is CD19, the tumor sensing receptor is SynNotch, and the expression cassette is Gal4-CD3-PGK-BFP.

[0021] Further provided is a method for producing tumor-activated alloreactive or xenoreactive T cells. The method comprises a) selecting a sample of T cells from a donor individual, or from a donor animal; b) optionally stimulating the sample of T cells to proliferate; c) abrogating the expression or function of at least one molecule necessary for TCR signaling and T cell activation in the T cells to render the T cells activation-incompetent; and d) modifying the T cells to (i) express a recombinant receptor that specifically binds to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and a tissue with blood cancer cell accumulation, wherein binding of the recombinant receptor with the target molecule releases or activates a transcription activator; and (ii) introduce an expression cassette that enables the transcription activator in (i) to drive the expression of the molecule abrogated in c), thereby restores the expression or function of the abrogated molecule, and thereby restores the ability of the T cells to activate through antigen recognition by TCR, thereby producing tumor-activated alloreactive or xenoreactive T cells. In the method, step d) is carried out before step c), step c) is carried out before step d), or steps c) and d) are performed at the same time.

[0022] In certain aspects of the method, the sample of T cells is from a donor individual, and wherein the donor individual has at least one HLA allele mismatch relative to an intended

recipient and the at least one HLA allele mismatch is located in a locus selected from the group consisting of: HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DPA1 and HLA-DPB1, thereby producing tumor-activated alloreactive T cells. In certain embodiments, the at least one HLA allele mismatch is located in a locus selected from the group consisting of: HLA-A, HLA-B, HLA-C, and HLA-DRB1. In certain embodiments, the HLA allele mismatch comprises four alleles of, HLA-A, HLA-B, HLA-C, and HLA-DRB1, six alleles of, HLA-A, HLA-B, HLA-C, and HLA-DRB1 or all eight alleles of HLA-A, HLA-B, HLA-C, and HLA-DRB1. In an embodiment, the HLA allele mismatch comprises all eight alleles of HLA-A, HLA-B, HLA-C, and HLA-DRB1.

10 **[0023]** In certain aspects of the method, step b) comprises: (i) co-culturing donor T cells with cells from an intended recipient; (ii) co-culturing donor T cells with cells from a second donor that has at least one HLA allele matched with the intended recipient, and (ii) is mismatched with the T cell donor; (iii) co-culturing donor T cells with a cell line expressing a least one HLA allele of the intended recipient; (iv) co-culturing donor T cells with an artificial surface coated with at least one protein encoded by at least one HLA allele of the intended recipient.

[0024] In certain embodiments of making the T cells, the at least one molecule necessary for TCR signaling and T cell activation is a cell surface molecule chosen from CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58, or an intracellular signaling molecule chosen from Lck, Zap70, calcineurin, PI3K, Fyn, PLC γ , NFAT, SLP76, PKC θ , NF κ B, AKT, and PDK1. In certain embodiments, the endogenous gene disrupted is selected from CD3 ϵ , CD3 ζ , CD3 γ , and CD3 δ . In an embodiment, the endogenous gene disrupted is CD3 ϵ .

25 **[0025]** In certain aspects of the method, step d) comprises introducing a nucleic acid encoding a tumor-sensing receptor into T cells, wherein the tumor-sensing receptor comprises (i) an extracellular domain that binds directly or indirectly to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and tissues with blood cancer cell accumulation; and (ii) an intracellular domain that activates or releases a transcription activator in response to extracellular domain binding to the target molecule.

[0026] In certain aspects of the method, step d) comprises introducing a nucleic acid encoding an expression cassette into T cells, wherein the expression cassette comprises (i) a transcription control element (TCE) that can be bound by the transcription activator activated or released by the tumor sensing receptor; and (ii) a DNA sequence that encodes a copy of the gene disrupted in step c), thereby enables the expression of the disrupted gene in response to tumor sensing receptor binding to target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and tissues with cancer cell accumulation.

[0027] In certain embodiments of making the T cells, the target molecule is enriched on tumor cells and/or in the tumor microenvironment and can be chosen, for example, from CD19, CD20, CD38, CD30, Her2/neu, ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2 (VEGFR2), high molecular weight- melanoma associated antigen (HMW-MAA), MAGE-A1, IL-13R-a2, GD2, 4-1BB, 5T4, adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), C-MET, CCR4, CD152, CD19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4, DRS, EGFR, EpCAM, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgG1, LI-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin $\alpha\beta 3$, MORAb-009, MS4A1, MUC1, mucin CanAg, N- glycolylneuraminic acid, NPC-IC, PDGF-Ra, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF- β , TRAIL-R1, TRAIL-R2, tumor antigen CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, mesothelin, NKG2D, CD147, NKR2, B7H3, and vimentin, or wherein the target molecule is enriched in a tissue with blood cancer cell accumulation, wherein the tissue is lymphoid and/or bone marrow tissue, and wherein the target molecule is chosen from CD45, CD19, CD20, CD4, CD8, CD2, CCR4, CD58, CD28, CD23, CD69, CD25, CD33, CD123 and CCL-1. In certain embodiments, the target molecule is selected from Her2/neu, EGFRvIII, CD19, IL-13RR-a2, mesothelin, MUCI, EpCAM, GD2 and CEA. In certain embodiments, the target molecule is selected from Her2/neu, EGFRvIII, CD19, IL-13RR-a2, mesothelin, and MUCI. In certain embodiments, the

target molecule is selected from Her2/neu, EGFRvIII, and CD19. In an embodiment, the target molecule is Her2/neu.

[0028] In certain embodiments, the expression cassette encoding the copy of the disrupted endogenous gene comprises a transcription control element driving expression of the copy of the disrupted endogenous gene is bound by a transcription activator selected from the group consisting of Gal4-VP64, Notch, tet transactivator, NFAT, AP-1, NFκB/Rel, NR4A1 (Nur77), T-Bet, IRF4, STATs and eomesodermin (Eomes). In certain embodiments, the expression cassette is selected from Gal4-CD3-PGK-BFP, NR4A-CD3-PGK-BFP, and NFAT-CD3-PGK-BFP. In certain embodiments, the tumor sensing receptor and expression cassette are selected from SynNotch and Gal4-CD3-PGK-BFP, CAR and NR4A-CD3-PGK-BFP, and CAR and NFAT-CD3-PGK-BFP.

[0029] In certain embodiments, the endogenous gene disrupted is CD3ε, the target molecule is selected from Her2/neu, EGFRvIII, and CD19, and the tumor sensing receptor is SynNotch. In certain embodiments, the endogenous gene disrupted is CD3ε, the target molecule is selected from Her2/neu, EGFRvIII, and CD19, the tumor sensing receptor is SynNotch, and the expression cassette is Gal4-CD3-PGK-BFP. In an embodiment, the endogenous gene disrupted is CD3ε, the target molecule is Her2/neu, the tumor sensing receptor is SynNotch, and the expression cassette is Gal4-CD3-PGK-BFP.

[0030] A method of treating cancer in a patient is also provided. The method comprises administering T cells as described above or herein or prepared by any method described above and herein, to a patient in need thereof. In certain aspects, the T cells are tumor-activated alloreactive T cells which are alloreactive with respect to the patient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The accompanying drawings are incorporated in and constitute a part of this specification and illustrate various methods and compositions disclosed herein. However, the disclosure not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

[0032] **FIGURE 1** depicts four steps of generating tumor-activated alloreactive T cells disclosed herein.

[0033] FIGURES 2A-2E illustrate the generation and use of a tumor-activated alloreactive T-cell that uses a SynNotch-based tumor-sensing receptor and a CD3 ϵ expression cassette. FIGURE 2A depicts an illustration of an alloreactive T cell including a schematic of a T cell receptor (TCR)/CD3 complex. A TCR is a heterodimer of an alpha (α) chain and a beta (β) chain. The TCR/CD3 complex comprises two CD3 epsilon (ϵ) chains, a CD3 gamma (γ) chain, a CD3 delta (δ) chain, and two CD3 zeta (ζ) chains. The gray boxes indicate ITAM domains in the CD3 chains. ITAM: immunoreceptor tyrosine-based activation motif. The alloreactive T cell is genetically altered to disrupt the expression of CD3 ϵ (ϵ knockout) using techniques such as CRISPR-Cas9 to generate an activation-incompetent T cell. The ϵ knockout T cell is depicted in FIGURE 2B and the TCR/CD3 complex comprises a CD3 γ chain, a CD3 δ chain, and two CD3 ζ chains. The activation-incompetent T cell illustrated in FIGURE 2B is genetically altered by introducing a tumor-associated antigen (TAA)-specific tumor-sensing receptor (in this embodiment a SynNotch-based tumor-sensing receptor) into the T cell, and a CD3 ϵ expression cassette in the nucleus of the cell. TCE: transcription control element. ϵ gene: CD3 ϵ gene. These steps produce the tumor-activated alloreactive T-cell depicted in FIGURE 2C. FIGURE 2D depicts the activities of the tumor-activated alloreactive T-cell (depicted in FIGURE 2C) in the presence of a tumor cell expressing the tumor cell expressing the tumor antigen recognized by the TAA-specific tumor-sensing receptor. Upon binding of the tumor antigen and the TAA-specific tumor sensing receptor, the transcription activator is cleaved from the sensor and activates transcription of the CD3 ϵ gene in the expression cassette. Resulting production of CD3 ϵ polypeptides restores functional TCR/CD3 expression on the T cell, which can then recognize and bind to allo-peptide-HLA on the tumor cells. This results in T cell activation and tumor cell killing (the first kill). Expression of TCR/CD3 expression on the T cell is expected to persist for a period of time, although signaling from the tumor sensing receptor will start to decay once the tumor cell is killed. As depicted in FIGURE 2E, T cells may therefore be able to kill other HLA+ tumor cells or stromal cells in the vicinity. The alloreactivity of the T cell may be sustained by encountering other TAA-expressing tumor cells. After exiting the tumor, the T cells will lose alloreactivity due to the decay of SynNotch signaling and the loss of CD3 ϵ expression. FIGURES 2A-C illustrate *ex vivo* e, and FIGUES 2D and E illustrate *in vivo* activities.

[0034] **FIGURE 3** depicts data for cytotoxicity of alloreactive T cells to U266 myeloma cells. SK1: CD8-specific monoclonal antibody SK1. % killing: specific killing calculated as $[1 - (\text{sample activity}) / (\text{max activity})] \times 100$. Data represents Mean $\pm$ SD ($n = 3$).

[0035] **FIGURE 4** depicts data for expression of TCR β and CD3 ϵ on modified D10 cells and wild type D10 cells. FIGURE 4 depicts flow cytometry data for wild type D10 cells and for D10 cells with CD3 zeta (ζ) knocked out (D10- ζ -KO) using a gRNA with the target specific sequence 5'-ctcctgggaaccgcacgtgg-3' (SEQ ID NO: 14). Cells were stained with antibodies specific for TCR β and CD3 ϵ and analyzed using flow cytometry.

[0036] **FIGURE 5** depicts HLA-I expression on wild-type MDA-MB-231 cells and MDA-MB-231 cells with β 2m knocked out. MDA-MB-231: human breast cancer cell line. β 2m KO: beta 2 microglobulin knock out. WT: wild type. Cells were stained with FITC-labeled anti-HLA-I monoclonal antibody clone W6/32.

[0037] **FIGURE 6** depicts flow cytometry data for proliferation of T cells co-cultured with MDA-MB-231 cells (wild type or with beta 2 microglobulin knock out). MDA-MB-231: human breast cancer cell line. β 2m KO: beta 2 microglobulin knock out. WT: wild type. CFSE: carboxyfluorescein diacetate succinimidyl ester.

[0038] **FIGURE 7** depicts flow cytometry data for CD3 ϵ and anti-CD19 SynNotch expression in primary human CD8 $^{+}$ T cells genetically modified to comprise anti-CD19 SynNotch receptor and CD3 ϵ expression cassettes. The anti-CD19 SynNotch receptor contains a cleavable artificial transcription activator Gal4-VP64; expression of the anti-CD19 SynNotch receptor is driven by a constitutively active PGK-1 promoter. The CD3 ϵ expression cassette includes a blue fluorescence protein (BFP)-encoding sequence driven by a constitutively active PGK-1 promoter. The genetically modified T cells were stimulated by co-culture with MDA-MB-231 cells; anti-human CD28 antibody was added to the co-culture with MDA-MB-231 cells. Anti-CD19 SynNotch expression was analyzed by staining with PE-conjugated anti-MycTag (Cell Signaling Technology, clone 9B11) (X-axis). The presence of CD3 ϵ expression cassette in T cells were marked by the expression of blue fluorescence protein (BFP) (Y-axis). FIGURES 7A and B shows data for the genetically modified primary human CD8 $^{+}$ T cells stimulated with MDA-MB-231 cells. FIGURE 7C shows data for the cells modified as in FIGURE 7B after CD3 ϵ knockout.

[0039] **FIGURE 8** depicts flow cytometry data for CD3 ϵ and TCR β expression of human CD8+ T cell. CD3 ϵ expression was knocked out (KO) in primary human CD8+ T cells using CRISPR-Cas9, and CD3 ϵ knocked out (CD3-KO) cells were purified using magnetic separation. FIGURE 8A depicts data for wild type T cells and FIGURE 8B depicts data for
 5 CD3-KO T cells, three days after electroporation with CRISPR-Cas9 complex (hCD3 ϵ sgRNA Hs.Cas9.CD3E.1.AC and Alt-R Sp Cas9 Nuclease V3). FIGURE 8C depicts data for purified CD3-KO cells.

[0040] **FIGURE 9** depicts a time course of flow cytometry data assessing proliferation of T cells subjected to CD3 ϵ knockout (CD3-KO cells). Primary human T cells were subjected
 10 to CD3 ϵ knockout via CRISPR. After knockout, the CD3 $^+$ T cells (i.e., wild type T cells) were not removed from the mixture. The mixed WT and CD3-KO T cells were cultured for 25 days and periodically assessed for CD3 ϵ and TCR β expression.

[0041] **FIGURE 10** depicts CD19 expression on wild type MDA-MB-231 cells (MDS-MG-231 WT) and modified MDA cells (MDA-MB-231-CD19).

15 [0042] **FIGURE 11** depicts flow cytometry data illustrating engagement of anti-CD19 SynNotch restores CD3 expression on CD3KO-19SN- ϵ CS T cells and enables the cells to activate in response to anti-CD3 antibody stimulation in terms of degranulation (CD107a) and IFN γ production. Cells were gated on BFP+ population.

[0043] **FIGURE 12** depicts data for cytotoxicity of alloreactive CD3KO-19SN- ϵ CS T cells to MDA-MB-231 cells. Alloreactive CD3KO-19SN- ϵ CS T cells display significant higher cytotoxicity toward MDA-MB-231 cells expressing CD19 (MDA-MB-231-CD19-luci) than toward WT MDA-MB-231 cells (MDA-MB-231-luci). Data are represented as mean $\pm$ SD ($n = 3$). ***: $P < 0.001$, Student's t-test.
 20

[0044] **FIGURE 13** depicts data for cytotoxicity of alloreactive wide type (WT) and
 25 CD3 ϵ knockout (CD3KO) T cells to MDA-MB-231 cells. Data are represented as mean $\pm$ SD ($n = 3$). ***: $P < 0.001$, Student's t-test.

[0045] **FIGURE 14** illustrates a CAR-based tumor sensing receptor that restores the expression of CD3 ϵ in an alloreactive CD3O T cell through signaling pathways and the activation of the transcription factor NFAT. The transcription control element (TCE) in the

CD3 expression cassette consists of NFAT binding sequences. CD3 expression cassettes with TCEs containing binding sequences for other activated transcription factors NR4A, NFκB and AP-1 can also be used. The re-expression of CD3ε restores the surface expression of TCR/CD3 complex.

5 [0046] **FIGURE 15** depicts a CD3ε expression cassette containing sequences for constitutive BFP expression. The first (left) part of the cassette drives the inducible expression of CD3ε, consisting of multiple copies of trans activator binding sites, followed by a minimal IL2 promoter and a CD3ε coding sequence. The second (right) part of the cassette drives the constitutive expression of BFP, consisting of a PGK-1 promoter followed
10 by a BFP coding sequence. The constitutive expression of GFP can be used as a marker for the presence of the whole cassette in the cell.

DETAILED DESCRIPTION

[0047] The present disclosure is directed to a tumor-activated alloreactive or xenoreactive T cell, a method of making the tumor-activated alloreactive or xenoreactive T cell, and
15 methods of using the tumor-activated alloreactive or xenoreactive T cell.

Definitions & Abbreviations

[0048] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It is also to be understood that the terminology used herein is for the
20 purpose of describing particular embodiments only, and is not intended to be limiting.

[0049] The following abbreviations/acronyms have the following meanings unless otherwise specified:

	cDNA	complementary DNA
	DNA	deoxyribonucleic acid
25	EDTA	ethylenediaminetetraacetic acid
	kDa	kiloDalton
	MW	molecular weight
	NCBI	National Center for Biotechnology Information
	PEG	polyethyleneglycol
30	pI	isoelectric point
	ppm	parts per million
	PVA	poly(vinyl alcohol)
	PVP	poly(vinylpyrrolidone)

	RNA	ribonucleic acid
	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
	w/v	weight/volume
	w/w	weight/weight
5	v/v	volume/volume
	wt%	weight percent
	°C	degrees Centigrade
	H ₂ O	water
	dH ₂ O or DI	deionized water
10	dH ₂ O	deionized water, Milli-Q filtration
	g or gm	grams
	µg	micrograms
	mg	milligrams
	kg	kilograms
15	µL and µl	microliters
	mL and ml	milliliters
	mm	millimeters
	µm	micrometer
	M	molar
20	mM	millimolar
	µM	micromolar
	U	units
	sec	seconds
	min(s)	minute/minutes
25	hr(s)	hour/hours
	ETOH	ethanol
	eq.	equivalents
	N	normal

30 [0050] Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry, and nucleic acid chemistry and hybridization are those well known and commonly employed in the art.

[0051] Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally performed according to conventional methods in the art and various general references (e.g., Sambrook and Russell, 2001, MOLECULAR CLONING, A LABORATORY APPROACH, Cold Spring Harbor Press, Cold Spring Harbor, NY, and Ausubel et al., 2002, Current Protocols in Molecular Biology, John Wiley & Sons, NY), which are provided throughout this document.

[0052] The nomenclature used herein and the laboratory procedures used in analytical chemistry and organic syntheses described below are those well known and commonly

employed in the art. Standard techniques or modifications thereof, are used for chemical syntheses and chemical analyses.

[0053] As used herein, each of the following terms has the meaning associated with it in this section.

5 [0054] The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

[0055] It must be noted that as used herein, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to
10 “an antibody” includes a plurality of such antibodies and reference to “the dosage” includes reference to one or more dosages and equivalents thereof known to those skilled in the art, and so forth.

[0056] “About” as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of +/- 20% or +/- 10%,
15 more preferably +/- 5%, even more preferably +/- 1%, and still more preferably +/- 0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

[0057] As used herein, the term “autologous” is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

[0058] An “effective amount” as used herein, means an amount of a therapeutic
20 compounds or combination thereof, when administered to a patient suffering from a malignancy provides a therapeutic benefit in alleviating one or more manifestations of the malignancy. It is understood, however, that the full therapeutic effect does not necessarily occur by administration of one dose, and may occur only after administration of a series of doses. Thus, an effective amount may be administered in one or more administrations. In the
25 context of therapeutic or prophylactic applications, the amount of active agent administered to the subject will depend on the type and severity of the disease or condition and on the characteristics of the subject, such as general health, age, sex, body weight and tolerance to drugs. It will also depend on the degree, severity and type of disease or condition. The

skilled artisan will be able to determine appropriate dosages depending on these and other factors.

[0059] As used herein, the term “individual” or “patient” or “subject” (as in the subject of the treatment) refers to any animal (e.g., a mammal), including, but not limited to humans, non-human primates, rodents, and the like. The individual is, in one embodiment, a human being. Typically, the terms “individual”, “subject” and “patient” are used interchangeably herein in reference to a human subject.

[0060] As used herein, the term “donor” refers to the individual person or animal from whom the T cells to be manipulated and used as therapeutics are obtained.

10 [0061] As used herein, a “recipient” refers to an individual, patient, or subject to whom the T-cells of the disclosure are intended to be administered and/or who receives the T-cells of the disclosure. A “recipient” may refer to a category of individual, patient or subject having a common characteristic, such as a particular HLA profile.

15 [0062] The term “HLA mismatched” refers to the condition that the HLA alleles expressed in the tissues of a first individual person are different from those in the tissues of a second individual person. The term specifically refers to the alleles in the highly polymorphic loci HLA-A, HLA-B, HLA-C, DRB1, DPA1, DPB1, DQA1, and DQB1.

[0063] The term “allogeneic” refers to the source of T cells used for manipulation and therapy are taken from a person other than the patient.

20 [0064] The term “alloreactive” refers to the ability of certain T cells of an individual person to react to cells and tissues of another individual person with mismatched HLA through TCR recognition of mismatched HLA and antigens presented by the mismatched HLA molecules.

25 [0065] The term “xenogeneic” refers to the source of T cells used for manipulation and therapy are taken from a non-human animal.

[0066] The term “xenoreactive” refers to the ability of certain T cells from an individual of a certain species to react to the cells and tissues of an individual of a different species through TCR recognition of mismatched HLA and antigens presented by mismatched HLA molecules.

[0067] As used herein, a “normal subject” or “control subject” refers, depending on the context, to a subject not suffering from a malignancy.

[0068] As used herein, a “control sample” refers to a sample from a control subject or a sample representative of a population of control subjects.

- 5 [0069] To “treat” a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject. Treating may include the postponement of further disease progression, or reduction in the severity of symptoms that have or are expected to develop, ameliorating existing symptoms and preventing additional symptoms.
- 10 [0070] An “antibody” (Ab) shall include, without limitation, an immunoglobulin which binds specifically to an antigen and comprises at least two heavy (H) chains and two light (L) chains interconnected by disulfide bonds, or an antigen-binding portion thereof. Each H chain comprises a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region comprises three constant domains, CH1,
- 15 CH2 and CH3. Each light chain comprises a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region comprises one constant domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL
- 20 comprises three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The antibodies may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, intracellular antibodies (“intrabodies”), Fv, Fab and F(ab)₂, as well as
- 25 single chain antibodies (scFv), heavy chain antibodies, such as camelid antibodies, and humanized antibodies (Harlow et al., 1999, *Using ANTIBODIES: A LABORATORY MANUAL*, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, *ANTIBODIES: A LABORATORY MANUAL*, Cold Spring Harbor, New York; Houston et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-5883; Bird et al., 1988, *Science* 242:423-426).

[0071] An “antigen binding portion” of an Ab (also called an “antigen-binding fragment”) or antigen binding portion thereof refers to one or more sequences of an Ab (full length or fragment of the full length antibody) that retain the ability to bind specifically to the antigen bound by the whole Ab. Examples of an antigen-binding fragment include intrabody,
5 bispecific antibody, Fab, F(ab')₂, scFv (single-chain variable fragment), Fab', dsFv, sc(Fv)₂, and scFv-Fc.

[0072] The term “nanobody” (Nb), as used herein, refers to the smallest antigen binding fragment or single variable domain (V_{HH}) derived from naturally-occurring heavy chain antibody and is known to the person skilled in the art. They are derived from heavy chain
10 only antibodies, seen in camelids (Hamers-Casterman et al., 1993; Desmyter et al., 1996). In the family of “camelids” immunoglobulins devoid of light polypeptide chains are found. “Camelids” comprise old world camelids (*Camelus bactrianus* and *Camelus dromedarius*) and new world camelids (for example, *Llama paccos*, *Llama glama*, *Llama guanicoe* and *Llama vicugna*). A single variable domain heavy chain antibody is referred to
15 herein as a nanobody or a V_{HH} antibody

[0073] As used herein, the term “variable domain” refers to immunoglobulin variable domains defined by Kabat et al., SEQUENCES OF IMMUNOLOGICAL INTEREST, 5th ed., U.S. Dept. Health & Human Services, Washington, D.C. (1991). The numbering and positioning of CDR amino acid residues within the variable domains is in accordance with the well-
20 known Kabat numbering convention. VH, “variable heavy chain” and “variable heavy chain domain” refer to the variable domain of a heavy chain. VL, “variable light chain” and “variable light chain domain” refer to the variable domain of a light chain.

[0074] A “humanized” antibody refers to an Ab in which some, most or all of the amino acids outside the CDR domains of a non-human Ab are replaced with corresponding amino
25 acids derived from human immunoglobulins. In one embodiment of a humanized form of an Ab, some, most or all of the amino acids outside the CDR domains have been replaced with amino acids from human immunoglobulins, whereas some, most or all amino acids within one or more CDR regions are unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they do not abrogate the ability of
30 the Ab to bind to a particular antigen. A “humanized” Ab retains an antigenic specificity similar to that of the original Ab.

[0075] By the term "synthetic antibody," as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

[0076] Reference to the wild-type protein is understood to include the mature form of the protein. A "mature" polypeptide means a wild-type polypeptide sequence from which a signal sequence has been cleaved during expression of the polypeptide. The mature protein can be a fusion protein between the mature polypeptide and a signal sequence polypeptide.

[0077] The term "variant," with respect to a polypeptide, refers to a polypeptide that differs from a specified wild-type, parental, or reference polypeptide in that it includes a man-made substitution, insertion, or deletion at one or more amino acid positions. Similarly, the term "variant," with respect to a polynucleotide, refers to a polynucleotide that differs in nucleotide sequence from a specified wild-type, parental, or reference polynucleotide. The identity of the wild-type, parental, or reference polypeptide or polynucleotide will be apparent from context.

