

**CHIMERIC ANTIGEN RECEPTOR T CELLS (CAR-T)
FOR THE TREATMENT OF CANCER**

[0001] This application claims the benefit of priority of United States Provisional Patent Application No. 62/799,513, filed on January 31st, 2019, and United States Provisional Patent Application No. 62/678,878 filed on May 31st, 2018, the disclosures of which are hereby incorporated by reference as if written herein in their entireties.

[0002] Disclosed herein are genome-edited chimeric antigen receptor T cells (CAR-T) and methods of using them for immunotherapy. In particular, the disclosure relates to T cells that can be genetically modified to express one or more chimeric antigen receptors (CARs) and methods of using the same for the treatment of cancer.

[0003] T cells, a type of lymphocyte, play a central role in cell-mediated immunity. They are distinguished from other lymphocytes, such as B cells and natural killer cells (NK cells), by the presence of a T-cell receptor (TCR) on the cell surface. T helper cells (T_H), also called CD4⁺ T or CD4 T cells, express CD4 glycoprotein on their surface. Helper T cells are activated when exposed to peptide antigens presented by MHC (major histocompatibility complex) class II molecules. Once activated, these cells proliferate rapidly and secrete cytokines that regulate immune response. Cytotoxic T cells (T_C), also known as CD8⁺ T cells or CD8 T cells, express CD8 glycoprotein on the cell surface. The CD8⁺ T cells are activated when exposed to peptide antigens presented by MHC class I molecules. Memory T cells, a subset of T cells, persist long term and respond to their cognate antigen, thus providing the immune system with "memory" against past infections and/or tumor cells. Gamma delta ($\gamma\delta$) T cells are the prototype of 'unconventional' T cells and represent a relatively small subset of T cells in peripheral blood. They are defined by expression of heterodimeric T-cell receptors (TCRs) composed of γ and δ chains. This sets them apart from the CD4⁺ helper T cells and CD8⁺ cytotoxic T cells. Viral-specific cytotoxic T lymphocytes are T cells with reactivity against viral antigens, notably Epstein-Barr virus (EBV) and cytomegalovirus (CMV).

[0004] The T cells described herein can be genetically modified to express chimeric antigen receptors (CARs), which are fusion proteins comprised of an antigen recognition moiety and T cell activation domains. T cells expressing CARs can recognize a specific protein, i.e., antigen

on tumor cells. These T cells expressing CARs can be expanded in the laboratory prior to infusion into a patient.

[0005] Clinical trials have shown high response rates after anti-CD19 CAR infusion in patients with B cell malignancies, including diffuse large B cell lymphoma (DLBCL) and B cell-precursor acute lymphoblastic leukemia (ALL), resulting in two FDA approved therapies Yescarta™ (axicabtagene ciiloleucel, Kite Pharma/Gilead) and Kymriah™ (tisagenlecleucel, Novartis). Despite these successes, the development of CAR-T cell therapy against T cell malignancies has proven problematic, in part due to the shared expression of target antigens between malignant T cells and effector T cells. Among the most general challenges are: (1) the antigen target(s) for the chimeric antigen receptor(s); (2) CAR design, i.e., mono CAR, dual CAR, tandem CAR; and (3) tumor heterogeneity, particularly the variance in the surface expression of tumor antigens. Therefore, there remains a need for improved chimeric antigen receptor (CAR)-based immunotherapies, which utilize genome-editing and construction of mono, dual, and tandem CARs, for more effective, safe, and efficient targeting of cancers, including T-cell associated malignancies.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] FIG. 1 shows a schematic of a dual CAR-T cell (dCAR-T cell).

[0007] FIG. 2 shows a schematic of a tandem CAR-T cell (tCAR-T cell).

[0008] FIG. 3 shows a schematic of dual and tandem CAR constructs.

[0009] FIG. 4 shows a schematic of tandem targeting CAR constructs.

[0010] FIG. 5 shows the purity of CAR-T product without mechanical depletion of CD3+ or CD2+ CAR-T cells. As shown through FACS analysis, there is a high purity