[0078] The term "recombinant," when used in reference to a subject cell, nucleic acid, protein or vector, indicates that the subject has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell, or express native genes at different levels or under different conditions than found in nature.

[0079] As used herein, the term "purified" refers to material (*e.g.*, an isolated polypeptide or polynucleotide) that is in a relatively pure state, *e.g.*, at least about 90% pure, at least about 95% pure, at least about 98% pure, or even at least about 99% pure.

[0080] As used herein, the term "amino acid sequence" is synonymous with the terms "polypeptide," "protein," and "peptide," and are used interchangeably. Where such amino

acid sequences exhibit activity, they may be referred to as an “enzyme.” The conventional one-letter or three-letter codes for amino acid residues are used, with amino acid sequences being presented in the standard amino-to-carboxy terminal orientation (*i.e.*, N→C).

[0081] The term “nucleic acid” encompasses DNA, RNA, heteroduplexes, and synthetic molecules capable of encoding a polypeptide. Nucleic acids may be single stranded or double stranded, and may be chemical modifications. The terms “nucleic acid” and “polynucleotide” are used interchangeably. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present compositions and methods encompass nucleotide sequences that encode a particular amino acid sequence. Unless otherwise indicated, nucleic acid sequences are presented in 5'-to-3' orientation.

[0082] “Encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

[0083] As used herein, “hybridization” refers to the process by which one strand of nucleic acid forms a duplex with, *i.e.*, base pairs with, a complementary strand, as occurs during blot hybridization techniques and PCR techniques. Stringent hybridization conditions are exemplified by hybridization under the following conditions: 65°C and 0.1X SSC (where 1X SSC = 0.15 M NaCl, 0.015 M Na₃ citrate, pH 7.0). Hybridized, duplex nucleic acids are characterized by a melting temperature (T_m), where one half of the hybridized nucleic acids are unpaired with the complementary strand. Mismatched nucleotides within the duplex lower the T_m . A nucleic acid encoding a variant α -amylase may have a T_m reduced by 1°C – 3°C or more compared to a duplex formed between the nucleotide of SEQ ID NO: 2 and its identical complement.

[0084] As used herein, a “synthetic” molecule is produced by *in vitro* chemical or enzymatic synthesis rather than by an organism.

[0085] As used herein, the terms “transformed,” “stably transformed,” and “transgenic,” used with reference to a cell means that the cell contains a non-native (e.g., heterologous) nucleic acid sequence integrated into its genome or carried as an episome that is maintained through multiple generations.

[0086] The term “introduced” in the context of inserting a nucleic acid sequence into a cell, means “transfection,” “transformation” or “transduction,” as known in the art.

[0087] The term “heterologous” with reference to a polynucleotide or protein refers to a polynucleotide or protein that does not naturally occur in a host cell.

[0088] The term “endogenous” with reference to a polynucleotide or protein refers to a polynucleotide or protein that occurs naturally in the host cell.

[0089] As used herein, the term “expression” refers to the process by which a polypeptide is produced based on a nucleic acid sequence. The process includes both transcription and translation.

[0090] A “selective marker” or “selectable marker” refers to a gene capable of being expressed in a host cell to facilitate selection of host cells carrying the gene. Examples of selectable markers include but are not limited to antimicrobials (*e.g.*, hygromycin, bleomycin, or chloramphenicol) and/or genes that confer a metabolic advantage over cells that lack the metabolic gene, such as a nutritional advantage on the host cell.

[0091] A “vector” refers to a polynucleotide sequence designed to introduce nucleic acids into one or more cell types. Examples of vectors include but are not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term “vector” includes an autonomously replicating plasmid or a virus. The term is also construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Vectors include cloning vectors, expression vectors, shuttle vectors, plasmids, phage particles, cassettes and the like.

[0092] An “expression vector” refers to a DNA construct comprising a DNA sequence encoding a polypeptide of interest, which coding sequence is operably linked to a suitable control sequence capable of effecting expression of the DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control transcription, a sequence encoding suitable ribosome binding sites on the mRNA, enhancers and sequences which control termination of transcription and translation. A control sequence is also referred to herein as a transcription control element (“TCE”). Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

[0093] The term “operably linked” means that specified components are in a relationship (including but not limited to juxtaposition) permitting them to function in an intended manner. For example, a regulatory sequence is operably linked to a coding sequence such that expression of the coding sequence is under control of the regulatory sequences.

[0094] Unless otherwise specified, a “nucleotide sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

[0095] The term “linker”, also referred to as a “spacer” or “spacer domain” as used herein interchangeably, refers to a an amino acid or sequence of amino acids that that is optionally located between two amino acid sequences in a fusion protein.

[0096] A “signal sequence” is a sequence of amino acids attached to the N-terminal portion of a protein, which facilitates the secretion of the protein outside the cell. The mature form of an extracellular protein lacks the signal sequence, which is cleaved off during the secretion process.

[0097] As used herein, “biologically active” refer to a sequence having a specified biological activity, such an enzymatic activity.

[0098] “Percent sequence identity” means that a variant has at least a certain percentage of amino acid residues identical to a wild-type protein, when aligned using the BLAST algorithm with default parameters. A variant with five amino acid substitutions or deletions of a 617 amino acid polypeptide, for example, would have a percent sequence identity of 99% (612 / 617 identical residues $\times$ 100, rounded to the nearest whole number) relative to the mature polypeptide. Such a variant would be encompassed by a variant having “at least 99% sequence identity” to the mature polypeptide.

[0099] As used herein, the term “fusion protein” or “fusion polypeptide” is a polypeptide comprised of at least two polypeptides, optionally also comprising a linking sequence, and that are operatively linked into one continuous protein. The two polypeptides linked in a fusion protein are typically derived from the at least two independent sources (i.e., not from the same parental polypeptide), and therefore a fusion protein comprises the at least two linked polypeptides not normally found linked in nature. Typically, the at least two polypeptides can be operably attached directly by a peptide bond, or may be connected by a linking group, such as a spacer domain. An example of a fusion polypeptide is a polypeptide that functions as a receptor for an antigen, wherein an antigen binding polypeptide forming an extracellular domain is fused to a different polypeptide, forming a “chimeric antigen receptor”. Also contemplated herein are fusion proteins comprising 3, 4, 5, 6, 7, 8, 9, or 10 or more heterologous polypeptides.

[00100] As used herein, “abrogating the expression” of a gene refers to the disruption the expression of the gene.

[00101] As used herein “abrogating the function” of a gene product refers to disrupting the function and activity of the gene product.

[00102] Ranges: throughout this disclosure, various aspects of the disclosure can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosure. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1

to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

[00103] An “isolated” compound as used herein means that the compound is removed from at least one component with which the compound is naturally associated with in nature.

[00104] As envisioned in the present invention with respect to the disclosed compositions of matter and methods, in one aspect the embodiments of the invention comprise the components and/or steps disclosed herein. In another aspect, the embodiments of the invention consist essentially of the components and/or steps disclosed herein. In yet another aspect, the embodiments of the invention consist of the components and/or steps disclosed herein.

DESCRIPTION

[00105] Embodiments of the present invention are described below. It is, however, expressly noted that the present invention is not limited to these embodiments, but rather the intention is that modifications that are apparent to the person skilled in the art and equivalents thereof are also included.

[00106] This disclosure exploits the alloreactivity or xenoreactivity of allogeneic T cells to broadly target cancer cells and stromal cells in tumors or tissues with blood cancer cell accumulation. Each T cell naturally expresses a unique type of T cell receptor (TCR) on the cell surface to recognize a specific antigen on target cells. In humans, the antigen is a short peptide (~10 amino acid residues) in complex with a cell surface protein termed human leukocyte antigen (HLA). HLA class I (HLA-I) proteins comprise an α heavy chain and a β 2 microglobulin (β 2M). Only the α -chain participates in peptide binding and TCR interaction. The α chain is encoded by three gene loci: HLA-A, B, and C, leading to the expression of three gene products on cell surfaces. All three loci are highly polymorphic, each with hundreds to thousands of different alleles among the human population. HLA-I molecules are expressed on virtually all human cells including many tumor cells. Peptide-HLA-I antigens are recognized by TCRs expressed on CD8⁺ cytotoxic T cells, which kill antigen-expressing cells upon activation. HLA class II (HLA-II) molecules are $\alpha\beta$ heterodimers and both chains take part in peptide presentation and TCR binding. HLA-II molecules are encoded by six gene loci

(HLA-DRA1, DRB1, DPA1, DPB1, DQA1 and DQB1), all of which are high polymorphic except DRA1. HLA class II are mostly expressed on B cells and specialized antigen presenting cells (APCs) but can also be induced on epithelial cells and on a variety of solid tumor cells (20). Peptide-HLA-II antigens activate CD4⁺ helper T cells, which release inflammatory cytokines and help the survival and function of CD8⁺ T cells. Due to the many polymorphisms in each HLA gene locus in the human population, it is rare for any two individuals to express the same set of HLA genes. A significant proportion (1-10%) of T cells of an individual can recognize and respond to cells from another individual through TCR recognition of mismatched HLAs and the antigen presented by the mismatched HLA molecules (21, 22). T cell alloreaction, i.e., reaction to cells and tissues from a different individual of the same species, plays a critical role in organ transplant rejection through host vs. graft (HvG) activity (23). If the recipient of transplantation is immunocompromised and cannot eliminate T cells from the graft through HvG, the grafted T cells may attack the recipient's tissues and cause severe, sometimes lethal graft vs host (GvH) disease (24). For these reasons, close matches in HLA genes between the organ donor and the recipient are necessary for successful transplantation. Matches at the HLA-A, HLA-B, HLA-C and HLA-DRB1 loci have been found to be most critical for successful transplantation, suggesting their higher degree of involvement in alloreactions than HLA-DP and HLA-DQ (25). Because of the diploid nature of human genome, a match at all eight HLA-A, HLA-B, HLA-C and HLA-DRB1 loci (8/8 match) has the best chance of success in transplantation. T cell reaction to cells from a different species, i.e., xenoreaction, tends to be stronger than alloreaction (26) and is the main roadblock for using animals such as pigs as sources of organs for human transplantation. It should be noted that the term "allogeneic" refers to the source of T cells being from an individual that is different from the recipient. "Alloreactive" may refer to the reactivity of transplanted allogeneic T cells to the recipient's tissues in GvH or the reactivity of the recipient's T cells to transplanted tissues, including transplanted allogeneic T cells, in HvG. Not all allogeneic T cells have alloreactivity to the recipient's tissues. On the other hand, the alloreactive T cells used for cancer treatment in this disclosure are all allogeneic in nature. Since the T cells of this disclosure are administered to a recipient with cancer, the terms "recipient" and "patient" are used interchangeably depending on the context.

[00107] Alloreactive T cells have been used for treating cancer in special settings. Donor lymphocyte infusion (DLI) is a well-established treatment for patients who have received an

allogeneic stem cell transplant for a hematological malignancy but have residual disease. In such cases, the patient receives strong chemotherapy or radiation therapy to kill cancer cells. Stem cells from a donor with partially matched HLAs are used to restore normal hematopoietic activity damaged by the radiation or chemotherapy. T cells from the same donor are then
5 infused to eliminate residual cancer cells through alloreaactions. Approximately 70% of these patients develop GvH diseases, which is correlated with lower risk of relapse of their malignancy (27, 28). A unique factor in this case is that the infused allogeneic T cells are unlikely to be eliminated by the patient through HvG because patient T cells are derived from stem cells of the same donor and tolerated to donor HLAs.

10 [00108] Using alloreactive or xenoreactive T cells to treat tumors therefore must overcome two main hurdles. The first is to avoid or minimize the patient's HvG activities to the allogeneic and xenogeneic T cells in order to allow the cells to survive and execute anti-tumor activities. In this regard, lympho-depletion caused by radiation or chemotherapy received by cancer patients compromises the patients' immune system and may create a window of reduced HvG
15 activities for treatment using allogeneic T cells (29). Progresses have also been made in making allogeneic T cells "stealthy" to the recipient's immune system. Using gene-editing technologies such as CRISPR-Cas9, HLA expression can be knocked out in allogeneic T cells, making them "invisible" to recipients' T cells (30, 31). The cells can be further modified to express non-classical HLA-I molecules such as HLA-E and HLA-G to protect them from
20 natural killer (NK) cells (32), which kill cells that do not express any HLA. The second hurdle is to restrict the destructive alloreactivity or xenoreactivity of T cells to tumor sites so that damages to normal tissues are minimized and severe GvH diseases are avoided.

[00109] This disclosure describes a method of generating genetically modified T cells with alloreactivities or xenoreactivities restricted to tumor sites or tissues with blood cancer cell
25 accumulation. The method comprises abrogating the ability of T cells to activate through TCR and introducing a mechanism that restores the ability at the tumor sites in response to molecular cues enriched on tumor cells or in the tumor microenvironment. This disclosure further describes the genetically modified T cells made and therapeutic uses thereof.

I. Method of generating Tumor-activated Alloreactive or Xenoreactive T cells

[00110] The disclosure describes the methods of generating tumor-activated alloreactive or xenoreactive T cells and their use in treating patients with cancer. The T cells are generated in four main steps (FIG. 1, depicting alloreactive T cells only for simplicity).

5 [00111] The first step is T cell collection. T cells are isolated from the blood of an HLA mismatched donor. The degree of HLA mismatch is determined by comparing the alleles of the donor and the recipient at the HLA-A, HLA-B, HLA-C, DRB1, DPA1, DPB1, DQA1 and DQB1 loci. Donors with at least one HLA allele mismatched with that of the recipient or patient are selected. Preferably the mismatch allele is at one of the HLA-A, HLA-B, HLA-C, DRB1 loci. Donors with mismatches at higher numbers of loci are preferred for strong T cell
10 alloreactivities. Xenogeneic T cells are isolated from an animal such as a pig. Total T cells as a mixture of CD4⁺ and CD8⁺ T cells, CD4⁺ T cells alone, or CD8⁺ T cells alone, can be isolated from the donor's blood using conventional methods known in the art or by using commercially available kits using purification columns or magnetic beads.

[00112] The second step is stimulation, which activates the T cells and drives them to
15 proliferate (i.e., drive the cells into cell cycle). This step aids genetic manipulation of the T cells in the following steps and expands the T cells. T cells can be stimulated nonspecifically or specifically. For nonspecific stimulation, T cells can be cultured with anti-CD3 and antibody and anti-CD28 antibodies coated on beads or plastic surfaces to activate all T cells regardless of their alloreactivity or xenoreactivity. For specific stimulation, T cells can be co-cultured
20 with cells from the patient, including peripheral blood mononuclear cells (PBMCs), cultured monocyte-derived dendritic cells (DCs), and cells isolated from resected tumors. Alternatively, T cells can be cultured with PBMCs or DCs from another donor who shares at least one common HLA allele the patient. Alloreactive T cells proliferate in response to PBMC from an HLA-mismatched individual as a result of T cell stimulation by antigen presenting cells (APCs)
25 in PBMCs through TCR-HLA interaction. This is the basis for mixed lymphocyte reaction (MLR), which has been used to determine the alloreactivity of T cells since 1964 (33, 34). To expand alloreactive T cells for the current disclosure, T cells from an HLA-mismatched donor can be labeled with the cell division tracking fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) and cultured with PBMCs from the patient. Prior to the culture, the patient
30 PBMCs are treated with irradiation (2,500 rads) or chemotherapy drugs, such as mitomycin C to abrogate their proliferation potential. Proliferated donor T cells with low CFSE levels are sorted using flow cytometry and used as the source of alloreactive T cells for downstream

genetic manipulations. The efficiency of alloreactive T cell expansion can be increased by using DCs. As professional antigen presenting cells (APCs), DCs express high levels of HLA-I, HLA-II and a host of costimulatory molecules such as CD40, CD80 and CD86. T cells expanded by DCs may also strongly activate T cells through engaging TCR and costimulatory receptors such as CD28. Monocyte-derived DCs (MoDCs) have been tested for immunotherapies against cancer, autoimmune and other disease for decades. As of August 2019, 120 human clinical trials were listed on ClinicalTrials.gov. Generation of MoDCs involves culturing enriched monocytes from an apheresis collection of PBMCs in the presence of granulocyte-macrophage colony-stimulation factor (GM-CSF) and interleukin-4 (IL-4) (35, 36). The processes of enriching monocyte, generating and cryopreserving MoDCs are well established and a number of commercial kits are available for generating clinical grade MoDCs (37, 38).

[00113] T cells can also be expanded by culturing with cell lines that express HLA proteins encoded by at least one of the patient's HLA alleles or artificial surfaces such as plastics that are coated with HLA proteins encoded by at least one of the patient's HLA alleles. Specific stimulation leads to selective activation and expansion of T cells that are alloreactive or xenoreactive to patient HLAs. Stimulated and expanded T cells can be cryo-preserved, for instance in DMSO at a suitable percentage, such as 10% DMSO or 7.5% DMSO, and thawed later for downstream genetic manipulations. The activation step may be omitted if genetic manipulations in the following steps can be achieved without T cell activation and T cell activation and expansion after infusion into the patients are preferred.

[00114] The third step is to abrogate the ability of T cells to activate through TCR signaling, thus making the T cells activation-incompetent. This is achieved through disrupting the expression or function of at least one molecule that is necessary- for TCR signaling and T cell activation (Fig. 1 and Figs. 2A and 2B). For instance, the expression of a gene encoding a critical protein can be disrupted using gene-editing technologies such as CRISPR-Cas9, transcription activator-like effector nuclease (TALENs), megaTALS, zinc-finger nucleases, or homing endonucleases (33). The disruption occurs on both copies of the target gene in a cell, leading to complete lack of expression in the cell and its progenies. In recent years, CRISPR-cas9 has become a routine technology in research labs and has seen numerous clinical applications (34) including in immunotherapies (35). A myriad of commercial products are available for guide RNA (gRNA) design and synthesis, recombinant cas9 proteins, and tools

for delivering gRNA and cas9 into many cell types. For primary human T cells, electroporation of gRNA-cas9 ribonucleoprotein (RNP) has become the method of choice for highly efficient gene knockout (36, 37). Genes that encode polypeptides that are necessary for TCR signaling and T cell activation can be transmembrane proteins. Exemplary transmembrane proteins expressed on the plasma membrane include CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58. Genes that encode polypeptides that are necessary for TCR signaling and T cell activation can encode intracellular signaling molecules involved in TCR signaling and T cell activation. Exemplary intracellular signaling molecules include but are not limited to Lck, Zap70, calcineurin, PI3K, Fyn, PLC γ , SLP76, PKC θ , AKT, NcK, and PDK1. The sequences of these exemplary molecules are readily available in public databases, such as National Institutes of Health GenBank® (U.S. Department of Health and Human Services) and UniProt. A number of free online tools are available for designing gRNA sequences to target any specific gene in the human genome. For example: URL:https://www.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM, URL:<https://zlab.bio/guide-design-resources>, and URL:<https://www.synthego.com/products/bioinformatics/crispr-design-tool>.

[00115] The fourth step is to generate tumor-activated alloreactive or xenoreactive T cells by equipping the activation-incompetent T cells with the ability to restore activation competency at tumor sites (FIG. 1 and FIG. 2C). This is achieved by introducing a tumor-sensing receptor and an expression cassette for the disrupted gene. The tumor-sensing receptor comprises an extracellular domain that binds to molecules enriched on tumor cells or in the tumor microenvironment and leads to the release or activation of a transcription activator from the intracellular domain. The extracellular domain can be, for instance, a single chain variable fragment (scFv), a Fab fragment, a designed ankyrin repeat protein (DARPin), a nanobody, a TCR, an Fc receptor, a growth factor receptor, a chemokine receptor, or a hormone receptor. The types of molecules the receptors recognize include, for example, tumor associated antigens (TAAs) expressed on tumor cells, including CD19, CD20, CD38, CD30, Her2/neu, ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2 (VEGFR2), high molecular weight- melanoma associated antigen (HMW-MAA), MAGE-A1, IL-13R-a2, GD2, and the like. Cancer-associated antigens also include,

e.g., 4-1BB, 5T4, adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), C-MET, CCR4, CD152, CD19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4, DRS, EGFR,

5 EpCAM, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgG1, LI-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin $\alpha\beta 3$, MORAb-009, MS4A1, MUC1, mucin CanAg, N- glycolylneuraminic acid, NPC-IC, PDGF-Ra, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, SCH 900105,

10 SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF- β , TRAIL-RI, TRAIL-R2, tumor antigen CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, mesothelin, NKG2D, CD147, NKR2, B7H3, and vimentin. The tumor-sensing receptor can also recognize soluble factors enriched in the tumor microenvironment such as chemokines (38), growth factors, and growth hormone (39). In an alternative embodiment, the tumor sensing receptor recognizes

15 molecules enriched in tissues with accumulation of blood cancer cells. For example, acute myeloid leukemia (AML) and myeloma cells tend to accumulate in bone marrow and lymphoid organs. The tumor sensing receptor may recognize molecules expressed on hematopoietic cells, especially lymphocytes that are abundant in these tissues. These molecules include CD45, CD19, CD20, CD4, CD8, CD2, CCR4, CD58, CD28, CD23,

20 CD69, CD25, CD33, CD123 and CCL-1.

[00116] The expression cassette comprises DNA sequences of a promoter-like transcriptional control element (TCE) operably linked to a DNA sequence encoding a copy of the disrupted gene (FIG. 15). Binding of the transcription activator to the transcriptional control element drives the expression of the gene disrupted in the third step and restores the T cell's

25 ability to activate and respond to antigens. Specifically, the TCE comprises multiple binding sites for the transactivation activator and a minimal promoter. Downstream of the TCE is the coding sequence of the gene disrupted in step 3. The coding sequence should encode the polypeptide of the gene but needs to be modified so that the gRNA used to disrupt the original gene can no longer function. This can be achieved by creating multiple silent mutations on the

30 sequence targeted by the gRNA, especially the PAM sequence. The expression cassette can additionally contain a coding sequence for a fluorescence protein such as BFP driven by a

constitutively active promoter. The expression of the fluorescence protein serves as an indicator for the existence of the expression cassette in the cell.

[00117] Two types of engineered receptors can be employed as tumor-sensing receptors. The first type releases a transcription activator from the intracellular domain upon ligand binding. The transcription activator then translocates to the nucleus where it activates the expression cassette. These receptors include, by way of example, synthetic Notch (SynNotch) (14, 40) and (US patent publication US20160264665A1 and US Patent No. 9834608B2), Modular Extracellular Sensor Architecture (MESA) (41) (US patent publication US20140234851A1), and Tango (42). A SynNotch receptor exploits the ability of Notch to, upon ligand binding, cleaves and release its intracellular domain, which acts as a transcription factor after translocating to the nucleus. A SynNotch receptor is constructed by replacing the extracellular recognition domain of the Notch with a binding domain for a specific target and replacing intracellular domain with a transcription activator that can bind to the TCE in the expression cassette and drive the expression of gene of interest. DNA constructs for the patented SynNotch receptors with the Gal4-VP64 transcription activator and extracellular binders for CD19 (Addgene Cat. #79125) and Her2 (Addgene Cat. #85424) can be readily modified to construct SynNotch receptors with other binding specificities and/or transcription activators. DNA construct for the patented expression cassette with the Gal4-VP64 TCE is also available from Addgene (Cat. #79123) and can be used to for expression of gene of interest in response to SynNotch activation. A CD3 ϵ expression cassette (Gal4-CD3-PKG-BFP) with an additional BFP coding sequence controlled by a constitutively active PGK-1 promoter is shown in SEQ ID NO: 1. Since SynNotch activation relies on surface-anchored ligands, it can be used to sense tumor antigens expressed on tumor cell surfaces such as Her2.

[00118] MESA can be used to sense soluble factors in the tumor microenvironment such as vascular endothelial growth factor (VEGF) or soluble tumor antigen shed from tumor cells. Target binding leads to receptor dimerization, which brings a protease on the intracellular domain of one monomer close to its substrate sequence on the other monomer. Cleavage of the substrate sequence leads to the release of a transcription activator linked to the cytosolic domain of the second monomer through the substrate sequence.

[00119] Tango is similar to MESA except that the protease is linked to an intracellular signaling molecule that is recruited to the receptor intracellular domain upon ligand binding.

Therefore, Tango can be used to sense a variety of soluble factors in the tumor microenvironment, including chemokines (38), growth factors, and growth hormone (39).

[00120] The second type of engineered tumor sensing receptors activates an endogenous transcription factor in T cells through signaling pathways. For example, a typical CAR with intracellular Immunoreceptor tyrosine-based activation motif (ITAM) domains can, in response to ligand binding, initiate multiple signaling pathways that lead to the activation of transcription factors NFAT, AP-1, NFκB/Rel, or NR4A1 (Nur77) (FIG. 14). Expression cassettes using TCE sequences containing binding sequence of these transcription factors to drive the expression of luciferase or GFP have been widely used in T cell activation reporter systems. The expression cassettes NFAT-CD3-PKG-BFP (SEQ ID NO: 2) AP1-CD3-PKG-BFP (SEQ ID NO: 3), NFκB-CD3-PKG-BFP (SEQ ID NO: 4) and NR4A-CD3-PKG-BFP (SEQ ID NO: 5) can be generated by replacing the Gal4 binding sites in the cassette Gal4-CD3-PKG-BFP with the binding sites for these transcription factors (TABLE 1). These expression cassettes can be used to drive the expression of downstream gene of interest as a result of ligand recognition by the CAR. The combination of CAR and transcription factor-driven expression cassette has been used to develop TRUCK (T cell redirected for universal cytokine-mediated killing), which secretes pro-inflammatory cytokines in response to CAR signaling to enhance CAR T cell function (43-45). TRUCK T cells have also entered clinical stage studies (NCT02498912 and NCT03721068).

TABLE 1. Transcription factor binding sites in expression cassettes for CAR-based tumor sensing receptors.

Transcription factor	Transcription factor binding motif	Transcription factor binding site in TCE	Number of motif repeats
AP-1	TTGAGTCA (SEQ ID NO: 6)	TTGAGTCAAGATTGAGTCATCGTTGAGTC AGACTTGAGTCACTATTGAGTCAACTTTG AGTCATGCTTGAGTCAGTATTGAGTCA (SEQ ID NO: 7)	8
NFAT	GGAGGAAAAA CTGTTTCATAC AGAAGGCGT (SEQ ID NO: 8)	GGAGGAAAAAAGTGTTCATACAGAAGGC GTGGAGGAAAAAAGTGTTCATACAGAAG GCGTGGAGGAAAAAAGTGTTCATACAGAA GGCGTGGAGGAAAAAAGTGTTCATACAGA AGGCGTGGAGGAAAAAAGTGTTCATACAG AAGGCGTGGAGGAAAAAAGTGTTCATACA GAAGGCGT (SEQ ID NO: 9)	6
NR4A1	AAAGGTCAC (SEQ ID NO: 10)	AAAGGTCACAGAAAAGGTCACCTCGAAAG GTCACGACAAAGGTCACCTAAAAGGTCAC ACTAAAGGTCACCTGCAAAGGTCACGTAAA AGGTCAC (SEQ ID NO: 11)	8
NFκB	GGGGAAATTC CCCT (SEQ ID NO: 12)	GGGGAAATTCCCCTAGAGGGGAAATTCCC CTTCGGGGGAAATTCCCCTGACGGGGGAAA TTCCCCTCTAGGGGAAATTCCCCTACTGG GGAAATTCCCCTTGCGGGGAAATTCCCCT GTA (SEQ ID NO: 13)	7

[00121] DNA sequences for the tumor-sensing receptor and the expression cassette will be introduced into T cells using retroviral or lentiviral vectors, or transposon vectors such as Sleeping Beauty (Addgene) and piggyBac (VectorBuilder), to facilitate their stable integration into the T cell genome. In practice, step three and step four can be carried out at the same time or in reverse order. T cells non-specifically or specifically stimulated may be restimulated. T cells may be cryopreserved after stimulation/expansion, after step 3, or after step 4. The tumor-activated alloreactive T cells can therefore be generated in a number of ways. Three examples are: (i) stimulation/expansion → SynNotch and expression cassette introduction → CD3ε KO → (cryopreservation) → treatment; (ii) stimulation/expansion → cryopreservation → restimulation → SynNotch and expression cassette introduction → CD3ε KO → (cryopreservation) → treatment; and (iii) stimulation/expansion → CD3ε KO → cryopreservation → restimulation with PMA and ionomycin → SynNotch and expression cassette introduction → (cryopreservation) → treatment.

II. Tumor-activated Alloreactive or Xenoreactive T cells

[00122] The present disclosure provides a tumor-activated alloreactive or xenoreactive T-cell. The T-cell originates from a healthy donor for whom the genotype of at least one of the HLA-A, B, C and DRB1 loci is known to mismatch that of the patient. The T cell is modified to be activation-incompetent. Specifically, the T cell is genetically modified to disrupt expression of at least one endogenous gene encoding a molecule that is critical for TCR signaling and T cell activation. The gene to be disrupted can encode a transmembrane protein expressed on the plasma membrane. Non-limiting examples of exemplary transmembrane proteins include CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58. The gene to be disrupted can encode an intracellular signaling molecule involved in TCR signaling and T cell activation. Non-limiting examples of exemplary intracellular signaling molecules include Lck, Zap70, calcineurin, PI3K, Fyn, PLC γ , SLP76, PKC θ , AKT, NcK and PDK1.

[00123] The activation-incompetent T cell further comprises an expression cassette comprising a copy of the gene encoding the molecule disrupted in the T cell. The expression vector comprises a transcriptional control element (TCE) operably linked to the gene, wherein binding of a cognate transcription activator to TCE results in expression of the gene encoding the molecule disrupted in the T cell. The T cell further comprises an exogenous tumor-sensing receptor. Binding of the tumor-sensing receptor to its cognate tumor antigen results in the release or activation of a transcriptional activator. A tumor antigen, as used herein, can be a tumor cell surface molecule, such as Her2, or a soluble factor present in a tumor microenvironment, such as vascular endothelial growth factor (VEGF), or a tumor antigen that is shed from tumor cells. In an alternative embodiment, the tumor-sensing receptor recognizes molecules enriched in tissues with accumulation of blood cancer cells. These include molecules expressed on hematopoietic cells that are abundant in bone marrow and lymphoid organs,

[00124] The genetically modified T cell comprising the above-described features are contemplated to provide at least one of the following beneficial properties of confined alloreactivity at tumor sites or tissues with blood cancer cell accumulation and being able to target both tumor cells and tumor stromal cells that express HLA.

[00125] In certain embodiments of the tumor-activated alloreactive or xenoreactive T-cell and the method of making the same, the endogenous gene disrupted is selected from CD3 ϵ , CD3 ζ , CD3 γ , and CD3 δ . In an embodiment, the endogenous gene disrupted is CD3 ϵ .

[00126] In certain embodiments, the target molecule is selected from Her2/neu, EGFRvIII, CD19, IL-13RR-a2, mesothelin, MUCI, EpCAM, GD2 and CEA. In certain embodiments, target molecule is selected from Her2/neu, EGFRvIII, CD19, IL-13RR-a2, mesothelin, and MUCI. In certain embodiments, the target molecule is selected from Her2/neu, EGFRvIII, and CD19. In an embodiment, the target molecule is Her2/neu.

[00127] In certain embodiments, the expression cassette is selected from Gal4-CD3-PGK-BFP, NR4A-CD3-PKG-BFP, and NFAT-CD3-PKG-BFP. In certain embodiments, the tumor sensing receptor and expression cassette are selected from SynNotch and Gal4-CD3-PGK-BFP, CAR and NR4A-CD3-PKG-BFP, and CAR and NFAT-CD3-PKG-BFP.

[00128] In certain aspects, the sample of T cells is from a donor individual, and wherein the donor individual has at least one HLA allele mismatch relative to an intended recipient and the at least one HLA allele mismatch is located in a locus selected from the group consisting of: HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DPA1 and HLA-DPB1, thereby producing tumor-activated alloreactive T cells. In certain embodiments, the at least one HLA allele mismatch is located in a locus selected from the group consisting of: HLA-A, HLA-B, HLA-C, and HLA-DRB1. In certain embodiments, the HLA allele mismatch comprises four alleles of, HLA-A, HLA-B, HLA-C, and HLA-DRB1, six alleles of, HLA-A, HLA-B, HLA-C, and HLA-DRB1 or all eight alleles of HLA-A, HLA-B, HLA-C, and HLA-DRB1. In an embodiment, the HLA allele mismatch comprises all eight alleles of HLA-A, HLA-B, HLA-C, and HLA-DRB1.

[00129] In certain embodiments, the endogenous gene disrupted is CD3 ϵ , the target molecule is selected from Her2/neu, EGFRvIII, and CD19, and the tumor sensing receptor is SynNotch.

[00130] In certain embodiments, the endogenous gene disrupted is CD3 ϵ , the target molecule is selected from Her2/neu, EGFRvIII, and CD19, the tumor sensing receptor is SynNotch, and the expression cassette is Gal4-CD3-PGK-BFP. In an embodiment, the endogenous gene disrupted is CD3 ϵ , the target molecule is Her2/neu, the tumor sensing receptor is SynNotch, and the expression cassette is Gal4-CD3-PGK-BFP. In an embodiment, the endogenous gene

disrupted is CD3 ϵ , the target molecule is CD19, the tumor sensing receptor is SynNotch, and the expression cassette is Gal4-CD3-PGK-BFP.

III. Therapeutic Methods and Uses

5 [00131] Tumor-activated alloreactive or xenoreactive T cells will be expanded in culture supplemented with cytokines such as IL-2 and IL-7. The T cells will be introduced to patients or cryo-preserved for later use. T cells from different donors or stimulations may be used for the same patient to achieve the best results.

10 [00132] A number of additional steps may be taken to reduce HvG and enhance T cell survival in the patient. First, the patient may be conditioned with lympho-depleting radiation or chemotherapy agent prior to infusion to suppress the immune system.

15 [00133] Second, T cells may be obtained from donors with partially matched HLAs to reduce HvG. For example, T cells from a donor with a 5/8 match of the HLA-A, B, C, and DRB1 loci may be stimulated with patient cells or cell lines expressing the patient's alleles at the three mismatched loci to expand T cells that are alloreactive to these alleles. Third, T cells may be additionally modified to become "stealthy" to the patient's immune system. For example, the expression of HLA-A, B or C on T cells may be disrupted using CRISPR-Cas9. It should be noted that only the mismatched HLA gene(s) needs to be disrupted. Alternatively, the expression of all HLA-I molecules on cell surfaces may be abrogated by knocking out the shared β 2M component, which is required for HLA-I to reach plasma membrane. This will be followed by introducing the α chain of HLA-E or HLA-G that is fused with β 2M in step four (25). HLA-E and HLA-G are non-classical HLA molecules that are not involved in alloreactions but can inhibit attacks from natural killer (NK) cells. Finally, for a patient with blood cancer and have received allogeneic hematopoietic stem cell transplantation, T cells may be obtained from the stem cell donor. In this case, the T cells will be tolerated by the patient's immune system. Moreover, in this case, T cells with restored alloreactivity will target only the patient's cells including cancer cells but avoid hematopoietic cells derived from the donor stem cells.

30 [00134] In the patient, due to deficiencies in critical components for TCR signaling, tumor-activated alloreactive or xenoreactive T cells cannot react to HLAs in normal tissues and should not cause GvH diseases. After migrating into tumors or tissues with blood cancer cell

accumulation, however, engagement of tumor-sensing receptors by cue molecules enriched on in the tumor or target tissues will restore the T cells' alloreactivity or xenoreactivity, leading to T cell killing of HLA-expressing tumor cells and stromal cells (FIG. 2D and 2E). The duration of reactivity depends on two factors. The first is the decaying rate of signals generated by the tumor sensing receptor, which determines how long the re-expression of the knocked out molecule can last after the receptor is disengaged from cue molecules. The half-life of expression driven by SynNotch receptors, for example, is around 8 hrs. The second is the distribution of tumor cue molecules within the tumor, which determines how often the tumor sensing receptor can be reengaged and activated. Figure 2A-2E illustrate the reactivity of an alloreactive T cell with CD3 ϵ expression controlled by a TAA-specific tumor sensing receptor. CD3 ϵ is a member of the TCR/CD3 complex. Loss of CD3 ϵ expression leads to the retention of other components in the endoplasmic reticulum (46). In this case, a tumor-activated alloreactive T cell enters a tumor in which only a fraction of tumor cells expressing TAA. The T cell's ability to activate will be restored by one of the TAA⁺ tumor cells through the interaction between TAA and the tumor-sending receptor that leads to CD3 ϵ expression from the cassette, which restores surface expression of the TCR/CD3 complex. A partial restoration of TCR/CD3 expression level may be sufficient to restore T cell alloreactivity (47). Alloreactivity to HLAs on the tumor cells will activate the T cell and leads to the killing of the tumor cell. Assuming that at least 50% CD3 ϵ expression is required for the T cell to retain alloreactivity, the T cell will be able to kill other TAA-negative tumor cells for 8 hrs before losing its killing activity. The T cell may regain alloreactivity if it encounters other TAA⁺ tumor cells before exiting the tumor. After exiting the tumor, the T cell will lose alloreactivity due to the decay of signaling from the tumor sensing receptor and CD3 ζ expression, although it may cause limited damage to the surrounding normal tissue because of residual CD3 ϵ expression. The scenario described above is consistent with a study of T cells expressing CD19-binding SynNotch that drives the expression of CARs specific tumor antigen ROR1 (48). When the T cells were administered into immunodeficient mice with bone marrow dissemination of lymphoma cells expressing both CD19 and ROR1, T cells expressed ROR1-specific CARs as the result of SynNotch-CD19 engagement and killed lymphoma cells in the mice. The T cells, however, killed CD19⁻/ROR⁺ bone marrow stromal cells as well, demonstrating that T cell toxicity extended to cells in the vicinity of SynNotch ligand-expressing cells.

[00135] The potency and specificity of tumor-activated alloreactive or xenoreactive T cells can be optimized in a number of ways. First, the potency of the cells may be controlled by selecting the level of HLA-mismatch between the donor and patient. For example, mismatches at all six HLA-I loci are expected to elicit stronger alloreaction than mismatch at only one.

5 [00136] Second, the ability of T cells to selectively target tumors and avoid normal tissues may be optimized by tuning the sensitivity of tumor-sensing receptors so that signals are generated only in response to high levels of tumor cue molecules. This may be achieved by adjusting the expression level, affinity, and extracellular linker length of the receptors.

[00137] Third, tumor selectivity of the T cells may be further controlled by using two tumor-
10 sensing receptors, one releasing the DNA-binding domain of the transcription activator and another one releasing the activation domain of the transcription activator. This way, only cells expressing both tumor antigens can restore the expression of the key molecule, thus the reactivity of the T cells. Alternatively, tumor selectivity may be controlled by using multiple tumor-sensing receptors each directing the expression of a distinct component critical for TCR
15 signaling. For instance, both CD3 ζ and CD3 ϵ may be knocked out and two tumor-sensing receptors will be introduced: one recognizes Her2 and directs the expression of CD3 ζ ; another recognizes a different tumor antigen MUC1 and directs the expression of CD3 ϵ . Since both CD3 ζ and CD3 ϵ are required for cell surface expression of the TCR/CD3 complex, only cells expressing both tumor antigens can activate the T cells.

20 [00138] Fourth, the balance between tumor cell killing and normal tissue damage may be tweaked by adjusting the duration of alloreactivity. This can be achieved by manipulating the stability of mRNA transcripts for the re-expressed key molecule or by using degrons (49) to control its rate of protein degradation. The balance may also be adjusted by enhancing or reducing T cell survival through controlling the degree of lympho-depletion in the patient, thus
25 the HvG activity against the allogeneic or xenogeneic T cells. Finally, the potency and specificity of tumor-activated alloreactive or xenoreactive T cells may be controlled by using a “universal” tumor-sensing receptor with an extracellular domain that binds to a peptide or chemical tag on a tumor antigen-binding soluble factor or with an extracellular domain derived from an Fc receptor that binds to tumor antigen-specific IgA, IgG or IgE antibodies. The
30 tumor-sensing receptor will be activated by the soluble factor or the antibodies bound on tumor

cells. The level of alloreactivity or xenoreactivity of the T cells can be manipulated by controlling the type and dose of the tumor-binding soluble factor or antibody.

5 [00139] Tumor-activated alloreactive or xenoreactive T cells are believed to have a number of advantages over CAR T cells in terms of efficacy and cost. First, once activated at the tumor site, allogenic T cells attack not only tumor cells expressing the tumor antigen, but also other tumor cells expressing HLA-I. Even though HLA-I downregulation is a common mechanism employed by tumor cells to escape immune surveillance, HLA-I expression is well preserved in many cancers. For example, 68% in gastric cancer (50), 57% in esophageal cancer (51), 45% in osteosarcoma (52), 34% in breast cancer (53), and 30% in lung cancer (54).

10 [00140] Second, alloreactive or xenoreactive T cells also attack HLA-I-expressing stromal cells such as carcinoma-associated fibroblasts, angiogenic vascular cells (55) and myeloid-derived suppressor cells (56) that play important roles in supporting tumor growth and in creating an immunosuppressive tumor microenvironment. Stromal cells have been found to strongly and uniformly express HLA-I even when tumor cells are HLA-negative (57-59). These
15 two features make it possible to use tumor-activated alloreactive or xenoreactive T cells to treat tumors with a low percentage of tumor antigen-expressing cells that may be considered unsuitable for CAR T cells. In this sense, tumor antigen-expressing cells not only serve as targets, but also designate the whole tumor for attacks by therapeutic T cells.

[00141] Third, since the sources of T cells are healthy donors, tumor-activated alloreactive
20 T cells that recognize certain popular HLA alleles or haplotypes may be produced in large quantities, cryo-preserved and offered as off-the-shelf products. T cell products with alloreactivities to each of the patient's HLA alleles can be selected, combined and administered for treatment. This will lower cost and enable repeated administration for better efficacy.

[00142] Taken together, tumor-activated alloreactive or xenoreactive T cells have the
25 potential to be more effective for a broader range of tumors than CAR T cells.

[00143] The T cells are administered to a subject in need of treatment for a cellular proliferative disorder, including but not limited to, cancer. The T cells may be administered either alone, or as a pharmaceutical composition in combination with one or more pharmaceutically acceptable carriers, diluents or excipients and/or with other components, such
30 as cytokines or other cell populations. Such compositions may comprise pharmaceutically

acceptable buffers such as neutral buffered saline, phosphate buffered saline and the like; pharmaceutically acceptable carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; pharmaceutically acceptable antioxidants; pharmaceutically acceptable chelating agents such as EDTA or glutathione; 5 pharmaceutically acceptable adjuvants (e.g., aluminum hydroxide); and pharmaceutically acceptable preservatives. Compositions are preferably formulated for intravenous administration.

[00144] Cancers that may be treated or prevented according to the present disclosure include a broad range of tumor types, including but not limited to: ovarian cancer, cervical cancer, 10 breast cancer, prostate cancer, testicular cancer, lung cancer, renal cancer, colorectal cancer, skin cancer, brain cancer, and tumors that may arise from hematological malignancies such as leukemias, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoid leukemia and chronic lymphoid leukemia. More particularly, cancers that may be treated by the compounds, compositions and methods of the disclosure include, but are not 15 limited to, the following: cardiac cancers, lung cancers, gastrointestinal cancers, genitourinary tract cancers, liver cancers, bone cancers, nervous system cancers, gynecological cancers, hematologic cancers, skin cancers, and adrenal gland cancers.

[00145] Cancers may comprise solid tumors that may or may not be metastatic. Cancers may also occur as a diffuse tissue. Thus, the term "tumor cell," as provided herein, includes a cell 20 afflicted by any one of the above identified disorders.

[00146] The T cells or pharmaceutical composition thereof may be administered by a route that results in the effective delivery of an effective amount of cells to the patient for pharmacological effect. Administration is typically parenteral. Intravenous administration is the preferred route, using infusion techniques that are commonly known in immunotherapy 25 (see, e.g., Rosenberg *et al.*, *New Eng. J. Med.* 319: 1676, 1988). The quantity of T cells and frequency of administration are determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials. An "effective amount" is determined by a physician with consideration of individual differences in age, weight, disease state, and disease severity of the patient. 30 Generally, the amount of T cells given in a single dosage will range from about 10^6 to 10^9 cells/kg body weight, including all integer values within those ranges. The T cells may be

administered multiple times at these dosages. The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

5 [00147] The T cells or composition comprising the T cells compositions may be provided in a pharmaceutical pack or kit comprising one or more containers or compartments filled with one or more compositions. Optionally associated with such container(s) is a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. Also optionally included with such
10 container(s) are instructions for carrying out the methods of the disclosure.

[00148] The instructional material may comprise a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the method. The package insert may comprise text housed in any physical medium, e.g., paper, cardboard, film, or may be housed in an electronic medium such as a diskette, chip, memory
15 stick or other electronic storage form. The instructional material of the kit of the disclosure may, for example, be affixed to a container which contains other contents of the kit, or be shipped together with a container which contains the kit. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the contents of the kit be used cooperatively by the recipient.

20

EXEMPLARY EMBODIMENTS

[00149] Among the embodiments provided herein are:

Embodiment 1. A method for producing tumor-activated alloreactive or xenoreactive T cells,
25 said method comprising:

- a) selecting a sample of T cells from an HLA-mismatched donor individual, or from a donor animal;
- b) optionally stimulating the sample of T cells to proliferate;

c) abrogating the expression or function of at least one molecule necessary for TCR signaling and T cell activation in the T cells to render the T cells activation-incompetent; and

d) modifying the T cells to (i) express a recombinant receptor molecule that
5 specifically binds to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and a tissue with blood cancer cell accumulation, wherein binding of the recombinant receptor with the target molecule releases or activates a transcription activator; and (ii) introduce an expression cassette that enables the transcription activator in (i) to drive the expression of the molecule abrogated in c). wherein step c) is performed before step
10 d), step d) is performed before step c), or steps c) and d) are performed at the same time, and

wherein binding of the recombinant receptor with the target molecule restores the expression or function of the abrogated molecule, and thereby restores the ability of the T cells to activate through antigen recognition by TCR,

thereby producing tumor-activated alloreactive or xenoreactive T cells.

15 Embodiment 2. The method of Embodiment 1, wherein step b) is not performed.

Embodiment 3. The method of Embodiment 1, wherein step b) is performed.

Embodiment 4. The method of any one of Embodiments 1 to 3, wherein step d) is carried out before step c).

Embodiment 5. The method of any one of Embodiments 1 to 3, wherein step c) is carried out
20 before step d).

Embodiment 6. The method of any one of Embodiments 1 to 4, wherein steps c) and d) are performed at the same time.

Embodiment 7. The method of any one of Embodiments 4 to 6, wherein the T cell stimulation step comprises culturing donor T cells with antibodies specific for CD3 and
25 CD28.

Embodiment 8. The method of any one of Embodiments 1 to 7, wherein the sample of T cells is from a donor individual, and wherein the donor individual has at least one HLA allele mismatch relative to an intended recipient and the at least one HLA allele mismatch is located in a locus selected from the group consisting of: HLA-A, HLA-B, HLA-C, HLA-

DRB1, HLA-DQA1, HLA-DQB1, HLA-DPA1 and HLA-DPB1, thereby producing tumor-activated alloreactive T cells.

Embodiment 9. The method of any one of Embodiments 1-8, wherein step b) comprises co-culturing donor T cells with cells from an intended recipient.

- 5 Embodiment 10. The method of Embodiment 9, where the cells from the intended recipient are peripheral blood mononuclear cells, dendritic cells, tumor cells, or a mixture of thereof.

Embodiment 11. The method of any one of Embodiments 1-8, wherein step b) comprises co-culturing donor T cells with cells from a second donor that (i) has at least one HLA allele matched with the intended recipient, and (ii) is mismatched with the T cell donor.

- 10 Embodiment 12. The method of Embodiment 11, where the cells from the second donor are peripheral blood mononuclear cells, dendritic cells, tumor cells, or a mixture of thereof.

Embodiment 13. The method of any one of Embodiments 1-8, wherein step b) comprises co-culturing donor T cells with a cell line expressing a least one HLA allele of the intended recipient.

- 15 Embodiment 14. The method of any one of Embodiments 9 to 13, the cells co-cultured with the donor T cells are treated with radiation or chemicals to block cell proliferation.

Embodiment 15. The method of any one of Embodiments 1-8, wherein step b) comprises co-culturing donor T cells with an artificial surface.

- Embodiment 16. The method of Embodiment 14, wherein the artificial surface is a plastic
20 coated with at least one protein encoded by at least one HLA allele of the intended recipient.

Embodiment 17. The method of any one of Embodiments 1 to 16, wherein step c) comprises abrogating the expression of at least one protein critical for TCR signaling and T cell activation by disrupting the gene encoding the protein.

- Embodiment 18. The method of Embodiment 17, wherein disrupting the gene uses a gene
25 editing technology chosen from CRISPR-Cas, a transcription activator-like effector nuclease (TALEN), amegaTALS, a zinc-finger nuclease, or a homing endonuclease.

- Embodiment 19. The method of any one of Embodiments 1 to 18, wherein the at least one molecule necessary for TCR signaling and T cell activation is a cell surface molecule chosen from CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1,
30 CD2, CD54, CD52, CD148, and CD58.

Embodiment 20. The method of any one of Embodiments 1 to 18, wherein the at least one molecule necessary for TCR signaling and T cell activation is an intracellular signaling molecule chosen from Lck, Zap70, calcineurin, PI3K, Fyn, PLC γ , NFAT, SLP76, PKC θ , NF κ B, AKT, and PDK1.

- 5 Embodiment 21. The method of any one Embodiments 1 to 20, wherein the activation-incompetent T cells of step c) are purified by first staining the cells using antibodies specific for the surface molecules, and then isolating the cells lacking antibody binding by flow cytometry, magnetic beads, and/or purification columns.

- Embodiment 22. The method of any one Embodiments 1 to 20, wherein the activation-
10 incompetent T cells of step c) are purified by using a live cell-specific DNA imaging technique.

Embodiment 23. The method of Embodiment 22, wherein the live cell-specific DNA imaging technique is CRISPR LiveFish.

- Embodiment 24. The method of any one of Embodiments 1 to 20, wherein the activation-
15 incompetent T cells of step c) are purified by selecting T cells unable to activate and proliferate after further stimulation through TCR.

Embodiment 25. The method of any one of Embodiments 1 to 24, wherein step d) comprises introducing a nucleic acid encoding a tumor-sensing receptor into T cells.

- Embodiment 26. The method of Embodiment 25, wherein the tumor-sensing receptor
20 comprises (i) an extracellular domain that binds directly or indirectly to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and tissues with blood cancer cell accumulation; and (ii) an intracellular domain that activates or releases a transcription activator in response to extracellular domain binding to the target molecule.

- Embodiment 27. The method of any one of Embodiments 1 to 26, wherein the target
25 molecule is enriched on tumor cells and/or in the tumor microenvironment and is chosen from CD19, CD20, CD38, CD30, Her2/neu, ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2 (VEGFR2), high molecular weight- melanoma associated antigen
30 (HMW-MAA), MAGE-A1, IL-13R-a2, GD2, 4-1BB, 5T4, adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX),

C-MET, CCR4, CD152, CD19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4, DRS, EGFR, EpCAM, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgG1, LI-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin $\alpha\beta 3$, MORAb-009, MS4A1, MUC1, mucin CanAg, N- glycolylneuraminic acid, NPC-IC, PDGF-Ra, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF- β , TRAIL-R1, TRAIL-R2, tumor antigen CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, mesothelin, NKG2D, CD147, NKR2, B7H3, and vimentin.

Embodiment 28. The method of any one of Embodiments 1 to 26, wherein the target molecule is enriched in a tissue with blood cancer cell accumulation, and wherein the tissue is lymphoid and/or bone marrow tissue.

Embodiment 29. The method of Embodiment 28, wherein the target molecule is chosen from CD45, CD19, CD20, CD4, CD8, CD2, CCR4, CD58, CD28, CD23, CD69, CD25, CD33, CD123 and CCL-1.

Embodiment 30. The method of any one of Embodiments 26 to 29, wherein the extracellular domain of the tumor-sensing receptor is a single chain variable fragment (scFv), a Fab fragment, a designed ankyrin repeat protein (DARPin), a TCR, a nanobody, a Fc receptor, a growth factor receptor, a chemokine receptor, or a hormone receptor.

Embodiment 31. The method of any one of Embodiments 26 to 29, wherein the tumor-sensing receptor is a version of the Synthetic Notch (SynNotch), Modular Extracellular Sensor Architecture (MESA), or Tango technology, wherein a transcription activator is released from the intracellular domain of the receptor in response to extracellular domain binding to the target molecule.

Embodiment 32. The method of any one of Embodiments 26 to 29, wherein the tumor-sensing receptor is a chimeric antigen receptor, wherein a downstream transcription factor is activated through signaling pathways activated in response to extracellular domain binding to the target molecule.

Embodiment 33. The method of any one of Embodiments 25 to 32, the nucleic acid comprises an expression cassette comprising a transcription control element operably linked

to a DNA sequence encoding a functional copy of the at least one molecule necessary for TCR signaling and T cell activation abrogated in step c), wherein binding of a transcription activator activates and/or releases from the tumor-sensing receptor to the transcription control element activates transcription of the encoded functional copy of the at least one molecule
5 necessary for TCR signaling and T cell activation.

Embodiment 34. The method of Embodiment 33, wherein the transcription control element is bound by a transcription activator selected from the group consisting of Gal4-VP64, Notch, tet transactivator, NFAT, AP-1, NF κ B/Rel, NR4A1 (Nur77), T-Bet, IRF4, STATs and comesodermin (Eomes).

10 Embodiment 35. The method of any one of Embodiments 1 to 34, wherein the potency and specificity of the tumor-activated alloreactive or xenoreactive T cells are regulated by controlling the affinity, specificity and expression level of tumor-sensing receptors or controlling the mRNA or protein stability of the critical molecule for T cell activation.

Embodiment 36. The method of any one of Embodiments 1 to 35, further comprising
15 modifying the T cells to reduce the potential of being detected and eliminated through HvG by the patient's immune system.

Embodiment 37. The method of Embodiment 36, wherein modifying the T cells comprises abrogating T cell expression of HLA alleles mismatched with the recipient by disrupting the genes encoding HLA class I α heavy chain.

20 Embodiment 38. The method of Embodiment 37, wherein disrupting the genes encoding the HLA class I α heavy chain uses a gene editing technology chosen from CRISPR-Cas, a transcription activator-like effector nuclease (TALEN), amegaTALS, a zinc-finger nuclease, or a homing endonuclease.

Embodiment 39. The method of Embodiment 36, wherein modifying the T cells comprises
25 abrogating T cell expression of all HLA class I on cell surface by disrupting the genes encoding beta-2-microglobulin (β 2M) thereby.

Embodiment 40. The method of Embodiment 39, wherein disrupting the genes encoding beta-2-microglobulin uses a gene editing technology chosen from CRISPR-Cas, a transcription activator-like effector nuclease (TALEN), amegaTALS, a zinc-finger nuclease,
30 or a homing endonuclease, and further comprising introduction of HLA-G or HLA-E α chain fused with β 2M using a non-viral or viral vector.

Embodiment 41. The method of any one of Embodiments 1 to 40, further comprising cryopreserving the tumor-activated T cells for later use.

Embodiment 42. A method of treating cancer in a patient by administering T cells prepared by the method of any one of Embodiments 1 to 41.

- 5 Embodiment 43. The method of Embodiment 42, wherein T cells generated from different donors or using different stimulations are administered to the same patient.

Embodiment 44. The method of Embodiment 42 or Embodiment 43, wherein prior to the administration of the T cells, the patient is conditioned with IFN γ (interferon gamma) to upregulate the expression of HLA on tumor cells and stromal cells.

- 10 Embodiment 45. The method of Embodiment 42 or Embodiment 43, wherein prior to the administration of the T cells, the patient is conditioned with lympho-depleting radiation or chemotherapy agent to suppress the immune system.

Embodiment 46. A genetically modified T cell (or a population thereof) comprising:

- 15 (i) genetic disruption of expression of at least one endogenous gene encoding a molecule necessary for TCR signaling and T cell activation,
- (ii) an exogenous nucleotide sequence encoding a tumor-sensing receptor that releases or activates a transcription activator in response to direct or indirect binding to molecules enriched on tumor cells, in the tumor microenvironment or in tissues with blood cancer cell accumulation, and
- 20 (iii) an exogenous nucleotide sequence comprising an expression cassette that expresses a copy of the disrupted endogenous gene of (i) in response to the released or activated transcription activator of (ii).

- Embodiment 47. The genetically modified T cell of Embodiment 46, wherein the at least one disrupted endogenous gene encoding a molecule necessary for TCR signaling and T cell
- 25 activation encodes a transmembrane protein selected from CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58, or encodes an intracellular signaling molecule chosen from Lck, Zap70, calcineurin, PI3K, Fyn, PLC γ , SLP76, PKC θ , AKT, and PDK1.

- Embodiment 48. The genetically modified T cell of Embodiment 46 or Embodiment 47,
- 30 wherein the tumor-sensing receptor comprises (i) an extracellular domain that binds directly

or indirectly to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and tissues with blood cancer cell accumulation; and (ii) an intracellular domain that activates or releases a transcription activator in response to extracellular domain binding to the target molecule.

- 5 Embodiment 49. The genetically modified T cell of Embodiment 48, wherein the tumor-sensing receptor is Synthetic Notch (SynNotch) receptor, a Modular Extracellular Sensor Architecture (MESA) receptor, or a Tango receptor.

- Embodiment 50. The genetically modified T cell of Embodiment 48 or Embodiment 49, wherein the target molecule is enriched on tumor cells and/or in the tumor microenvironment
- 10 and is chosen from CD19, CD20, CD38, CD30, Her2/neu, ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2 (VEGFR2), high molecular weight- melanoma associated antigen (HMW-MAA), MAGE-A1, IL-13R-a2, GD2, 4-1BB, 5T4,
- 15 adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), C-MET, CCR4, CD152, CD19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4, DRS, EGFR, EpCAM, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75,
- 20 GPNMB, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgG1, LI-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin $\alpha\beta 3$, MORAb-009, MS4A1, MUC1, mucin CanAg, N- glycolylneuraminic acid, NPC-IC, PDGF-Ra, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF- β , TRAIL-R1, TRAIL-R2, tumor antigen
- 25 CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, mesothelin, NKG2D, CD147, NKR2, B7H3, and vimentin.

Embodiment 51. The genetically modified T cell of Embodiment 48 or Embodiment 49, wherein the target molecule is enriched in a tissue with blood cancer cell accumulation, and wherein the tissue is lymphoid and/or bone marrow tissue.

- 30 Embodiment 52. The genetically modified T cell of Embodiment 51, wherein the target molecule is chosen from CD45, CD19, CD20, CD4, CD8, CD2, CCR4, CD58, CD28, CD23, CD69, CD25, CD33, CD123 and CCL-1.

Embodiment 53. The genetically modified T cell of any one of Embodiments 48 to 52, wherein the extracellular domain of the tumor-sensing receptor is a single chain variable fragment (scFv), a Fab fragment, a designed ankyrin repeat protein (DARPin), a TCR, a nanobody, a Fc receptor, a growth factor receptor, a chemokine receptor, or a hormone
5 receptor.

Embodiment 54. The genetically modified T cell of Embodiment 46 or Embodiment 48 wherein the tumor-sensing receptor is a chimeric antigen receptor, wherein a downstream transcription factor is activated through signaling pathways activated in response to extracellular domain binding to the target molecule.

10 Embodiment 55. The genetically modified T cell of any one of Embodiments 46 to 54, wherein the transcription control element driving expression of the copy of the disrupted endogenous gene is bound by a transcription activator selected from the group consisting of Gal4-VP64, Notch, tet transactivator, NFAT, AP-1, NFκB/Rel, NR4A1 (Nur77), T-Bet, IRF4, STATs and eomesodermin (Eomes).

15 Embodiment 56. A pharmaceutical composition comprising an effective amount of genetically modified T cells of any one of Embodiments 46 to 55 and a pharmaceutically acceptable carrier.

Embodiment 57. A kit comprising genetically modified T cells of any one of Embodiments 46 to 55 and instructional material for the use of the cells in a therapeutic method.

20 Embodiment 58. A method of treating cancer in a patient by administering T cells of any one of Embodiments 46 to 55 or the pharmaceutical composition of Embodiment 56.

Embodiment 59. The method of Embodiment 58, wherein T cells generated from different donors or using different stimulations are administered to the same patient.

25 Embodiment 60. The method of Embodiment 58 or Embodiment 59, wherein prior to the administration of the T cells, the patient is conditioned with INFγ to upregulate the expression of HLA on tumor cells and stromal cells.

Embodiment 61. The method of Embodiment 58 or Embodiment 59, wherein prior to the administration of the T cells, the patient is conditioned with lympho-depleting radiation or chemotherapy agent to suppress the immune system.

Embodiment 62. Use of a genetically modified T cell according to any one of Embodiments 46 to 55 in the treatment of cancer in a patient in need thereof.

Embodiment 63. Use of a genetically modified T cell according to any one of Embodiments 46 to 55 in the manufacture of a medicament to treat cancer.

5 **[00150]** Further embodiments provided herein are:

Embodiment P1: A method for producing tumor-activated alloreactive or xenoreactive T cells, said method comprising:

- a. selecting a sample of T cells from an HLA-mismatched donor individual or from a donor animal;
- 10 b. stimulating the said T cells to drive the cells into cell cycle (proliferate);
- c. generating activation-incompetent T cells by abrogating the expression or function of at least one molecule critical for TCR signaling and T cell activation; and
- 15 d. generating tumor-activated alloreactive or xenoreactive T cells by introducing a mechanism that recognizes molecules enriched on tumor cells or in the tumor microenvironment, and as the result of the recognition, restores the expression or function of the molecule abrogated in the previous step, thus restores the ability of T cells to activate through antigen recognition by TCR.

20 Embodiment P2: The method of Embodiment P1, wherein steps c) and d) are carried out at the same time or in reverse order.

Embodiment P3: The method of Embodiment P1, wherein T cell samples are from donor individuals with HLA genes mismatched with the patient at a single locus or at multiple loci selected from the group consisting of: HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DPA1 and HLA-DPB1 locus.

25 Embodiment P4: The method of Embodiment P1, wherein the T cell stimulation step comprises culturing donor T cells with antibodies specific for CD3 and CD28.

Embodiment P5: The method of Embodiment P1, wherein the T cell stimulation step comprises co-culturing donor T cells with cells from the patient, including peripheral blood mononuclear cells, dendritic cells, tumor cells, or a mixture of these cells.

Embodiment P6: The method of Embodiment P1, wherein the T cell stimulation step comprises culturing donor T cells with peripheral blood mononuclear cells, dendritic cells, or a mixture of these cells from another donor who has at least one HLA allele matched with the patient but mismatched with the T cell donor.

- 5 Embodiment P7: The method of Embodiment P1, wherein the T cell stimulation step comprises culturing donor T cells with cell lines expressing at least one HLA allele of the patient.

Embodiment P8: The method of Embodiment P1, wherein the T cell stimulation step comprises culturing donor T cells with artificial surfaces such as plastics coated with proteins
10 encoded by at least one HLA allele of the patient.

Embodiment P9: The method of Embodiments P5, P6, or P7, wherein the stimulator cells (not the T cells) are treated with radiation or chemicals to block cell proliferation.

Embodiment P10: The method of Embodiment P1, wherein the step of generating activation-incompetent T cells comprises abrogating the expression of at least one protein critical for
15 TCR signaling and T cell activation by disrupting the gene encoding the protein using gene-editing technologies including CRISPR-Cas, transcription activator-like effector nuclease (TALENs), megaTALS, zinc-finger nucleases, and homing endonucleases.

Embodiment P11: The method of Embodiment P10, wherein the proteins critical for TCR signaling and T cell activation include cell surface molecules CD3ε, CD3ζ, CD3γ, CD3δ,
20 CD4, CD8α, CD8β, LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58, and intracellular signaling molecules Lck, Zap70, calcineurin, PI3K, Fyn, PLCγ, NFAT, SLP76, PKCθ, NFκB, AKT, and PDK1.

Embodiment P12: The method of Embodiment P10, wherein T cells with abrogated expression of surface molecules are purified by staining the cells using antibodies specific for
25 the surface molecules, followed by isolation of cells without antibody binding using technologies including flow cytometry, magnetic beads, and purification columns.

Embodiment P13: The method of Embodiment P10, wherein T cells with abrogated expression of intracellular signaling molecules are isolated using live cell specific DNA imaging techniques including CRISPR LiveFISH.

Embodiment P14: The method of Embodiment P10, wherein T cells with at least one molecule critical for TCR signaling and activation disrupted are purified based on the inability of the T cells to activate and proliferate after further stimulation through TCR.

Embodiment P15: The method of Embodiment P1, wherein the generating tumor-activated
5 alloreactive or xenoreactive T cells step comprises introducing nucleic acids for a tumor-sensing receptor into T cells.

Embodiment P16: The method of Embodiment P15, wherein the tumor-sensing receptor comprises an extracellular domain that binds directly or indirectly to molecules enriched on tumor cells or in the tumor microenvironment and an intracellular domain that, in response to
10 extracellular domain binding, activates or releases a transcription activator.

Embodiment P17: The method of Embodiment P16, wherein the extracellular domain of the tumor-sensing receptor is a single chain variable fragment (scFv), a Fab fragment, a designed ankyrin repeat protein (DARPin), a TCR, a nanobody, a Fc receptor, a growth factor receptor, a chemokine receptor, or a hormone receptor.

Embodiment P18: The method of Embodiment P15, where the tumor-sensing receptor is
15 based on the Synthetic Notch (SynNotch), Modular Extracellular Sensor Architecture (MESA), or Tango technology.

Embodiment P19: The method of Embodiment P1, wherein the step of generating tumor-activated alloreactive or xenoreactive T cells comprises introducing nucleic acids for an
20 expression cassette into T cells, wherein the expression cassette comprises a transcription control element operably linked to a DNA sequence that encodes the gene disrupted in Embodiment P10 and Embodiment P11, wherein binding of the transcription activator activated and/or released from the tumor-sensing receptor in Embodiment P16 to the transcription control element activates the transcription of the disrupted gene.

Embodiment P20: The method of Embodiment P1, wherein the potency and specificity of the tumor-activated alloreactive or xenoreactive T cells are regulated by controlling the affinity, specificity and expression level of tumor-sensing receptors or controlling the mRNA or protein stability of the critical molecule for T cell activation.

Embodiment P21: The method of Embodiment P1, wherein the tumor activated alloreactive
30 or xenoreactive T cells are further modified to reduce the potential of being detected and eliminated through HvG by the patient's immune system.

Embodiment P22: The method of Embodiment P21 wherein T cell expression of HLA alleles mismatched with the patient are abrogated by disrupting the genes encoding HLA class I α heavy chain using gene-editing technologies including CRISPR-Cas, transcription activator-like effector nuclease (TALENs), megaTALS, zinc-finger nucleases, and homing
5 endonucleases.

Embodiment P23: The method of Embodiment P21 wherein the expression of all HLA class I on cell surface is abrogated by disrupting the genes encoding β 2M using gene-editing technologies, followed by the introduction of HLA-G or HLA-E α chain fused with β 2M using non-viral or viral vectors.

10 Embodiment P24: The method of Embodiment P1, wherein the tumor-activated alloreactive or xenoreactive T cells are cryopreserved for later use.

Embodiment P25: A method of treating cancer in a patient by administering T cells prepared by the method of Embodiment 1.

15 Embodiment P26: The method of Embodiment P25, wherein T cells generated from different donors or using different stimulations are administered to the same patient.

Embodiment P27: The method of Embodiment P25, wherein prior to the administration of the T cells, the patient is conditioned with $\text{INF}\gamma$ to upregulate the expression of HLA on tumor cells and stromal cells.

20 Embodiment P28: The method of Embodiment P25, wherein prior to the administration of the T cells, the patient is conditioned with lympho-depleting radiation or chemotherapy agent to suppress the immune system.

Embodiment P29: A genetically modified tumor-activated alloreactive or xenoreactive T cell.

25 Embodiment P30: The T cell of Embodiment P29, comprising a genetic modification to disrupt expression of at least one endogenous gene encoding a molecule that is critical for TCR signaling and T cell activation.

Embodiment P31: The T cell of Embodiment P30, wherein the at least one endogenous gene disrupted encodes a transmembrane protein or an intracellular signaling molecule.

Embodiment P32: The T cell of Embodiment P31, wherein the disrupted endogenous gene is a transmembrane protein selected from CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58.

Embodiment P33: The T cell of Embodiment P31, wherein the disrupted endogenous gene is an intracellular signaling molecule selected from Lck, Zap70, calcineurin, PI3K, Fyn, PLC γ , NFAT, SLP76, PKC θ , NF κ B, AKT, NcK and PDK1.

Embodiment P34: A pharmaceutical composition comprising an effective amount of genetically modified tumor-activated T cells of Embodiment P29 and a pharmaceutically acceptable carrier.

Embodiment P35: A method of treating cancer in a patient by administering genetically modified tumor-activated T cells of Embodiment P29.

Embodiment P36: The method of Embodiment P35, wherein T cells generated from different donors or stimulations are administered to the same patient.

Embodiment P37: The method of Embodiment P35, wherein prior to the administration of the T cells, the patient is conditioned with INF γ to upregulate the expression of HLA on tumor cells and stromal cells.

Embodiment P38: The method of Embodiment P35, wherein prior to the administration of the T cells, the patient is conditioned with lympho-depleting radiation or chemotherapy agent to suppress the immune system.

Embodiment P39: A kit comprising genetically modified tumor-activated T cells of Embodiment P29 and instructional material for the use of the cells in a therapeutic method.

EXAMPLES

[00151] The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1: T cell alloreactivity to U266 myeloma cells

[00152] The cytotoxicity of alloreactive T cells to U266 myeloma cells was determined as followed. T cells from a healthy donor were activated with anti-CD3/CD28 beads for 72 hrs and expanded for additional 5 days in medium supplemented with 100 U/ml recombinant human IL2. 2.5x10⁵ T cells were incubated with 2.5x10⁴ U266 cells expressing luciferase at a 10:1 ratio for 16 hrs in the absence or presence of 10 µg/ml CD8-specific monoclonal antibody SK1. Luciferase activities in the remaining live U266-luciferase cells were determined using the Bright-Glo™ luciferase assay system (Promega, Madison, WI). The luciferase activities of 2.5 × 10⁴ U266-luciferase cells cultured without T cells were determined as the maximum activity. Specific killing was calculated as {1-(sample activity)/(max activity)} × 100.

[00153] The data are shown in Figure 3. U266, a cell line established from an IgE myeloma patient, expresses high levels of HLA class I (60). Co-culturing U266 cells with pre-activated HLA-I-mismatched primary human CD8+ T cells led to significant killing of U266 cells. The killing was largely inhibited by the anti-CD8 antibody SK1, which has been shown to effectively block T cell activation through TCR-HLA class I interaction, in which CD8 is critically involved (61, 62).

Example 2: Reduced TCR/CD3 complex expression on mouse D10 T cells after CD3ζ knockout using CRISPR-Cas9

[00154] The experiment was carried out as follows. CD3ζ is required for the assembly and cell surface expression of TCR/CD3 complex, which also consists of TCRα, TCRβ, CD3γ and CD3ε chains. Guide RNA (gRNA) designed to target the extracellular domain of mouse CD3ζ was synthesized using a GeneArt™ Precision gRNA Synthesis Kit (Invitrogen). The gRNA and Cas9 ribonucleoproteins (RNPs) were introduced into D10 cells using an electroporation-based Neon Transfection System (Invitrogen). Cells were stained with antibodies specific for TCRβ and CD3ε and analyzed using flow cytometry.

[00155] The data are shown in Figure 4. CD3ζ was knocked out in more than 60% of D10 cells, as indicated by the much lower surface expression levels of TCRβ and CD3ε.

Example 3: Non-specific primary T cell activation and expansion using anti-CD3/CD28 magnetic beads.

[00156] Five hundred thousand (500,000) purified primary human CD8⁺ T cells were cultured with human T-activator CD3/CD28 Dynabeads (Life Technologies) at a 1:1 cell to bead ratio in a 48-well plate. The cells were cultured in OpTmizer culture medium (Thermo Fisher; CTSTM OpTmizer™ T cell Expansion SFM, 6 mM GlutaMAX™, and 2% CTSTM Immune Cell SR) supplemented with 10 ng/ml recombinant human IL2 (rhIL-2; R&D Systems). Beads were removed after 3 days of stimulation.

[00157] The T cells were maintained at 0.5×10^6 /ml to 2×10^6 /ml with change of medium and rhIL2 every 1 to 2 days.

Example 4: Specific stimulation and expansion of alloreactive T cells using the human breast cancer cell line MDA-MB-231 cell line

[00158] The human breast cancer cell line MDA-MB-231 (ATCC) expresses relatively high levels of HLA-I (A02:17; A02:01; B41:01; B40:02; C17:01; C02:02) (PMID: 29331515). Figure 5, MDA-MB-231 WT. To generate a negative control without HLA-I expression, beta-2-microglobulin (β 2m) was knocked out by transfecting the cells with *Streptococcus pyogenes* (S.p.) Cas9 nuclease V3 (IDT) and single guide RNA (sgRNA; IDT Hs.Cas9.B2M.1.AA; target specific sequence: 5'- CGTGAGTAAACCTGAATCTT-3' SEQ ID NO: 15) using a Neon Transfection System (ThermoFisher). MDA-MB-231 cells with β 2m knocked out showed minimal levels of HLA-I expression (Figure 5, MDA-MB-231 β 2m KO).

[00159] To specifically stimulate and expand alloreactive T cells, 0.125×10^6 MDA-MB-231 cells were seeded in a 48 well plate and cultured overnight. 0.75×10^6 purified primary human CD8 T cells from a donor with mismatched HLA-I (A 34:02, A 68:02; B 07:02, B 57:03; C 07:01, C 07:02) were added and cultured in OpTmizer culture medium supplemented with 10 ng/ml rhIL-2 and 1 μ g/ml Ultra-LEAF™ purified anti-CD28 monoclonal antibody (Biolegend, clone CD28.2). After 5 days of culture, T cells were moved to a flask and cultured at a density of 0.5×10^6 to 2×10^6 cells/ml.

[00160] The data are shown in Figure 6. CFSE (carboxyfluorescein diacetate succinimidyl ester) labeled T cells proliferated rapidly when co-cultured with WT MDA cells but showed limited proliferation with MDA cells with HLA-I knocked out. See Figure 6A and 6B, respectively.

Example 5: Lentiviral transduction of primary human T cells to introduce anti-CD19 SynNotch and CD3 ϵ expression cassette

[00161] Lentiviral vectors for anti-CD19 SynNotch were packaged using the transfer vector pHR_PGK_antiCD19_synNotch_Gal4VP64 (Addgene Cat. #79125). The vector drives the expression of anti-CD19 SynNotch receptor downstream of a constitutively active PGK-1 promoter. The anti-CD19 SynNotch receptor comprises a N-terminal MycTag followed by a CD19-specific extracellular single chain variable fragment (scFv), a Notch core sequence including the transmembrane domain, and an intracellular domain containing a cleavable artificial transcription activator Gal4-VP64, which consists of a Gal4 DNA binding domain and a VP64 transcription activation domain (PMID: 26830878, PMID: 26830879).

[00162] To prepare lentiviral vectors for the CD3 ϵ expression cassette, the transfer vector pHR_Gal4UAS_IRES_mC_pGK_tBFP (Addgene #79123) (PMID: 26830878, PMID: 26830879) was modified by inserting a human CD3 ϵ coding sequence downstream the Gal4 binding sites and removing the IRES-mCherry sequence. The human CD3 ϵ coding sequence was codon optimized so that the target-specific sequence of the gRNA for CD3 ϵ knockout no longer functions through recognizing the CD3 ϵ coding sequence in the cassette and working with Cas9 to cleave it. The resulting transfer vector encodes a Gal4-CD3-PKG-BFP cassette (SEQ ID NO: 1) in which CD3 ϵ expression is controlled by the Gal4 binding sequence and the expression of blue fluorescence protein (BFP) is driven by a constitutively active PGK-1 promoter (FIG. 15). BFP expression therefore serves as a marker for the presence the cassette in transduced cells.

[00163] Lentiviral vectors were packaged in Lenti-X 293T cells (Takara) and concentrated 100-fold using the Lentivirus Precipitation Solution (ALSTEM). To transduce T cells, 50 μ l concentrated lentiviral vectors each for anti-CD19 SynNotch and Gal4-CD3-PKG-BFP cassette were added to T cells stimulated by anti-CD3/CD28 beads or MDA-MB-231 cells at day 1 post stimulation. After 24 hours of incubation in the presence of 10 μ g/ml protamine, media were changed to dilute protamine sulfate and 10 ng/ml rhIL-2 was added. For co-culture with MDA-MB-231 cells, anti-human CD28 antibody (Biolegend, clone CD28.2) was added to 1 μ g/ml. Anti-CD19 synNotch expression was analyzed by staining with PE-conjugated anti-MycTag (Cell Signaling Technology, clone 9B11). The presence of CD3 ϵ expression cassette in T cells was marked by the expression of BFP.

[00164] As shown in Fig. 7A and 7B, 7 days after the transduction of primary human CD8+ T cells stimulated with MDA-MB-231 cells, more than 50%, 20% and 17% of cells expressed the Gal4-CD3-PKG-BFP alone, anti-CD19 SynNotch alone, and both, respectively.

Example 6: CD3 ϵ knockout (KO) using CRISPR-Cas9 and isolation purification of CD3-KO cells using magnetic separation

[00165] To knock out CD3 ϵ expression, 4 to 5 days after stimulation with anti-CD3/CD28 beads or MDA-MB-231 cells, primary human CD8+ T cells were electroporated using the Neon Transfection System (ThermoFisher). Specifically, ribonucleoprotein (RNP) was formed by incubating 7.5 pmol of hCD3 ϵ Alt-R CRISPR-Cas9 single guide RNA (sgRNA, IDT # Hs.Cas9.CD3E.1.AC, target-specific sequence: 5'-agggcattgtcaattactg-3'; SEQ ID NO: 16) and 7.5 pmol of Alt-R S.p Cas9 Nuclease V3 (IDT) in 5 μ l Buffer R for 15 mins. The RNP was then mixed with 0.2X10⁶ T cells in Buffer R, loaded into a 10 μ l tip and electroporated using the program #24 (1600 v, 10 ms, 3 pulses). As shown in Fig. 8A and Fig. 8B, three days post electroporation, ~94% T cells were stained negative for both CD3 ϵ and TCR β , indicating efficient knockout of CD3 ϵ and the intracellular retention of the TCR/CD3 complex. To purify the CD3-KO cells, the CD3+ T cells were removed using an EasySep Human CD3 Positive Selection Kit II (StemCell Technologies). The purity of CD-KO cells reached to more than 99% after two rounds of removal with the kit (Fig. 8C).

[00166] To determine whether the lack of CD3 ϵ expression impacts the T cells' proliferation potential, CD3 ϵ knockout was performed as described above but without removing CD3+ T cells and the mixed WT and CD3-KO T cells were cultured for 25 days.

[00167] As shown in Fig. 9, the ratio of CD3-KO to WT T cells stayed relatively constant over time, indicating that CD3-KO T cells had the similar proliferation potential as WT T cells. As shown in Fig. 7C, CD3 knockout did not significantly impact the expression of SynNotch and expression cassette in transduced T cells.

Example 7: CD3 knockout abrogated T cell alloreactive killing

[00168] To determine the cytotoxicity of alloreactive T cells and the impact of CD3 knockout, T cells from an HLA-mismatched donor (A29:02, A30:01; B35:01, B53:01; C04:01) were stimulated with MDA-MB-231 cells and electroporated to knock out CD3 ϵ as in

EXAMPLE 6. Wild type (WT; intact CD3 ϵ) or purified CD3KO cells were co-cultured with MDA-MB-231 cells expressing firefly luciferase (MDA-MB-231-luci) at a 3:1 ratio for 6 hrs. MDA-MB-231-luci cells cultured alone were used as controls. The luciferase activities of the cultures were determined using the Bright-Glo reagent (Promega) and read on a Victor plate reader (PerkinElmer). The percentages of specific % killing were calculated as $100 \times [1 - (\text{luciferase activity of sample} / \text{luciferase activity of control})]$.

[00169] As shown Fig. 13, the WT T cells showed >60% specific killing of MDA-MB-231 cells. In contrast, a negative percentage of specific killing was seen for CD3KO cells. The negative value was a result of slightly more luciferase activities in the co-cultured cells than in the control cells.

Example 8: Engagement of anti-CD19 SynNotch restored CD3 ϵ expression on CD3-KO T cells expressing anti-CD19 SynNotch and Gal4-CD3-PKG-BFP cassette and enabled the cells to activate in response to anti-CD3 stimulation

[00170] To generate CD3-KO T cells expressing anti-CD19 SynNotch and Gal4-CD3-PKG-BFP cassette (CD3KO-19SN- ϵ CS T cells), primary human CD8 $^{+}$ T cells were stimulated with MDA-MB-231 cells, transduced with lentiviral vectors encoding the anti-CD19 SynNotch and Gal4-CD3-PKG-BFP cassette, electroporated to knockout CD3 ϵ , and CD3-negative T cells were purified as described in EXAMPLES 4, 5 and 6. To test the cells' ability to restore CD3 expression and react to anti-CD3 stimulation, 200,000 CD3KO-19SN- ϵ CS T cells were stimulated for 16 hours with 80,000 WT MDA-MB-231 cells or MDA-MB-231 cells expressing human CD19 (**Fig. 10**) in a 96-well tissue culture plate. The T cells were then transferred to a separate well containing anti-CD3/CD28 Dynabeads (Life Technologies) at a 1:1 bead to T cell ratio and incubated for 6 hours in the presence of monensin (Biolegend) and FITC- conjugated anti-human-CD107a antibody (Biolegend, clone H4A3). Beads were then removed, and the cells were fixed in 4% formaldehyde, permeabilized with permeabilization buffer (0.5% BSA, 0.1% saponin, in DPBS/azide), stained with anti-human-CD3 and anti-human-IFN γ (Biolegend clone 4S.B3) antibodies and analyzed using a NovoCyte Quanteon flow cytometer.

[00171] The data are shown in Fig. 11A significant number of BFP $^{+}$ CD3KO-19SN- ϵ CS T cells restored CD3 expression in response to MDA-MB-231-CD19 cells but not to WT MDA-MB-231 cells. Among the T cells with restored CD3 expression, most degranulated as indicated

by the positive CD107a staining (Fig. 11A) and produced IFN γ (Fig. 11B). The result demonstrated that engagement of anti-CD19 SynNotch on CD3KO-19SN- ϵ CS T cells restored CD3 expression and the cells' ability to activate through TCR/CD3 signaling.

Example 9: Alloreactive killing of CD19+ target cells by CD3KO-19SN- ϵ CS T cells

5 [00172] To determine the cytotoxicity of alloreactive CD3KO-19SN- ϵ CS T cells against target cells expressing CD19, primary CD8+ T cells from a donor with HLA-I (A11:01, A30:02; B18:01, B51:01; C05:01, C15:02) mismatched with MDA-MB-231 cells were stimulated with MDA-MB-231 cells, transduced and electroporated to knock out CD3 ϵ as described in EXAMPLES 4, 5 and 6. The cells were sorted and CD3 ϵ BFP⁺MycTag⁺ cells
10 were collected as CD3KO-19SN- ϵ CS T cell. T cells were cultured with either MDA-MB-231 expressing firefly luciferase (MDA-MB-231-luci) or MDA-MB-231-CD19 expressing firefly luciferase (MDA-MB-231-CD19-luci) at a 1.5:1 ratio for 16 hrs. MDA-MB-231-luci and MDA-MB-231-CD19-luci cultured alone were used as respective controls. The luciferase activities of the cultures were determined using the Bright-Glo reagent (Promega) and read
15 on a Victor plate reader (PerkinElmer). The percentages of specific % killing were calculated as $100 \times [1 - (\text{luciferase activity of sample} / \text{luciferase activity of control})]$.

[00173] As shown Fig. 12, alloreactive CD3KO-19SN- ϵ CS T cells displayed a significant higher level of cytotoxicity to MDA-MB-231 cells expressing CD19 than the WT cells. Taken together with Fig. 11, the data demonstrate the ability of alloreactive CD3KO-19SN- ϵ CS T cells to restore TCR/CD3 expression in response to SynNotch engagement and to kill
20 HLA-I mismatched target cells through alloreaction.

Example 10: Alloreactive T cell stimulation and expansion using patient PBMCs or MoDCs

[00174] Based on a previously published protocol (63), PBMCs will be isolated from a
25 patient leukapheresis using density-gradient centrifugation over Ficoll-Paque (MP Biomedicals, Aurora, OH, USA) and washed in OpTmizer culture medium (Thermo Fisher). To generate MoDCs, PBMCs from the patient will be resuspended in culture medium at a final concentration of 3 to 5×10^6 cells/mL and incubated in a standard tissue culture flask for 2 hours at 37°C in a 5% CO₂-containing atmosphere. Nonadherent cells will be removed by
30 vigorous pipetting. The remaining adherent cells will be cultured in medium supplemented with 200 ng/mL recombinant human GM-CSF (R&D Systems, Minneapolis, MN, USA) and 4

ng/mL recombinant human interleukin IL-4 (R&D Systems). Fresh cytokines will be added every 2 to 3 days. For the maturation of DCs, culture medium will be replaced on day 6, and 1100 U/mL recombinant human TNF- α (R&D Systems) is added for 24 hours. The PBMCs and DCs will be irradiated (2500 rads) using a RS2000 irradiator (Radsource) prior to co-culture with T cells. T cells will be purified from the PBMCs of an HLA-mismatched donor using an EasySep human T cell enrichment kit (StemCell Technologies). The T cells will be labeled with 2.5 μ M CFSE in labeling buffer (DPBS with 5% FBS) for 5 mins at room temperature and washed with labeling buffer three times. To stimulate and expand alloreactive T cells, CFSE-labeled T cells will be mixed with either PBMCs or matured DCs at a 1:1 ratio and cultured for 4 days in the presence of 10 ng/ml rh IL2 (R&D Systems). The cells will be harvested, stained with anti-CD3-APC and sorted for the CD3⁺CFSE^{low} population. The cells will be further expanded in culture, used for downstream genetic manipulations immediately, or cryopreserved for future use.

Example 11: Method of in vivo functional study of tumor-activated alloreactive T cells in mouse models

[00175] To study the efficacy and toxicity of tumor activated alloreactive T cells for tumors *in vivo*, MDA-MB-231 cells will be used to form subcutaneous tumors in severely immunocompromised NSG mice (Jax). To mimic the heterogeneity of TAA expression in tumor tissues, the tumors will be formed with a mixture of MDA-MB-231 expressing firefly luciferase and tumor antigen Her2 (MDA-MB-231-luci-Her2) and MDA-MB-231 cells expressing luciferase only (MDA-MB-231-luci) at varying ratios. Tumor development will be monitored by imaging the luciferase activity of the tumors using a IVIS Lumina LT imager (PerkinElmer). After the tumors become detectable, the mice will be treated with tumor activated alloreactive T cells (CD3KO-19SN- ϵ CS T cells), conventional Her2-specific 2nd generation CAR T cells, anti-CD4/CD28 beads-activated but unmodified T cells or left untreated. Tumor activated alloreactive T cells will be prepared using primary human T cells from a donor with HLA-I that mismatches the HLA-I of MDA-MB-231 cells. The T cells will be stimulated with MDA-MB-231 cells, transduced with lentiviral vectors encoding Her2-specific SynNotch and Gal4-CD3-PKG-BFP cassette, and electroporated to knock out CD3 ϵ as described in EXAMPLES 4, 5 and 6. The T cells will be administered through i.v. (intravenous) injection and their efficacy in controlling tumor growth will be monitored by imaging the tumor luciferase activities over time and by comparing the survival curves of

each treatment. The CD3KO-19SN-εCS T cells are predicted to be the most effective in controlling tumor growth, followed by conventional Her2-specific 2nd generation CAR T cells, and followed by anti-CD4/CD28 beads-activated but unmodified T cells. To check the toxicities of therapeutic T cells toward MDA-MB-231-luci-Her2 vs MDA-MB-231-luci, tumors will be removed after treatment, and the ratio and distribution of the two target cell types will be determined using immunohistochemistry by staining human HER2. Tumors treated with CD3KO-19SN-εCS T cells are predicated to have higher MDA-MB-231-luci-Her2 to MDA-MB-231-luci ratio than tumors treated with the conventional Her2-specific 2nd generation CAR T cells. To monitor GvH side effects caused by the therapeutic T cells, non-tumor tissues from the skin, liver, lung and heart will be examined for T cell infiltration using immunohistochemistry. The GvH side effects will also be compared by treating NSG mice without tumors with tumor activated alloreactive T cells, conventional Her2-specific 2nd generation CAR T cells, anti-CD4/CD28 beads-activated but unmodified T cells or left untreated. Weight loss and survival will be monitored and the levels of serum inflammatory cytokine including INF γ , IL2, IL-12, IL-17, IL5 and TNF- α will be monitored and compared. Mice treated with CD3KO-19SN-εCS T cells are predicated to display lower levels of GvH than mice treated with conventional Her2-specific 2nd generation CAR T cells or anti-CD4/CD28 beads-activated but unmodified T cells.

[00176] The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

[00177] Although the present embodiments have been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of these embodiments, and would readily be known to the skilled artisan. The appended Embodiments are intended to be construed to include all such embodiments and equivalent variations.

LIST OF REFERENCES

1. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med.* 2011;365(8):725-33. Epub
5 2011/08/13. doi: 10.1056/NEJMoa1103849. PubMed PMID: 21830940; PMCID: 3387277.
2. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, Stetler-Stevenson M, Phan GQ, Hughes MS, Sherry RM, Yang JC, Kammula US, Devillier L, Carpenter R, Nathan DA, Morgan RA, Laurencot C, Rosenberg SA. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-
10 CD19 chimeric-antigen-receptor-transduced T cells. *Blood.* 2012;119(12):2709-20. Epub 2011/12/14. doi: blood-2011-10-384388 [pii] 10.1182/blood-2011-10-384388. PubMed PMID: 22160384; PMCID: 3327450.
3. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, Milone MC, Levine BL, June CH. Chimeric antigen receptor-
15 modified T cells for acute lymphoid leukemia. *N Engl J Med.* 2013;368(16):1509-18. Epub 2013/03/27. doi: 10.1056/NEJMoa1215134. PubMed PMID: 23527958.
4. Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, Bartido S, Stefanski J, Taylor C, Olszewska M, Borquez-Ojeda O, Qu J, Wasielewska T, He Q, Bernal Y, Rijo IV, Hedvat C, Kobos R, Curran K, Steinherz P, Jurcic J, Rosenblat T, Maslak P, Frattini M,
20 Sadelain M. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci Transl Med.* 2013;5(177):177ra38. Epub 2013/03/22. doi: 5/177/177ra38 [pii] 10.1126/scitranslmed.3005930. PubMed PMID: 23515080.
5. Kochenderfer JN, Wilson WH, Janik JE, Dudley ME, Stetler-Stevenson M, Feldman
25 SA, Maric I, Raffeld M, Nathan DA, Lanier BJ, Morgan RA, Rosenberg SA. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood.* 2010;116(20):4099-102. Epub 2010/07/30. doi: blood-2010-04-281931 [pii] 10.1182/blood-2010-04-281931. PubMed PMID: 20668228; PMCID: 2993617.
- 30 6. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a

- chimeric antigen receptor recognizing ERBB2. *Mol Ther.* 2010;18(4):843-51. Epub 2010/02/25. doi: mt201024 [pii]
10.1038/mt.2010.24. PubMed PMID: 20179677; PMCID: 2862534.
7. Hosonaga M, Arima Y, Sampetean O, Komura D, Koya I, Sasaki T, Sato E, Okano
5 H, Kudoh J, Ishikawa S, Saya H, Ishikawa T. HER2 Heterogeneity Is Associated with Poor Survival in HER2-Positive Breast Cancer. *Int J Mol Sci.* 2018;19(8). doi: 10.3390/ijms19082158. PubMed PMID: 30042341; PMCID: PMC6121890.
 8. Fennemann FL, de Vries IJM, Figdor CG, Verdoes M. Attacking Tumors From All Sides: Personalized Multiplex Vaccines to Tackle Intratumor Heterogeneity. *Front Immunol.*
10 2019;10:824. doi: 10.3389/fimmu.2019.00824. PubMed PMID: 31040852; PMCID: PMC6476980.
 9. Fargion S, Carney D, Mulshine J, Rosen S, Bunn P, Jewett P, Cuttitta F, Gazdar A, Minna J. Heterogeneity of cell surface antigen expression of human small cell lung cancer detected by monoclonal antibodies. *Cancer Res.* 1986;46(5):2633-8. PubMed PMID:
15 3008997.
 10. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrisette JJD, Martinez-Lage M, Brem S, Maloney E, Shen A, Isaacs R, Mohan S, Plesa G, Lacey SF, Navenot JM, Zheng Z, Levine BL, Okada H, June CH, Brogdon JL, Maus MV. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces
20 adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med.* 2017;9(399). doi: 10.1126/scitranslmed.aaa0984. PubMed PMID: 28724573; PMCID: PMC5762203.
 11. Liu X, Jiang S, Fang C, Yang S, Olalere D, Pequignot EC, Cogdill AP, Li N, Ramones M, Granda B, Zhou L, Loew A, Young RM, June CH, Zhao Y. Affinity-Tuned ErbB2 or EGFR Chimeric Antigen Receptor T Cells Exhibit an Increased Therapeutic Index
25 against Tumors in Mice. *Cancer Res.* 2015;75(17):3596-607. doi: 10.1158/0008-5472.CAN-15-0159. PubMed PMID: 26330166; PMCID: PMC4560113.
 12. Kloss CC, Condomines M, Cartellieri M, Bachmann M, Sadelain M. Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. *Nat Biotechnol.* 2013;31(1):71-5. doi: 10.1038/nbt.2459. PubMed PMID:
30 23242161; PMCID: PMC5505184.
 13. Wilkie S, van Schalkwyk MC, Hobbs S, Davies DM, van der Stegen SJ, Pereira AC, Burbidge SE, Box C, Eccles SA, Maher J. Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. *J*

- Clin Immunol. 2012;32(5):1059-70. doi: 10.1007/s10875-012-9689-9. PubMed PMID: 22526592.
14. Roybal KT, Rupp LJ, Morsut L, Walker WJ, McNally KA, Park JS, Lim WA. Precision Tumor Recognition by T Cells With Combinatorial Antigen-Sensing Circuits. *Cell*. 2016;164(4):770-9. doi: 10.1016/j.cell.2016.01.011. PubMed PMID: 26830879; PMCID: PMC4752902.
15. Krenciute G, Prinzing BL, Yi Z, Wu MF, Liu H, Dotti G, Balyasnikova IV, Gottschalk S. Transgenic Expression of IL15 Improves Antiglioma Activity of IL13Ralpha2-CAR T Cells but Results in Antigen Loss Variants. *Cancer Immunol Res*. 2017;5(7):571-81. doi: 10.1158/2326-6066.CIR-16-0376. PubMed PMID: 28550091; PMCID: PMC5746871.
16. Cummins KD, Gill S. Chimeric antigen receptor T-cell therapy for acute myeloid leukemia: how close to reality? *Haematologica*. 2019;104(7):1302-8. doi: 10.3324/haematol.2018.208751. PubMed PMID: 31221785; PMCID: PMC6601074.
17. Ehninger A, Kramer M, Rolig C, Thiede C, Bornhauser M, von Bonin M, Wermke M, Feldmann A, Bachmann M, Ehninger G, Oelschlagel U. Distribution and levels of cell surface expression of CD33 and CD123 in acute myeloid leukemia. *Blood Cancer J*. 2014;4:e218. doi: 10.1038/bcj.2014.39. PubMed PMID: 24927407; PMCID: PMC4080210.
18. Testa U, Pelosi E, Frankel A. CD 123 is a membrane biomarker and a therapeutic target in hematologic malignancies. *Biomark Res*. 2014;2(1):4. doi: 10.1186/2050-7771-2-4. PubMed PMID: 24513123; PMCID: PMC3928610.
19. Friedman KM, Garrett TE, Evans JW, Horton HM, Latimer HJ, Seidel SL, Horvath CJ, Morgan RA. Effective Targeting of Multiple B-Cell Maturation Antigen-Expressing Hematological Malignancies by Anti-B-Cell Maturation Antigen Chimeric Antigen Receptor T Cells. *Hum Gene Ther*. 2018;29(5):585-601. doi: 10.1089/hum.2018.001. PubMed PMID: 29641319; PMCID: PMC5930946.
20. Gaida MM, Welsch T, Herpel E, Tschaharganeh DF, Fischer L, Schirmacher P, Hansch GM, Bergmann F. MHC class II expression in pancreatic tumors: a link to intratumoral inflammation. *Virchows Arch*. 2012;460(1):47-60. doi: 10.1007/s00428-011-1175-x. PubMed PMID: 22120497.
21. Suchin EJ, Langmuir PB, Palmer E, Sayegh MH, Wells AD, Turka LA. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J Immunol*. 2001;166(2):973-81. doi: 10.4049/jimmunol.166.2.973. PubMed PMID: 11145675.

22. DeWolf S, Grinshpun B, Savage T, Lau SP, Obradovic A, Shonts B, Yang S, Morris H, Zuber J, Winchester R, Sykes M, Shen Y. Quantifying size and diversity of the human T cell alloresponse. *JCI Insight*. 2018;3(15). doi: 10.1172/jci.insight.121256. PubMed PMID: 30089728; PMCID: PMC6129121.
- 5 23. Heeger PS. T-cell allorecognition and transplant rejection: a summary and update. *Am J Transplant*. 2003;3(5):525-33. doi: 10.1034/j.1600-6143.2003.00123.x. PubMed PMID: 12752308.
24. Shlomchik WD. Graft-versus-host disease. *Nat Rev Immunol*. 2007;7(5):340-52. doi: 10.1038/nri2000. PubMed PMID: 17438575.
- 10 25. Lee SJ, Klein J, Haagenson M, Baxter-Lowe LA, Confer DL, Eapen M, Fernandez-Vina M, Flomenberg N, Horowitz M, Hurley CK, Noreen H, Oudshoorn M, Petersdorf E, Setterholm M, Spellman S, Weisdorf D, Williams TM, Anasetti C. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood*. 2007;110(13):4576-83. doi: 10.1182/blood-2007-06-097386. PubMed
15 PMID: 17785583.
26. Tahara H, Ide K, Basnet N, Tanaka Y, Ohdan H. Determination of the precursor frequency and the reaction intensity of xenoreactive human T lymphocytes. *Xenotransplantation*. 2010;17(3):188-96. doi: 10.1111/j.1399-3089.2010.00575.x. PubMed PMID: 20636539.
- 20 27. Kolb HJ, Schmid C, Barrett AJ, Schendel DJ. Graft-versus-leukemia reactions in allogeneic chimeras. *Blood*. 2004;103(3):767-76. doi: 10.1182/blood-2003-02-0342. PubMed PMID: 12958064.
28. Scarisbrick JJ, Dignan FL, Tulpule S, Gupta ED, Kolade S, Shaw B, Evison F, Shah G, Tholouli E, Mufti G, Pagliuca A, Malladi R, Raj K. A multicentre UK study of GVHD following DLI: rates of GVHD are high but mortality from GVHD is infrequent. *Bone Marrow Transplant*. 2015;50(1):62-7. doi: 10.1038/bmt.2014.227. PubMed PMID: 25310308.
- 25 29. Marcus A, Eshhar Z. Allogeneic adoptive cell transfer therapy as a potent universal treatment for cancer. *Oncotarget*. 2011;2(7):525-6. doi: 10.18632/oncotarget.300. PubMed
30 PMID: 21719916; PMCID: PMC3248176.
30. Ren J, Liu X, Fang C, Jiang S, June CH, Zhao Y. Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition. *Clin Cancer Res*.

- 2017;23(9):2255-66. doi: 10.1158/1078-0432.CCR-16-1300. PubMed PMID: 27815355; PMCID: PMC5413401.
31. Torikai H, Reik A, Soldner F, Warren EH, Yuen C, Zhou Y, Crossland DL, Huls H, Littman N, Zhang Z, Tykodi SS, Kebriaei P, Lee DA, Miller JC, Rebar EJ, Holmes MC, Jaenisch R, Champlin RE, Gregory PD, Cooper LJ. Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. *Blood*. 2013;122(8):1341-9. doi: 10.1182/blood-2013-03-478255. PubMed PMID: 23741009; PMCID: PMC3750336.
32. Gornalusse GG, Hirata RK, Funk SE, Riobos L, Lopes VS, Manske G, Prunkard D, Colunga AG, Hanafi LA, Clegg DO, Turtle C, Russell DW. HLA-E-expressing pluripotent stem cells escape allogeneic responses and lysis by NK cells. *Nat Biotechnol*. 2017;35(8):765-72. doi: 10.1038/nbt.3860. PubMed PMID: 28504668; PMCID: PMC5548598.
33. Gaj T, Sirk SJ, Shui SL, Liu J. *Genome-Editing Technologies: Principles and Applications*. Cold Spring Harb Perspect Biol. 2016;8(12). doi: 10.1101/cshperspect.a023754. PubMed PMID: 27908936; PMCID: PMC5131771.
34. Hirakawa MP, Krishnakumar R, Timlin JA, Carney JP, Butler KS. Gene editing and CRISPR in the clinic: current and future perspectives. *Biosci Rep*. 2020;40(4). doi: 10.1042/BSR20200127. PubMed PMID: 32207531; PMCID: PMC7146048.
35. Bailey SR, Maus MV. Gene editing for immune cell therapies. *Nat Biotechnol*. 2019;37(12):1425-34. doi: 10.1038/s41587-019-0137-8. PubMed PMID: 31160723.
36. Osborn MJ, Webber BR, Knipping F, Lonetree CL, Tennis N, DeFeo AP, McElroy AN, Starker CG, Lee C, Merkel S, Lund TC, Kelly-Spratt KS, Jensen MC, Voytas DF, von Kalle C, Schmidt M, Gabriel R, Hippen KL, Miller JS, Scharenberg AM, Tolar J, Blazar BR. Evaluation of TCR Gene Editing Achieved by TALENs, CRISPR/Cas9, and megaTAL Nucleases. *Mol Ther*. 2016;24(3):570-81. doi: 10.1038/mt.2015.197. PubMed PMID: 26502778; PMCID: PMC4786913.
37. Choi BD, Yu X, Castano AP, Darr H, Henderson DB, Bouffard AA, Larson RC, Scarfo I, Bailey SR, Gerhard GM, Frigault MJ, Leick MB, Schmidts A, Sagert JG, Curry WT, Carter BS, Maus MV. CRISPR-Cas9 disruption of PD-1 enhances activity of universal EGFRvIII CAR T cells in a preclinical model of human glioblastoma. *J Immunother Cancer*. 2019;7(1):304. doi: 10.1186/s40425-019-0806-7. PubMed PMID: 31727131; PMCID: PMC6857271.

38. Nagarsheth N, Wicha MS, Zou W. Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. *Nat Rev Immunol.* 2017;17(9):559-72. doi: 10.1038/nri.2017.49. PubMed PMID: 28555670; PMCID: PMC5731833.
39. Chesnokova V, Melmed S. Growth hormone in the tumor microenvironment. *Arch Endocrinol Metab.* 2019;63(6):568-75. doi: 10.20945/2359-3997000000186. PubMed PMID: 31939481; PMCID: PMC7025769.
40. Morsut L, Roybal KT, Xiong X, Gordley RM, Coyle SM, Thomson M, Lim WA. Engineering Customized Cell Sensing and Response Behaviors Using Synthetic Notch Receptors. *Cell.* 2016;164(4):780-91. doi: 10.1016/j.cell.2016.01.012. PubMed PMID: 26830878; PMCID: PMC4752866.
41. Schwarz KA, Daringer NM, Dolberg TB, Leonard JN. Rewiring human cellular input-output using modular extracellular sensors. *Nat Chem Biol.* 2017;13(2):202-9. doi: 10.1038/nchembio.2253. PubMed PMID: 27941759.
42. Barnea G, Strapps W, Herrada G, Berman Y, Ong J, Kloss B, Axel R, Lee KJ. The genetic design of signaling cascades to record receptor activation. *Proc Natl Acad Sci U S A.* 2008;105(1):64-9. doi: 10.1073/pnas.0710487105. PubMed PMID: 18165312; PMCID: PMC2224232.
43. Chmielewski M, Abken H. CAR T cells transform to trucks: chimeric antigen receptor-redirectioned T cells engineered to deliver inducible IL-12 modulate the tumour stroma to combat cancer. *Cancer Immunol Immunother.* 2012;61(8):1269-77. doi: 10.1007/s00262-012-1202-z. PubMed PMID: 22274776.
44. Liu Y, Di S, Shi B, Zhang H, Wang Y, Wu X, Luo H, Wang H, Li Z, Jiang H. Armored Inducible Expression of IL-12 Enhances Antitumor Activity of Glypican-3-Targeted Chimeric Antigen Receptor-Engineered T Cells in Hepatocellular Carcinoma. *J Immunol.* 2019;203(1):198-207. doi: 10.4049/jimmunol.1800033. PubMed PMID: 31142602.
45. Chmielewski M, Abken H. CAR T Cells Releasing IL-18 Convert to T-Bet(high) FoxO1(low) Effectors that Exhibit Augmented Activity against Advanced Solid Tumors. *Cell Rep.* 2017;21(11):3205-19. doi: 10.1016/j.celrep.2017.11.063. PubMed PMID: 29241547.
46. Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu Rev Immunol.* 1988;6:629-62. doi: 10.1146/annurev.iy.06.040188.003213. PubMed PMID: 3289580.

47. Labrecque N, Whitfield LS, Obst R, Waltzinger C, Benoist C, Mathis D. How much TCR does a T cell need? *Immunity*. 2001;15(1):71-82. doi: 10.1016/s1074-7613(01)00170-4. PubMed PMID: 11485739.
48. Srivastava S, Salter AI, Liggitt D, Yechan-Gunja S, Sarvothama M, Cooper K,
5 Smythe KS, Dudakov JA, Pierce RH, Rader C, Riddell SR. Logic-Gated ROR1 Chimeric Antigen Receptor Expression Rescues T Cell-Mediated Toxicity to Normal Tissues and Enables Selective Tumor Targeting. *Cancer Cell*. 2019;35(3):489-503 e8. doi: 10.1016/j.ccell.2019.02.003. PubMed PMID: 30889382; PMCID: PMC6450658.
49. Natsume T, Kanemaki MT. Conditional Degrons for Controlling Protein Expression
10 at the Protein Level. *Annu Rev Genet*. 2017;51:83-102. doi: 10.1146/annurev-genet-120116-024656. PubMed PMID: 29178817.
50. Ishigami S, Natsugoe S, Nakajo A, Arigami T, Kitazono M, Okumura H, Matsumoto M, Uchikado Y, Setoyama T, Sasaki K, Aikou T. HLA-class I expression in gastric cancer. *J Surg Oncol*. 2008;97(7):605-8. doi: 10.1002/jso.21029. PubMed PMID: 18459158.
- 15 51. Mizukami Y, Kono K, Maruyama T, Watanabe M, Kawaguchi Y, Kamimura K, Fujii H. Downregulation of HLA Class I molecules in the tumour is associated with a poor prognosis in patients with oesophageal squamous cell carcinoma. *Br J Cancer*. 2008;99(9):1462-7. doi: 10.1038/sj.bjc.6604715. PubMed PMID: 18841157; PMCID: PMC2579690.
- 20 52. Tsukahara T, Kawaguchi S, Torigoe T, Asanuma H, Nakazawa E, Shimozaawa K, Nabeta Y, Kimura S, Kaya M, Nagoya S, Wada T, Yamashita T, Sato N. Prognostic significance of HLA class I expression in osteosarcoma defined by anti-pan HLA class I monoclonal antibody, EMR8-5. *Cancer Sci*. 2006;97(12):1374-80. doi: 10.1111/j.1349-7006.2006.00317.x. PubMed PMID: 16995877.
- 25 53. Kaneko K, Ishigami S, Kijima Y, Funasako Y, Hirata M, Okumura H, Shinchii H, Koriyama C, Ueno S, Yoshinaka H, Natsugoe S. Clinical implication of HLA class I expression in breast cancer. *BMC Cancer*. 2011;11:454. doi: 10.1186/1471-2407-11-454. PubMed PMID: 22014037; PMCID: PMC3214195.
54. Kikuchi E, Yamazaki K, Torigoe T, Cho Y, Miyamoto M, Oizumi S, Hommura F,
30 Dosaka-Akita H, Nishimura M. HLA class I antigen expression is associated with a favorable prognosis in early stage non-small cell lung cancer. *Cancer Sci*. 2007;98(9):1424-30. doi: 10.1111/j.1349-7006.2007.00558.x. PubMed PMID: 17645781.

55. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 2012;21(3):309-22. doi: 10.1016/j.ccr.2012.02.022. PubMed PMID: 22439926.
56. Veglia F, Perego M, Gabrilovich D. Myeloid-derived suppressor cells coming of age. *Nat Immunol*. 2018;19(2):108-19. doi: 10.1038/s41590-017-0022-x. PubMed PMID: 29348500; PMCID: PMC5854158.
57. Park HS, Cho U, Im SY, Yoo CY, Jung JH, Suh YJ, Choi HJ. Loss of Human Leukocyte Antigen Class I Expression Is Associated with Poor Prognosis in Patients with Advanced Breast Cancer. *J Pathol Transl Med*. 2019;53(2):75-85. doi: 10.4132/jptm.2018.10.11. PubMed PMID: 30424591; PMCID: PMC6435992.
58. Cabrera T, Lopez-Nevot MA, Gaforio JJ, Ruiz-Cabello F, Garrido F. Analysis of HLA expression in human tumor tissues. *Cancer Immunol Immunother*. 2003;52(1):1-9. doi: 10.1007/s00262-002-0332-0. PubMed PMID: 12536234.
59. Connor ME, Stern PL. Loss of MHC class-I expression in cervical carcinomas. *Int J Cancer*. 1990;46(6):1029-34. doi: 10.1002/ijc.2910460614. PubMed PMID: 2174412.
60. Okamoto M, Inaba T, Yamada N, Uchida R, Fuchida SI, Okano A, Shimazaki C, Taniwaki M. Expression and role of MHC class I-related chain in myeloma cells. *Cytotherapy*. 2006;8(5):509-16. doi: 10.1080/14653240600957586. PubMed PMID: 17050256.
61. Campanelli R, Palermo B, Garbelli S, Mantovani S, Lucchi P, Necker A, Lantelme E, Giachino C. Human CD8 co-receptor is strictly involved in MHC-peptide tetramer-TCR binding and T cell activation. *Int Immunol*. 2002;14(1):39-44. doi: 10.1093/intimm/14.1.39. PubMed PMID: 11751750.
62. Wooldridge L, Hutchinson SL, Choi EM, Lissina A, Jones E, Mirza F, Dunbar PR, Price DA, Cerundolo V, Sewell AK. Anti-CD8 antibodies can inhibit or enhance peptide-MHC class I (pMHCI) multimer binding: this is paralleled by their effects on CTL activation and occurs in the absence of an interaction between pMHCI and CD8 on the cell surface. *J Immunol*. 2003;171(12):6650-60. doi: 10.4049/jimmunol.171.12.6650. PubMed PMID: 14662868.
63. Scheinberg P, Price DA, Ambrozak DR, Barrett AJ, Douek DC. Alloreactive T cell clonotype recruitment in a mixed lymphocyte reaction: implications for graft engineering. *Exp Hematol*. 2006;34(6):788-95. doi: 10.1016/j.exphem.2006.03.001. PubMed PMID: 16728284.

CLAIMS

What is Claimed is:

1. A genetically modified alloreactive or xenoreactive T cell comprising:
 - (i) genetic disruption of expression of at least one endogenous gene encoding
5 a molecule necessary for T cell receptor (TCR) signaling and T cell activation,
 - (ii) an exogenous nucleotide sequence encoding a tumor-sensing receptor that releases or activates a transcription activator in response to direct or indirect binding to molecules enriched on tumor cells, present in the tumor microenvironment or present in tissues with blood cancer cell accumulation, and
10 (iii) an exogenous nucleotide sequence comprising an expression cassette that expresses a copy of the disrupted endogenous gene of (i) in response to the released or activated transcription activator of (ii),
wherein the genetically modified T cell is alloreactive or xenoreactive.
- 15 2. The genetically modified T cell of Claim 1, wherein the at least one disrupted endogenous gene encoding a molecule necessary for TCR signaling and T cell activation encodes a transmembrane protein selected from CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58, or encodes an intracellular signaling molecule chosen from Lck, Zap70, calcineurin, PI3K, Fyn,
20 PLC γ , SLP76, PKC θ , AKT, and PDK1.
3. The genetically modified T cell of Claim 1 or Claim 2, wherein the tumor-sensing receptor comprises (i) an extracellular domain that binds directly or indirectly to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and tissues
25 with blood cancer cell accumulation; and (ii) an intracellular domain that activates or releases a transcription activator in response to extracellular domain binding to the target molecule.
4. The genetically modified T cell of Claim 3, wherein the tumor-sensing receptor is a Synthetic Notch (SynNotch) receptor, a Modular Extracellular Sensor Architecture (MESA)

receptor, or a Tango receptor, and wherein an intracellular transcription activator is released from the receptor in response to extracellular domain binding to the target molecule.

5 5. The genetically modified T cell of Claim 3, wherein the tumor-sensing receptor is a chimeric antigen receptor (CAR) with intracellular ITAM domains, wherein an endogenous transcription factor is activated through signaling pathways in response to extracellular domain binding to the target molecule.

10 6. The genetically modified T cell of any one of Claims 1 to 5, wherein the extracellular domain of the tumor-sensing receptor is a single chain variable fragment (scFv), a Fab fragment, a designed ankyrin repeat protein (DARPin), a TCR, a nanobody, a Fc receptor, a growth factor receptor, a chemokine receptor, or a hormone receptor.

15 7. The genetically modified T cell of any one of Claims 3 to 6, wherein the target molecule is enriched on tumor cells and/or in the tumor microenvironment and is chosen from CD19, CD20, CD38, CD30, Her2/neu, ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2 (VEGFR2), high molecular weight- melanoma associated antigen
20 (HMW-MAA), MAGE-A1, IL-13R-a2, GD2, 4-1BB, 5T4, adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), C-MET, CCR4, CD152, CD19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4, DRS, EGFR, EpCAM, FAP, fibronectin extra domain-B, folate receptor
25 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgG1, LI-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin $\alpha\beta$ 3, MORAb-009, MS4A1, MUC1, mucin CanAg, N- glycolylneuraminic acid, NPC-IC, PDGF-Ra, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF- β ,
30 TRAIL-R1, TRAIL-R2, tumor antigen CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, mesothelin, NKG2D, CD147, NKR2, B7H3, and vimentin, or .

wherein the target molecule is enriched in a tissue with blood cancer cell accumulation, wherein the tissue is lymphoid and/or bone marrow tissue, and wherein the target molecule is chosen from CD45, CD19, CD20, CD4, CD8, CD2, CCR4, CD58, CD28, CD23, CD69, CD25, CD33, CD123 and CCL-1.

5

8. The genetically modified T cell of any one of Claims 1 to 7, wherein the expression cassette comprises a transcription control element operably linked to a copy of the disrupted gene of Claim 1(ii), wherein the expression of the disrupted gene is driven by the binding of the transcription control unit by a transcription activator selected from the group consisting of Gal4-VP64, Notch, tet transactivator, NFAT, AP-1, NFκB/Rel, NR4A1 (Nur77), T-Bet, IRF4, STATs and comesodermin (Eomes).

10

9. A method for producing tumor-activated alloreactive or xenoreactive T cells, said method comprising:

15

- a) selecting a sample of T cells from a donor individual, or from a donor animal;
- b) optionally stimulating the sample of T cells to proliferate;
- c) abrogating the expression or function of at least one molecule necessary for TCR signaling and T cell activation in the T cells to render the T cells activation-incompetent; and

20

d) modifying the T cells to (i) express a recombinant receptor that specifically binds to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and a tissue with blood cancer cell accumulation, wherein binding of the recombinant receptor with the target molecule releases or activates a transcription activator; and (ii) introduce an expression cassette that enables the transcription activator in (i) to drive the expression of the molecule abrogated in c), thereby restores the expression or function of the abrogated molecule, and thereby restores the ability of the T cells to activate through antigen recognition by TCR, thereby producing tumor-activated alloreactive or xenoreactive T cells.

25

10. The method of Claim 9, wherein step d) is performed before step c), step c) is performed before step d), or steps c) and d) are performed at the same time.

30

11. The method of Claim 9 or 10, wherein the sample of T cells is from a donor individual, and wherein the donor individual has at least one HLA allele mismatch relative to an intended recipient and the at least one HLA allele mismatch is located in a locus selected from the group consisting of: HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DPA1 and HLA-DPB1.

12. The method of any one of Claims 9 to 11, wherein step b) comprises: (i) co-culturing donor T cells with cells from an intended recipient; (ii) co-culturing donor T cells with cells from a second donor that (1) has at least one HLA allele matched with the intended recipient, and (2) is mismatched with the T cell donor; (3) co-culturing donor T cells with at least one cell line expressing a least one HLA allele of the intended recipient; (4) co-culturing donor T cells with an artificial surface coated with at least one protein encoded by at least one HLA allele of the intended recipient.

13. The method of any one of Claims 9 to 12, wherein the at least one molecule necessary for TCR signaling and T cell activation is a cell surface molecule chosen from CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58, or an intracellular signaling molecule chosen from Lck, Zap70, calcineurin, PI3K, Fyn, PLC γ , SLP76, PKC θ , AKT, and PDK1.

14. The method of any one of Claims 9 to 13, wherein step d) comprises introducing a nucleic acid encoding a tumor-sensing receptor into T cells, wherein the tumor-sensing receptor comprises (i) an extracellular domain that binds directly or indirectly to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and tissues with blood cancer cell accumulation; and (ii) an intracellular domain that activates or releases a transcription activator in response to extracellular domain binding to the target molecule.

15. The method of Claim 14, wherein the tumor-sensing receptor is a Synthetic Notch (SynNotch) receptor, a Modular Extracellular Sensor Architecture (MESA) receptor, or a Tango receptor, and wherein an intracellular transcription activator is released from the receptor in response to extracellular domain binding to the target molecule.

16. The method of Claim 14, wherein the tumor-sensing receptor is a chimeric antigen receptor (CAR) with intracellular ITAM domains, wherein an endogenous transcription factor is activated through signaling pathways activated in response to extracellular domain binding to the target molecule.

17. The method of Claims 9 to 16, where in the tumor-sensing receptor is a single chain variable fragment (scFv), a Fab fragment, a designed ankyrin repeat protein (DARPin), a nanobody, a TCR, a Fc receptor, a growth factor receptor, a chemokine receptor, or a hormone receptor.

18. The method of any one of Claims 9 to 17, wherein the target molecule is enriched on tumor cells and/or in the tumor microenvironment and is chosen from CD19, CD20, CD38, CD30, Her2/neu, ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2 (VEGFR2), high molecular weight- melanoma associated antigen (HMW-MAA), MAGE-A1, IL-13R-a2, GD2, 4-1BB, 5T4, adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), C-MET, CCR4, CD152, CD19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4, DRS, EGFR, EpCAM, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgG1, LI-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin $\alpha\beta$ 3, MORAb-009, MS4A1, MUC1, mucin CanAg, N- glycolylneuraminic acid, NPC-IC, PDGF-Ra, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF- β , TRAIL-R1, TRAIL-R2, tumor antigen CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, mesothelin, NKG2D, CD147, NKR2, B7H3, and vimentin,

or

wherein the target molecule is enriched in a tissue with blood cancer cell accumulation, wherein the tissue is lymphoid and/or bone marrow tissue, and wherein the target molecule is chosen from CD45, CD19, CD20, CD4, CD8, CD2, CCR4, CD58, CD28, CD23, CD69, CD25, CD33, CD123 and CCL-1.

5

19. The method of any one of Claims 9 to 18, wherein step d) comprises introducing an expression cassette comprising a transcription control element operably linked to a copy of the disrupted gene of Claim 1(ii), wherein the expression of the disrupted gene is driven by the binding of the transcription control unit by a transcription activator selected from the
10 group consisting of Gal4-VP64, Notch, tet transactivator, NFAT, AP-1, NFκB/Rel, NR4A1 (Nur77), T-Bet, IRF4, STATs and eomesodermin (Eomes).

20. A method of treating cancer in a patient by administering T cells of any one of Claims 1 to 8 or prepared by the method of any one of Claims 9 to 19.

15

21. The method of Claim 20, wherein the T cells are tumor-activated alloreactive T cells prepared by the method of Claim 11.

AMENDED CLAIMS

received by the International Bureau on 21 December 2021 (21.12.2021)

1. A genetically modified alloreactive or xenoreactive T cell comprising:

(i) genetic disruption of expression of at least one endogenous gene encoding a molecule necessary for T cell receptor (TCR) signaling and T cell activation,

(ii) an exogenous nucleotide sequence encoding a tumor-sensing receptor that releases or activates a transcription activator in response to direct or indirect binding to molecules enriched on tumor cells, present in the tumor microenvironment or present in tissues with blood cancer cell accumulation, and

(iii) an exogenous nucleotide sequence comprising an expression cassette that expresses a copy of the disrupted endogenous gene of (i) in response to the released or activated transcription activator of (ii),

wherein the genetically modified T cell is alloreactive or xenoreactive.

2. The genetically modified T cell of Claim 1, wherein the at least one disrupted endogenous gene encoding a molecule necessary for TCR signaling and T cell activation encodes a transmembrane protein selected from CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58, or encodes an intracellular signaling molecule chosen from Lck, Zap70, calcineurin, PI3K, Fyn, PLC γ , SLP76, PKC θ , AKT, and PDK1.

3. The genetically modified T cell of Claim 1 or Claim 2, wherein the tumor-sensing receptor comprises (i) an extracellular domain that binds directly or indirectly to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and tissues with blood cancer cell accumulation; and (ii) an intracellular domain that activates or releases a transcription activator in response to extracellular domain binding to the target molecule.

4. The genetically modified T cell of Claim 3, wherein the tumor-sensing receptor is a Synthetic Notch (SynNotch) receptor, a Modular Extracellular Sensor Architecture (MESA) receptor, or a Tango receptor, and wherein an intracellular transcription activator is released from the receptor in response to extracellular domain binding to the target molecule.

5. The genetically modified T cell of Claim 3, wherein the tumor-sensing receptor is a chimeric antigen receptor (CAR) with intracellular ITAM domains, wherein an endogenous transcription factor is activated through signaling pathways in response to extracellular domain binding to the target molecule.

6. The genetically modified T cell of any one of Claims 1 to 5, wherein the extracellular domain of the tumor-sensing receptor is a single chain variable fragment (scFv), a Fab fragment, a designed ankyrin repeat protein (DARPin), a TCR, a nanobody, a Fc receptor, a growth factor receptor, a chemokine receptor, or a hormone receptor.

7. The genetically modified T cell of any one of Claims 3 to 6, wherein the target molecule is enriched on tumor cells and/or in the tumor microenvironment and is chosen from CD19, CD20, CD38, CD30, Her2/neu, ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2 (VEGFR2), high molecular weight- melanoma associated antigen (HMW-MAA), MAGE-A1, IL-13R-a2, GD2, 4-1BB, 5T4, adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), C-MET, CCR4, CD152, CD19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4, DRS, EGFR, EpCAM, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgG1, LI-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin $\alpha\beta$ 3, MORAb-009, MS4A1, MUC1, mucin CanAg, N- glycolylneuraminic acid, NPC-IC, PDGF-Ra, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF- β ,

TRAIL-R1, TRAIL-R2, tumor antigen CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, mesothelin, NKG2D, CD147, NKR2, B7H3, and vimentin, or .

wherein the target molecule is enriched in a tissue with blood cancer cell accumulation, wherein the tissue is lymphoid and/or bone marrow tissue, and wherein the target molecule is chosen from CD45, CD19, CD20, CD4, CD8, CD2, CCR4, CD58, CD28, CD23, CD69, CD25, CD33, CD123 and CCL-1.

8. The genetically modified T cell of any one of Claims 1 to 7, wherein the expression cassette comprises a transcription control element operably linked to a copy of the disrupted gene of Claim 1(ii), wherein the expression of the disrupted gene is driven by the binding of the transcription control unit by a transcription activator selected from the group consisting of Gal4-VP64, Notch, tet transactivator, NFAT, AP-1, NFκB/Rel, NR4A1 (Nur77), T-Bet, IRF4, STATs and eomesodermin (Eomes).

9. A method for producing tumor-activated alloreactive or xenoreactive T cells, said method comprising:

- a) selecting a sample of T cells from a donor individual, or from a donor animal;
- b) optionally stimulating the sample of T cells to proliferate;
- c) abrogating the expression or function of at least one molecule necessary for TCR signaling and T cell activation in the T cells to render the T cells activation-incompetent; and
- d) modifying the T cells to (i) express a recombinant receptor that specifically binds to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and a tissue with blood cancer cell accumulation, wherein binding of the recombinant receptor with the target molecule releases or activates a transcription activator; and (ii) introduce an expression cassette that enables the transcription activator in (i) to drive the expression of the molecule abrogated in c), thereby restores the expression or function of the abrogated molecule, and thereby restores the ability of the T cells to activate through antigen recognition by TCR, thereby producing tumor-activated alloreactive or xenoreactive T cells.

10. The method of Claim 9, wherein step d) is performed before step c), step c) is performed before step d), or steps c) and d) are performed at the same time.

11. The method of Claim 9 or 10, wherein the sample of T cells is from a donor individual, and wherein the donor individual has at least one HLA allele mismatch relative to an intended recipient and the at least one HLA allele mismatch is located in a locus selected from the group consisting of: HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DPA1 and HLA-DPB1.

12. The method of any one of Claims 9 to 11, wherein step b) comprises: (i) co-culturing donor T cells with cells from an intended recipient; (ii) co-culturing donor T cells with cells from a second donor that (1) has at least one HLA allele matched with the intended recipient, and (2) is mismatched with the T cell donor; (3) co-culturing donor T cells with at least one cell line expressing a least one HLA allele of the intended recipient; (4) co-culturing donor T cells with an artificial surface coated with at least one protein encoded by at least one HLA allele of the intended recipient.

13. The method of any one of Claims 9 to 12, wherein the at least one molecule necessary for TCR signaling and T cell activation is a cell surface molecule chosen from CD3 ϵ , CD3 ζ , CD3 γ , CD3 δ , CD4, CD8 α , CD8 β , LAT, TRIM, CD45, CD28, LFA-1, CD2, CD54, CD52, CD148, and CD58, or an intracellular signaling molecule chosen from Lck, Zap70, calcineurin, PI3K, Fyn, PLC γ , SLP76, PKC θ , AKT, and PDK1.

14. The method of any one of Claims 9 to 13, wherein step d) comprises introducing a nucleic acid encoding a tumor-sensing receptor into T cells, wherein the tumor-sensing receptor comprises (i) an extracellular domain that binds directly or indirectly to a target molecule enriched on or in at least one of tumor cells, tumor microenvironment, and tissues with blood cancer cell accumulation; and (ii) an intracellular domain that activates or releases a transcription activator in response to extracellular domain binding to the target molecule.

15. The method of Claim 14, wherein the tumor-sensing receptor is a Synthetic Notch (SynNotch) receptor, a Modular Extracellular Sensor Architecture (MESA) receptor, or a

Tango receptor, and wherein an intracellular transcription activator is released from the receptor in response to extracellular domain binding to the target molecule.

16. The method of Claim 14, wherein the tumor-sensing receptor is a chimeric antigen receptor (CAR) with intracellular ITAM domains, wherein an endogenous transcription factor is activated through signaling pathways activated in response to extracellular domain binding to the target molecule.

17. The method of Claims 9 to 16, where in the tumor-sensing receptor is a single chain variable fragment (scFv), a Fab fragment, a designed ankyrin repeat protein (DARPin), a nanobody, a TCR, a Fc receptor, a growth factor receptor, a chemokine receptor, or a hormone receptor.

18. The method of any one of Claims 9 to 17, wherein the target molecule is enriched on tumor cells and/or in the tumor microenvironment and is chosen from CD19, CD20, CD38, CD30, Her2/neu, ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2 (VEGFR2), high molecular weight- melanoma associated antigen (HMW-MAA), MAGE-A1, IL-13R-a2, GD2, 4-1BB, 5T4, adenocarcinoma antigen, alpha-fetoprotein, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA-IX), C-MET, CCR4, CD152, CD19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4, DRS, EGFR, EpCAM, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF-I, IgG1, LI-CAM, IL-13, IL-6, insulin-like growth factor I receptor, integrin $\alpha\beta$ 3, MORAb-009, MS4A1, MUC1, mucin CanAg, N- glycolylneuraminic acid, NPC-IC, PDGF-Ra, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF- β , TRAIL-R1, TRAIL-R2, tumor antigen CTAA16.88, VEGF-A, VEGFR-1, VEGFR2, mesothelin, NKG2D, CD147, NKR2, B7H3, and vimentin,

or

wherein the target molecule is enriched in a tissue with blood cancer cell accumulation, wherein the tissue is lymphoid and/or bone marrow tissue, and wherein the target molecule is chosen from CD45, CD19, CD20, CD4, CD8, CD2, CCR4, CD58, CD28, CD23, CD69, CD25, CD33, CD123 and CCL-1.

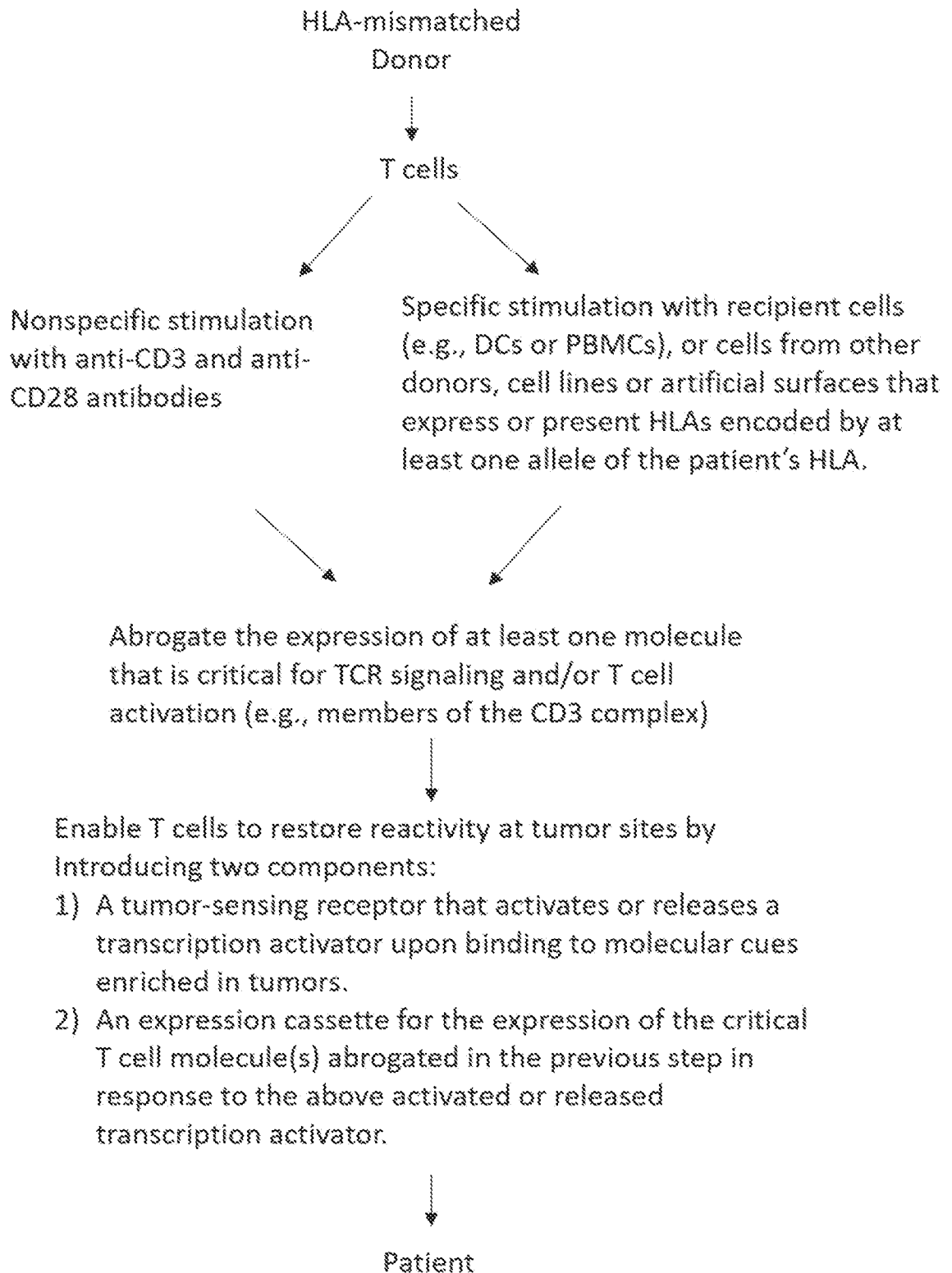
19. The method of any one of Claims 9 to 18, wherein step d) comprises introducing an expression cassette comprising a transcription control element operably linked to a copy of the disrupted gene of Claim 9c), wherein the expression of the disrupted gene is driven by the binding of the transcription control unit by a transcription activator selected from the group consisting of Gal4-VP64, Notch, tet transactivator, NFAT, AP-1, NFκB/Rel, NR4A1 (Nur77), T-Bet, IRF4, STATs and eomesodermin (Eomes).

20. A method of treating cancer in a patient by administering T cells of any one of Claims 1 to 8 or prepared by the method of any one of Claims 9 to 19.

21. The method of Claim 20, wherein the T cells are tumor-activated alloreactive T cells prepared by the method of Claim 11.

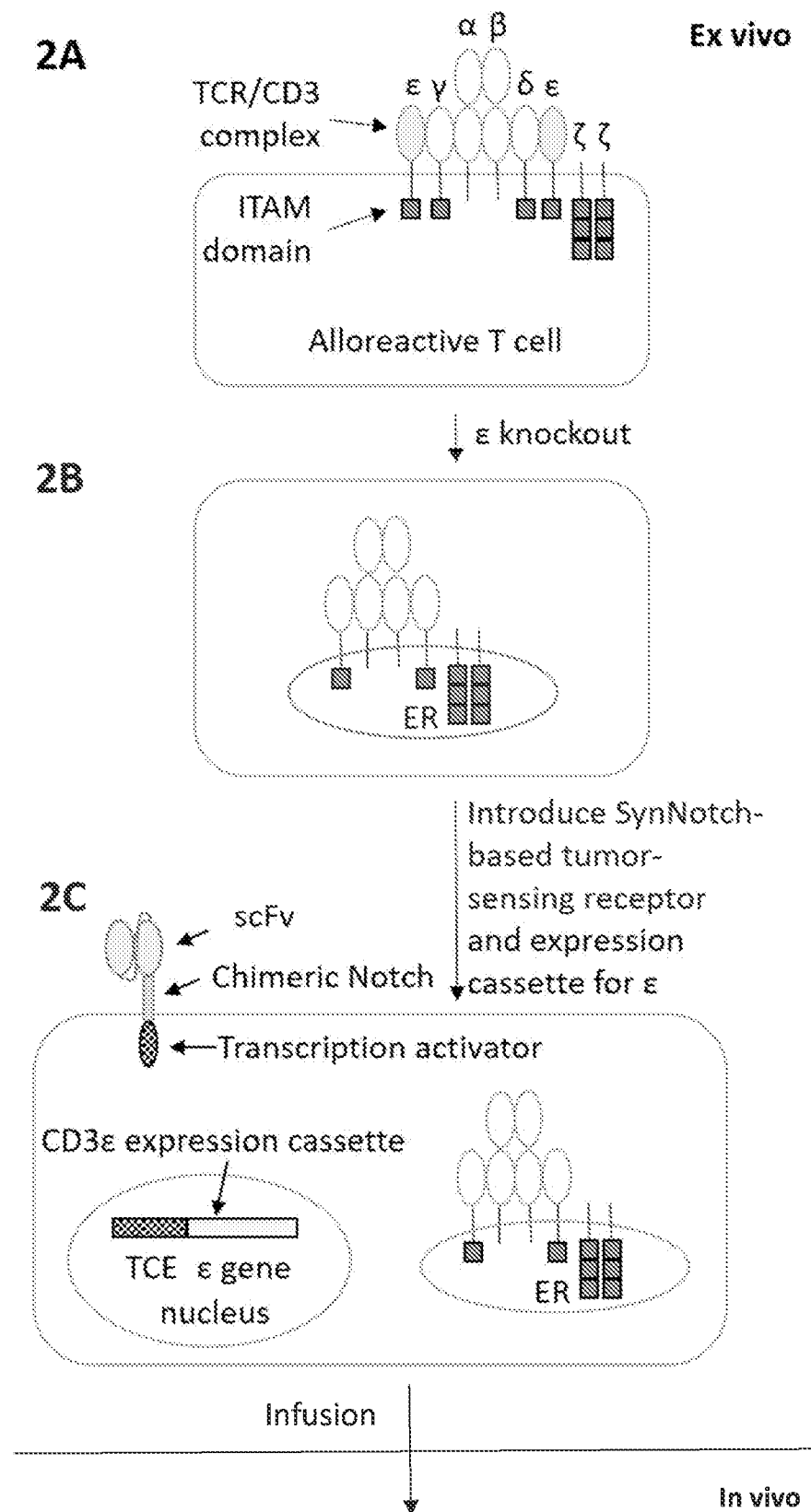
1/16

FIGURE 1

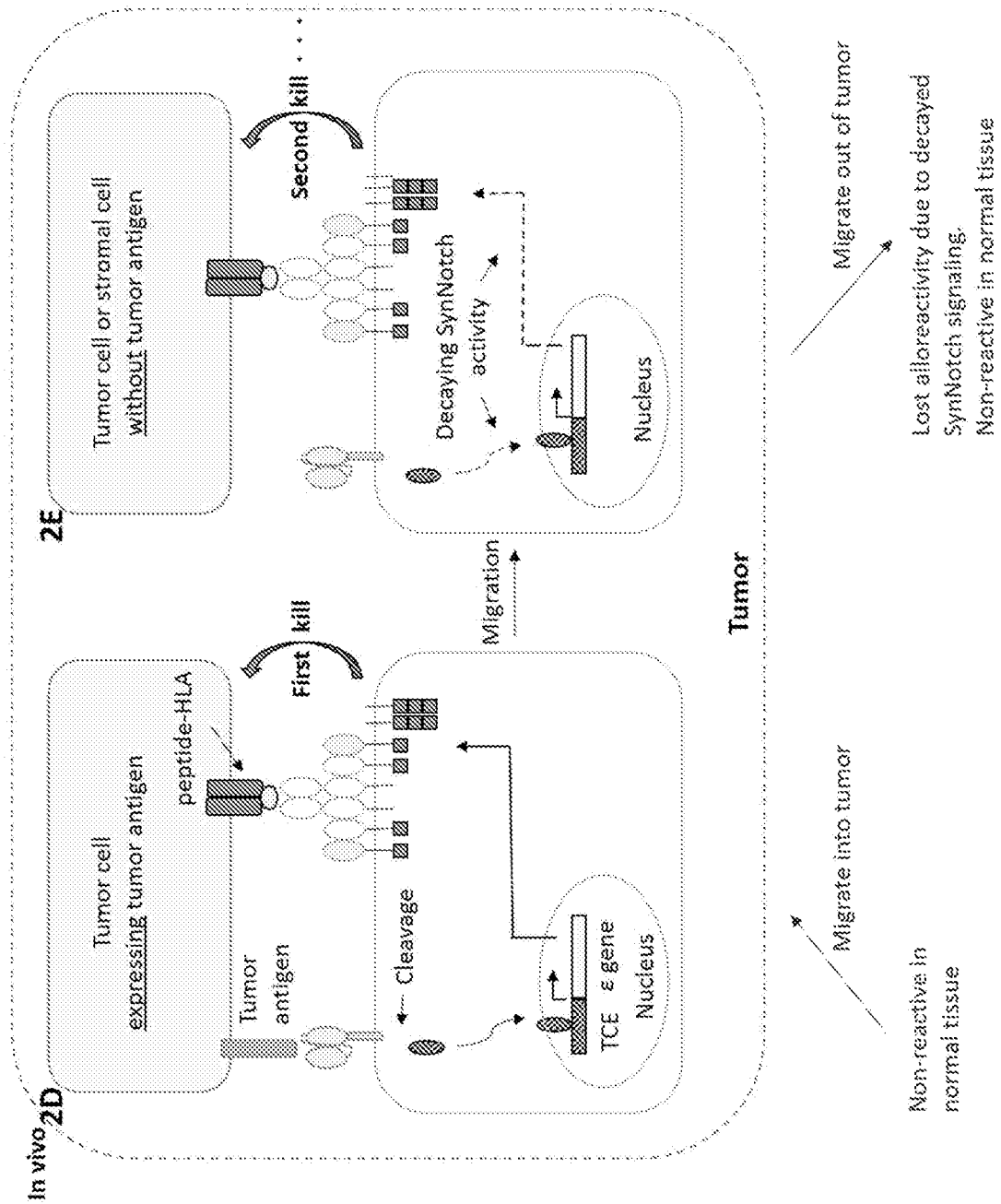


2/16

FIGURES 2A-2C



FIGURES 2D-2E



4/16

FIGURE 3

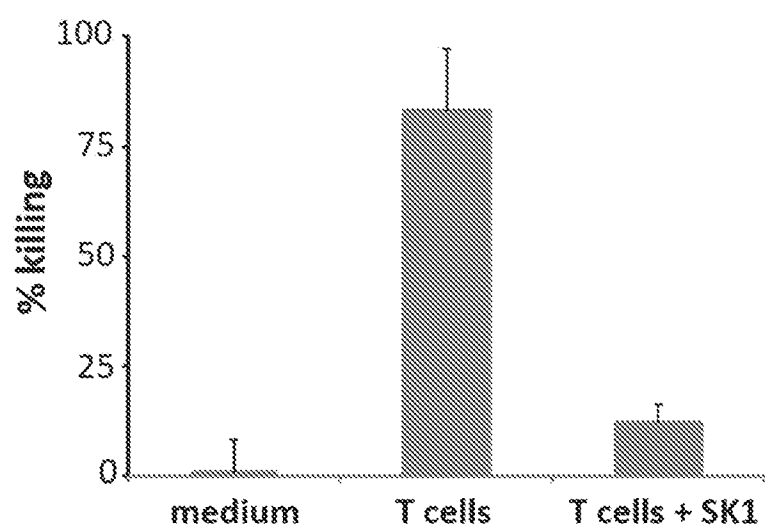


FIGURE 4

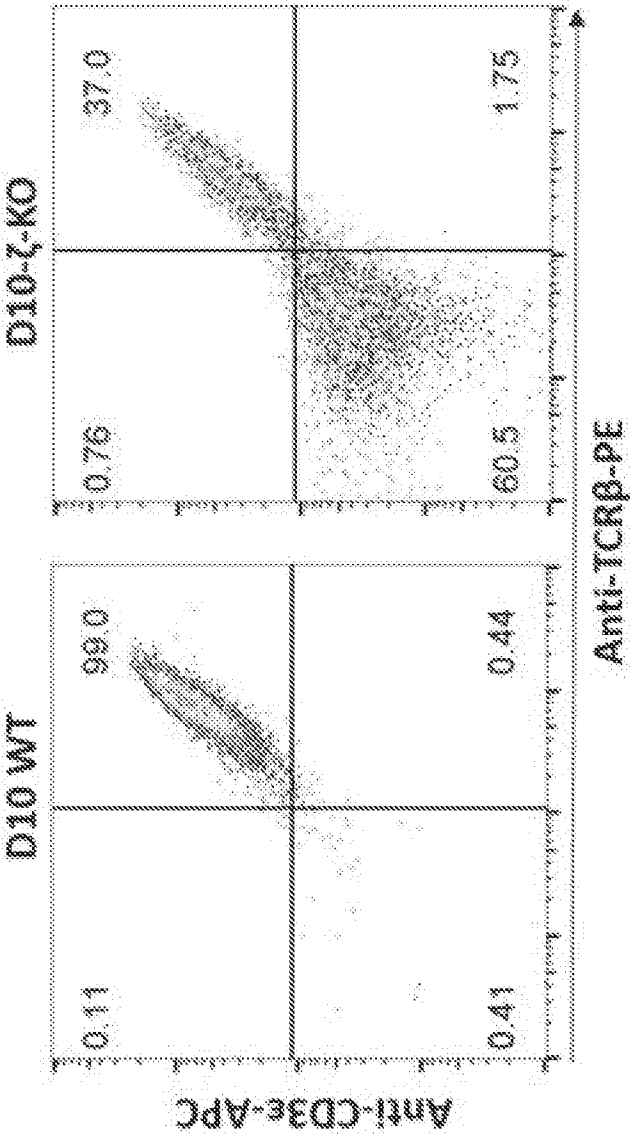
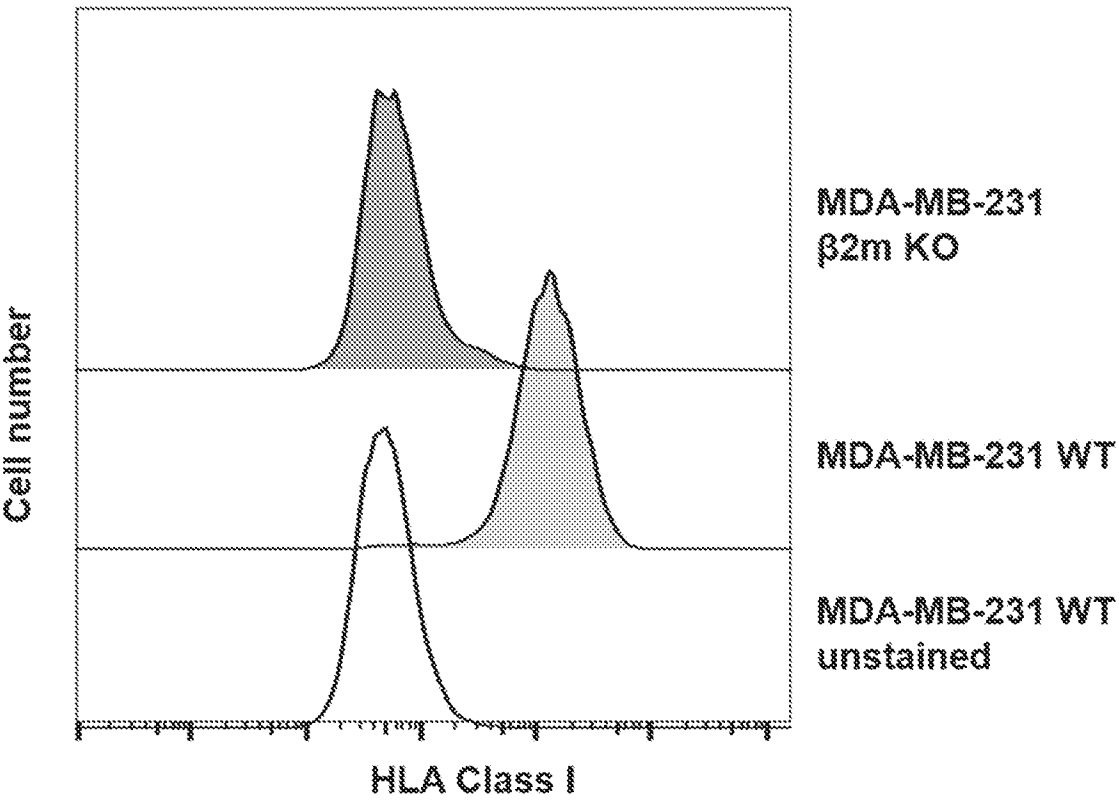


FIGURE 5



7/16

FIGURE 6

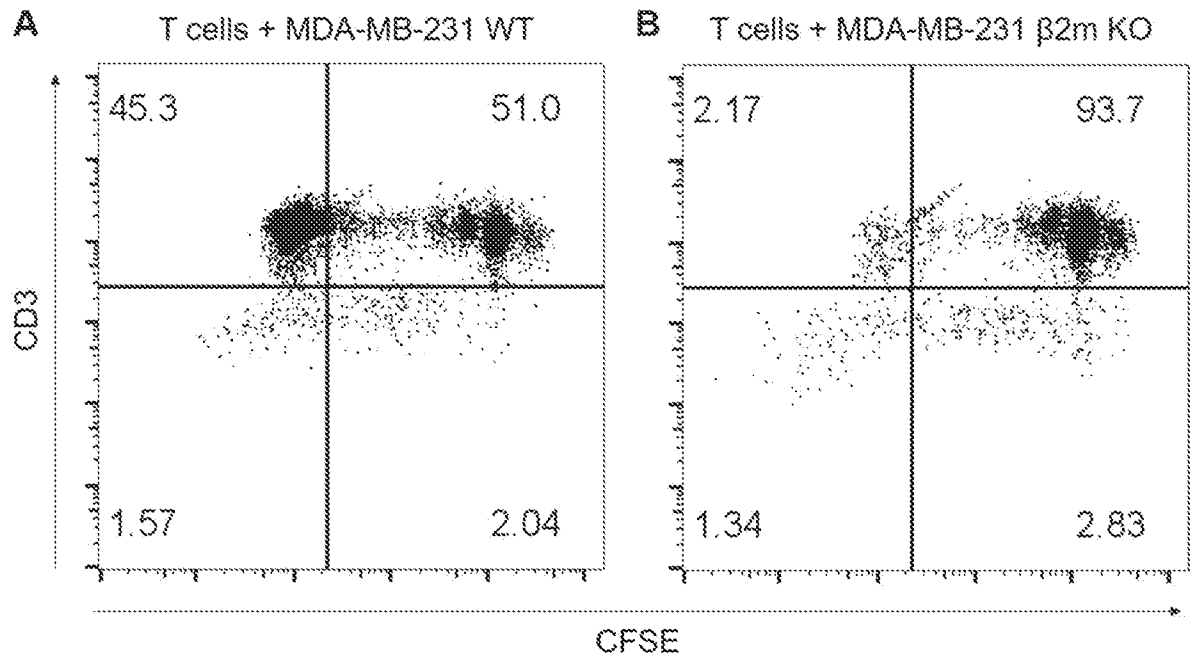


FIGURE 7

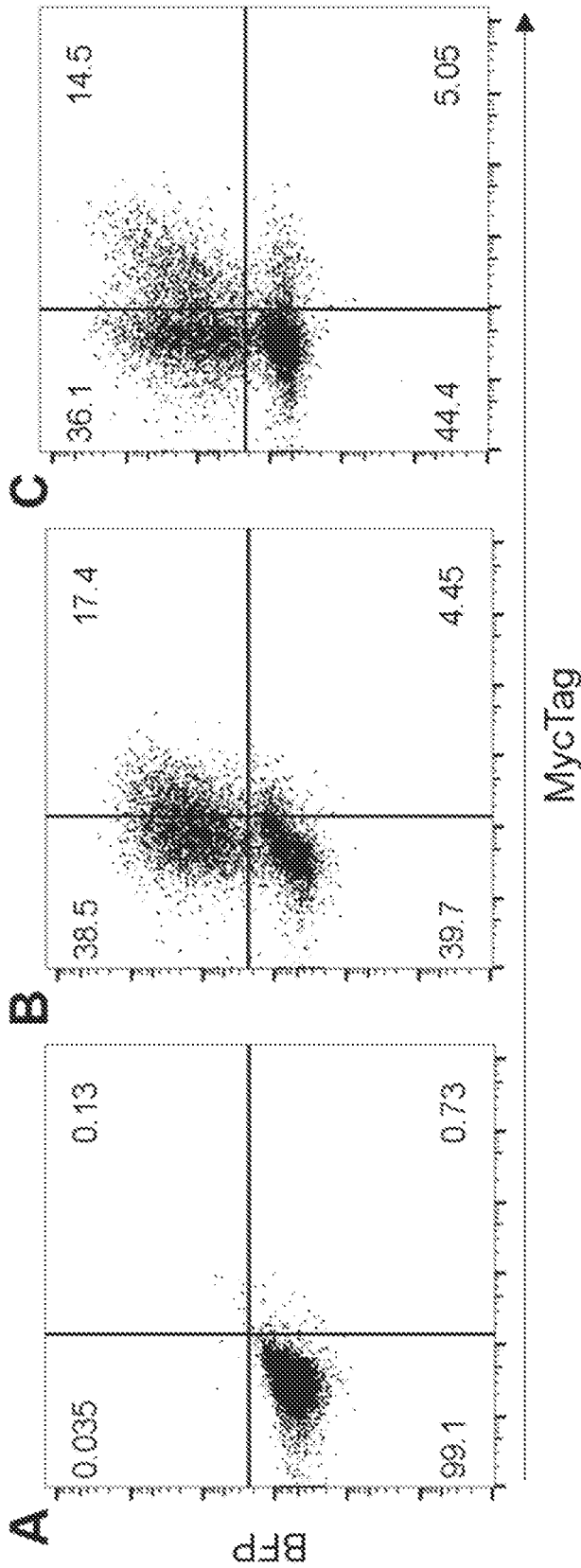
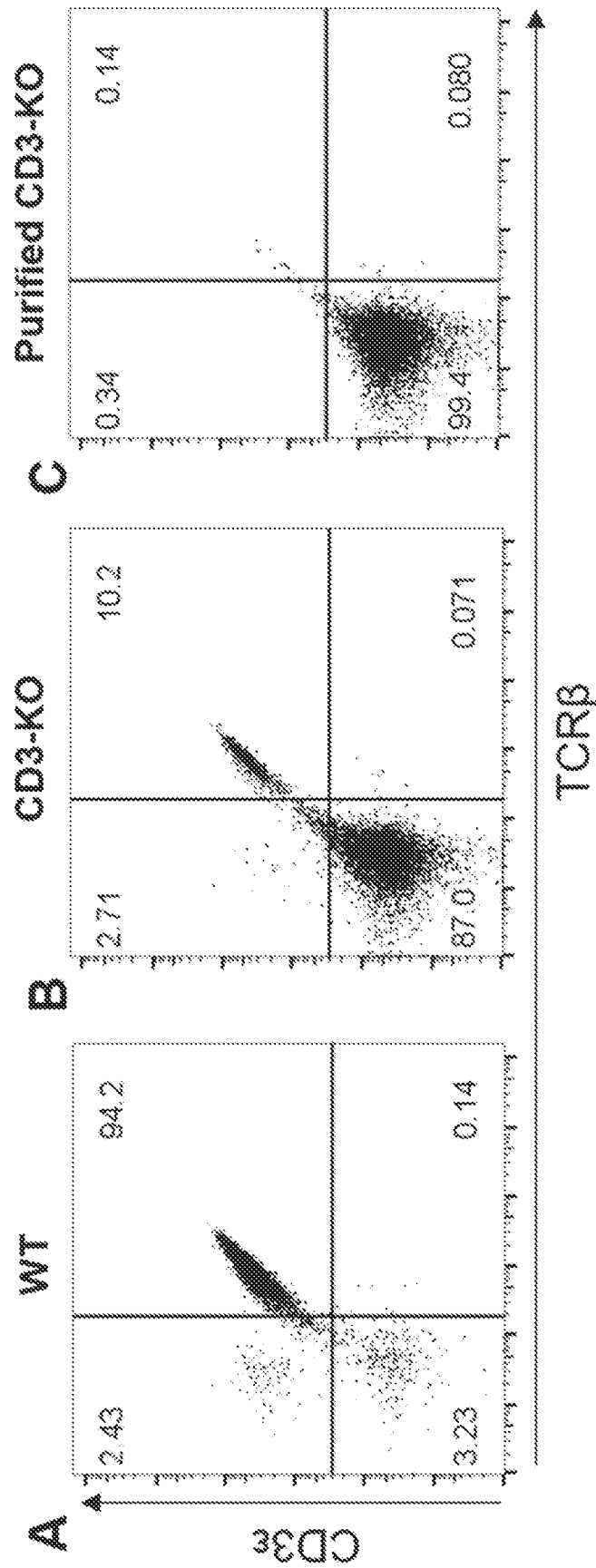
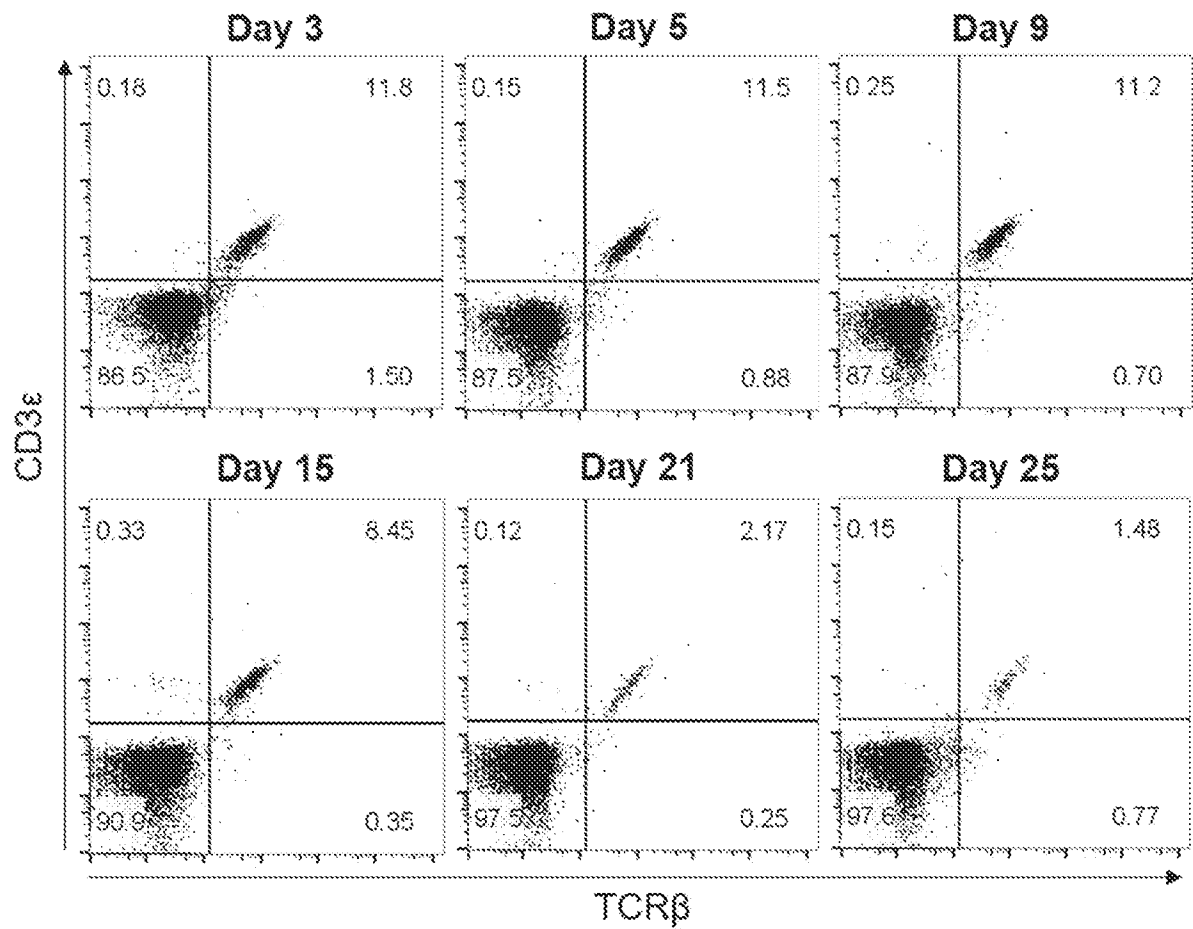


FIGURE 8



10/16

FIGURE 9



11/16

FIGURE 10

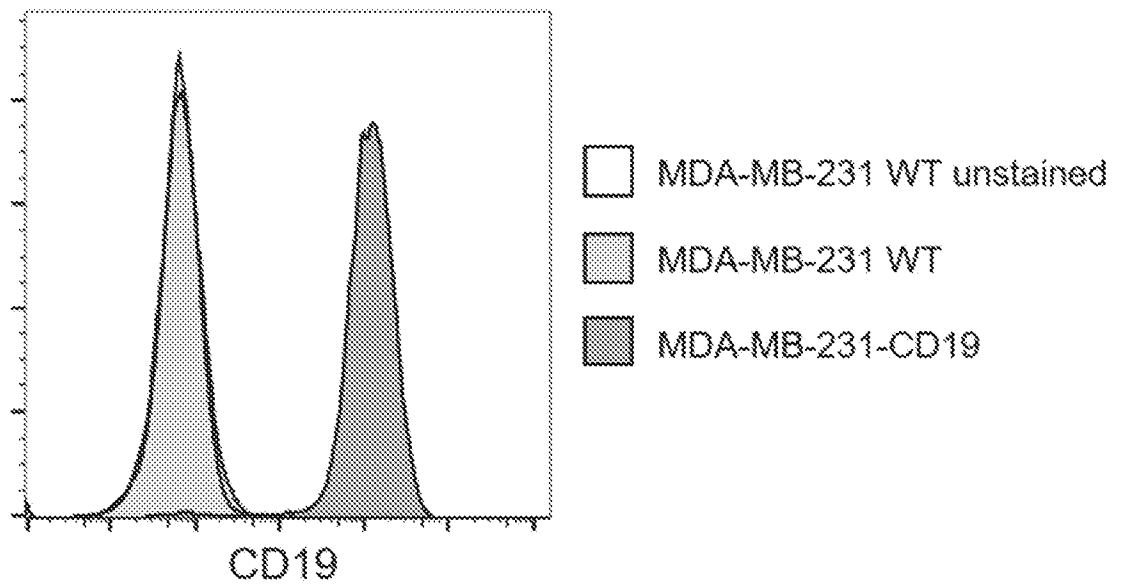
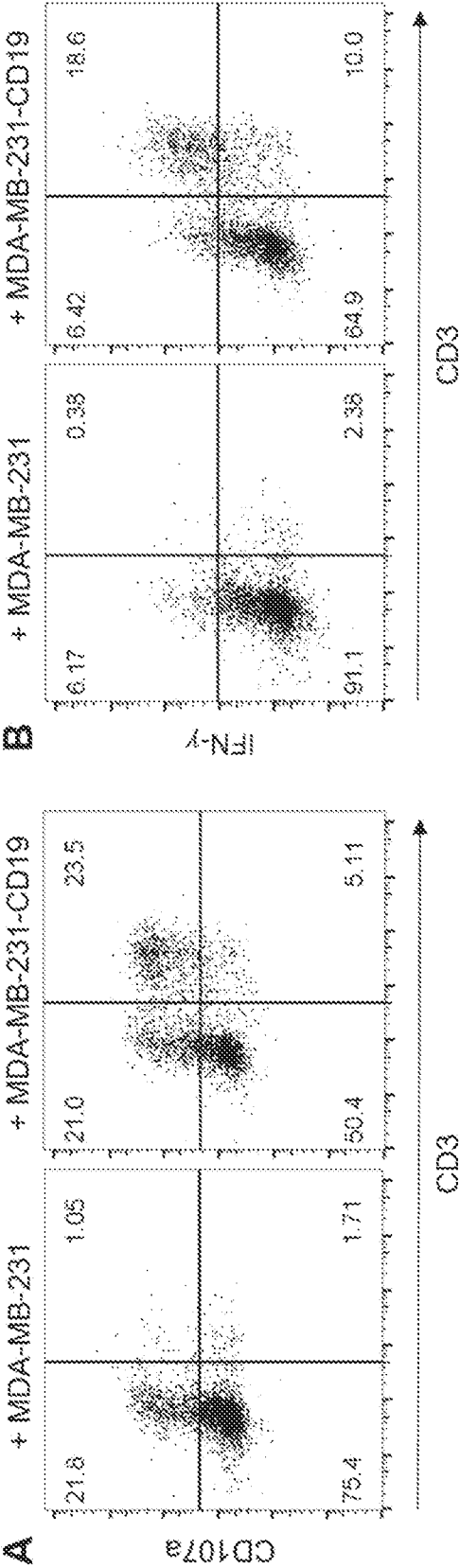
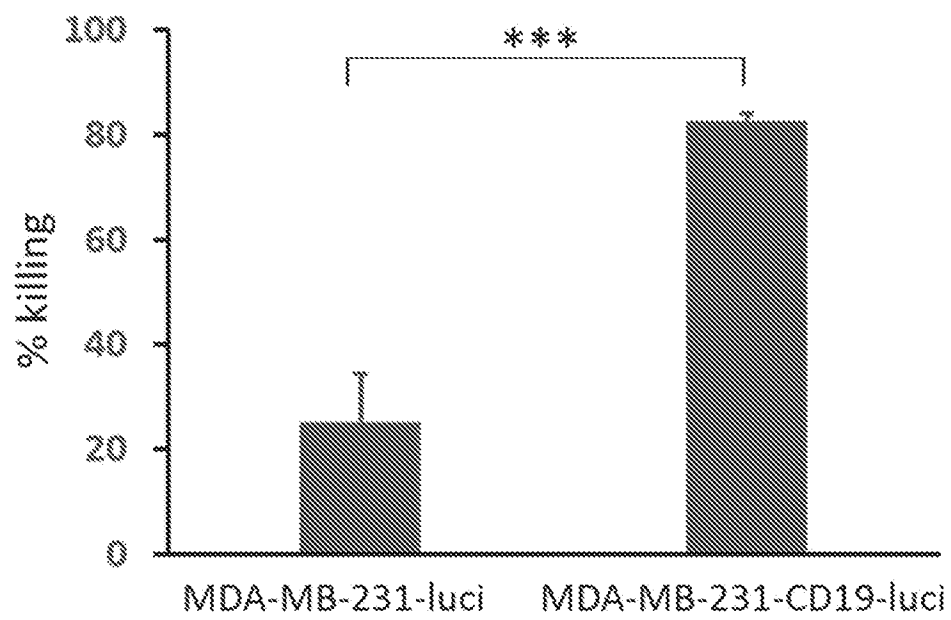


FIGURE 11



13/16

FIGURE 12



14/16

FIGURE 13

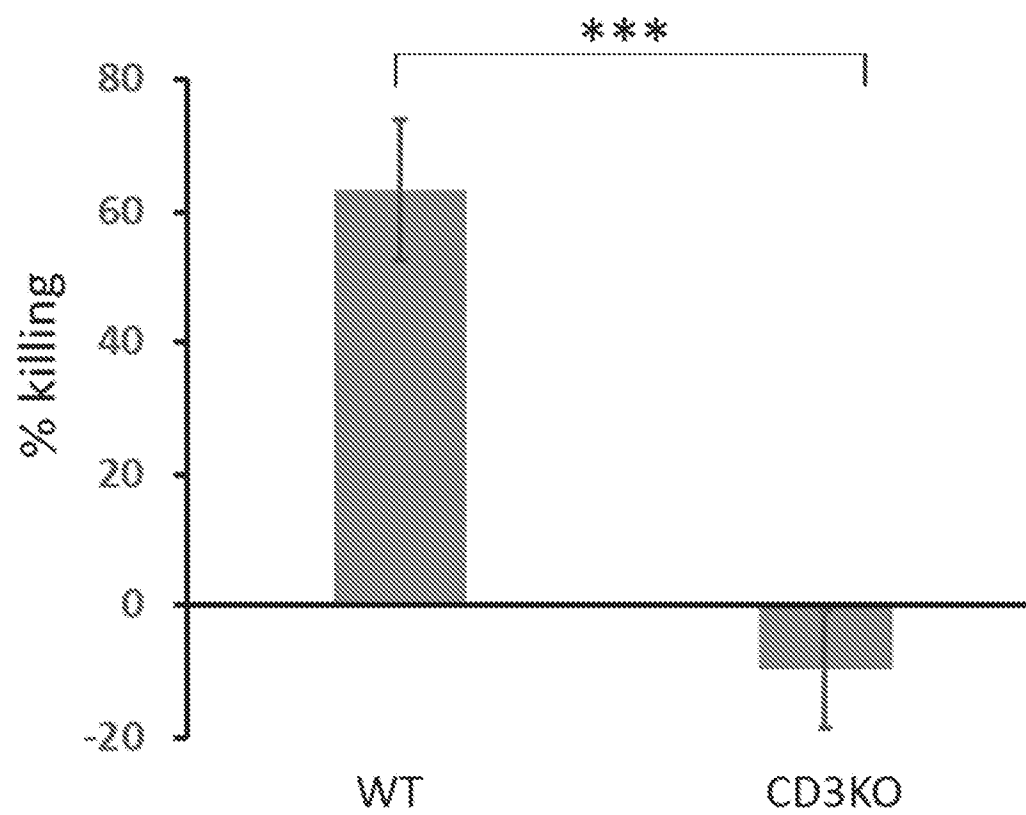


FIGURE 14

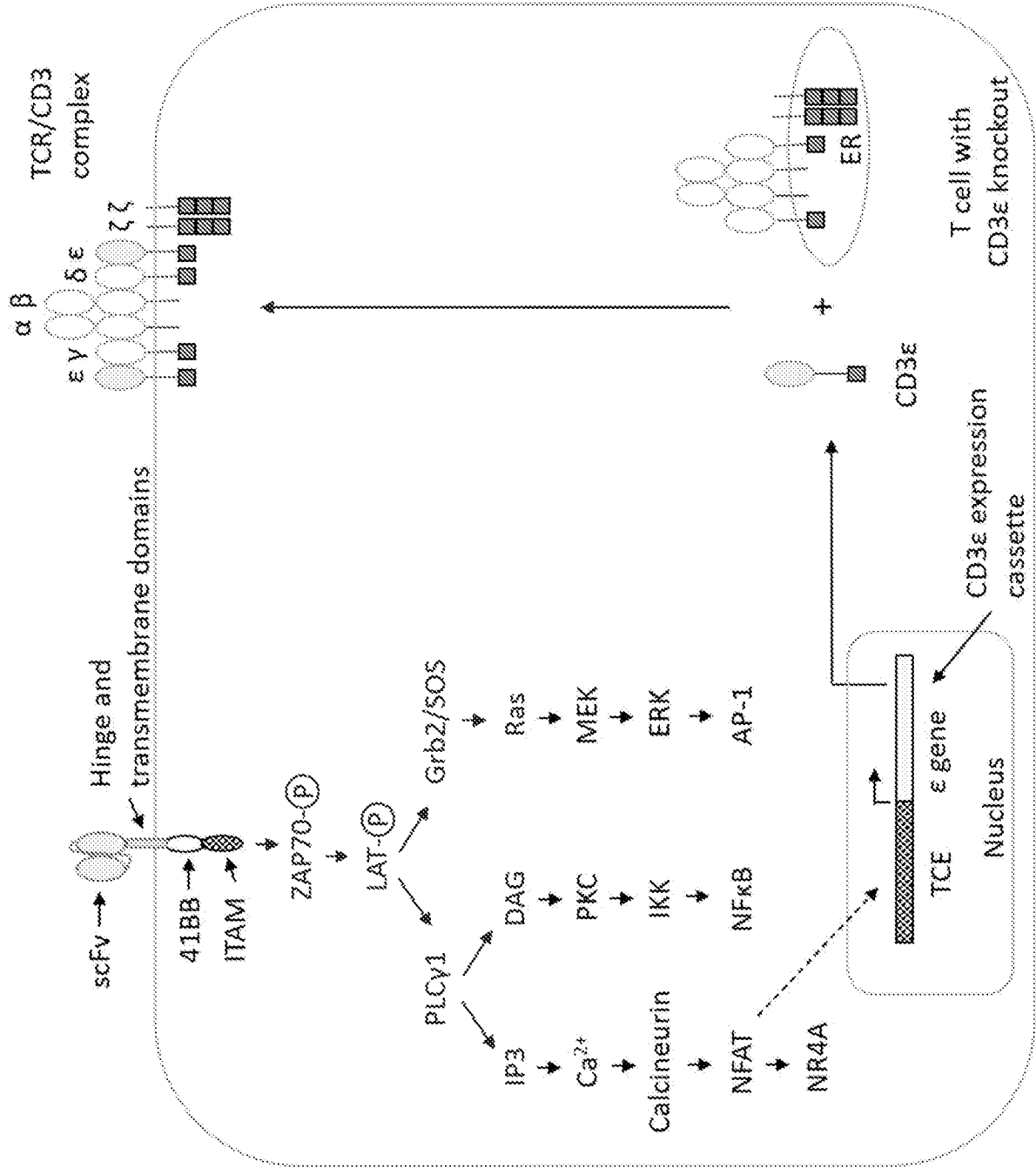
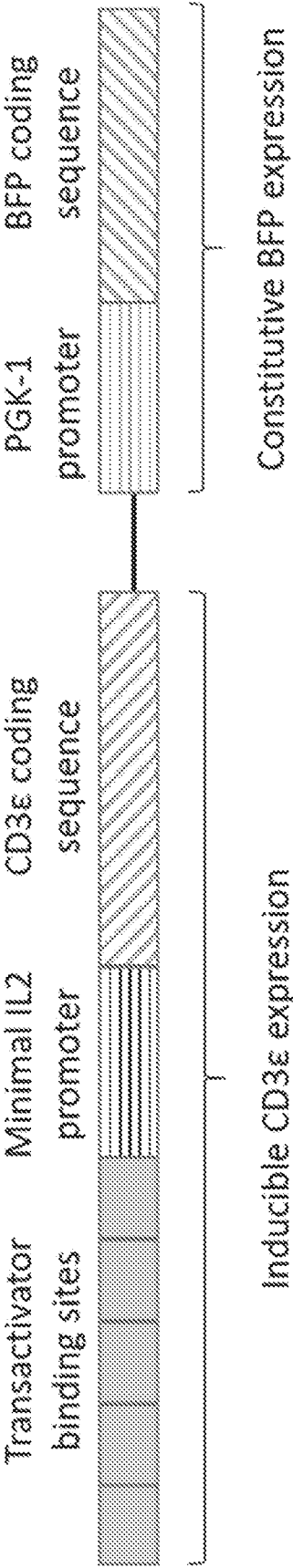


FIGURE 15



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/040765

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0783 A61K35/17
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

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

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

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROYBAL KOLE T ET AL: "Engineering T Cells with Customized Therapeutic Response Programs Using Synthetic Notch Receptors", CELL, ELSEVIER, AMSTERDAM NL, vol. 167, no. 2, 29 September 2016 (2016-09-29), page 419, XP029761104, ISSN: 0092-8674, DOI: 10.1016/J.CELL.2016.09.011 abstract figure 1 ----- -/--	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 October 2021

Date of mailing of the international search report

22/10/2021

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Brero, Alessandro

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/040765

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROYBAL KOLE T ET AL: "Precision Tumor Recognition by T Cells With Combinatorial Antigen-Sensing Circuits", CELL, ELSEVIER, AMSTERDAM NL, vol. 164, no. 4, 28 January 2016 (2016-01-28), pages 770-779, XP029416808, ISSN: 0092-8674, DOI: 10.1016/J.CELL.2016.01.011 cited in the application abstract figure 1	1-21
A	----- US 2017/233474 A1 (LIM WENDELL A [US] ET AL) 17 August 2017 (2017-08-17) abstract figures 60-65	1-21
A	----- WO 2019/199689 A1 (UNIV PENNSYLVANIA [US]) 17 October 2019 (2019-10-17) abstract figure 1	1-21
A	----- WO 2020/018964 A1 (HUTCHINSON FRED CANCER RES [US]) 23 January 2020 (2020-01-23) abstract example 5	1-21
A	----- WO 2017/193059 A1 (UNIV CALIFORNIA [US]; UNIV PRINCETON [US]; THE SIMONS FOUND INC [US]) 9 November 2017 (2017-11-09) abstract [0044] ff.	1-21
A	----- SHENGNAN YU ET AL: "Next generation chimeric antigen receptor T cells: safety strategies to overcome toxicity", MOLECULAR CANCER, vol. 18, no. 1, 20 August 2019 (2019-08-20), pages 1-13, XP055661102, DOI: 10.1186/s12943-019-1057-4 Retrieved from the Internet: URL:https://molecular-cancer.biomedcentral.com/articles/10.1186/s12943-019-1057-4> figure 1 page 8	1-21
	----- -/--	

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/040765

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KELLIHER MICHELLE A. ET AL: "NOTCH Signaling in T-Cell-Mediated Anti-Tumor Immunity and T-Cell-Based Immunotherapies", FRONTIERS IN IMMUNOLOGY, vol. 9, 20 August 2018 (2018-08-20), pages 1-6, XP055783125, DOI: 10.3389/fimmu.2018.01718 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6109642/pdf/fimmu-09-01718.pdf> page 4 -----	1-21
A	Choe Joseph H ET AL: "Engineering T Cells to Treat Cancer: The Convergence of Immuno-Oncology and Synthetic Biology", Annu. Rev. Cancer Biol, 5 February 2020 (2020-02-05), pages 51121-39, XP055849398, DOI: 10.1146/annurev-cancerbio-030419- Retrieved from the Internet: URL:https://www.annualreviews.org/doi/pdf/10.1146/annurev-cancerbio-030419-033657 [retrieved on 2021-10-08] figure 2 page 124 - page 125 -----	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2021/040765

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2017233474	A1	17-08-2017	
		AU 2016222887 A1	03-08-2017
		BR 112017017884 A2	10-04-2018
		CA 2973890 A1	01-09-2016
		CN 108064283 A	22-05-2018
		EP 3262166 A1	03-01-2018
		HK 1255666 A1	23-08-2019
		JP 6784687 B2	11-11-2020
		JP 2018506293 A	08-03-2018
		JP 2021019625 A	18-02-2021
		KR 20170126897 A	20-11-2017
		US 2016264665 A1	15-09-2016
		US 2017233474 A1	17-08-2017
		US 2018079812 A1	22-03-2018
		US 2018208636 A1	26-07-2018
		US 2018355011 A1	13-12-2018
		US 2021107965 A1	15-04-2021
		WO 2016138034 A1	01-09-2016
WO 2019199689	A1	17-10-2019	
		AU 2019253562 A1	19-11-2020
		EP 3781176 A1	24-02-2021
		US 2021032661 A1	04-02-2021
		WO 2019199689 A1	17-10-2019
		WO 2020210235 A2	15-10-2020
WO 2020018964	A1	23-01-2020	NONE
WO 2017193059	A1	09-11-2017	
		US 2019134093 A1	09-05-2019
		WO 2017193059 A1	09-11-2017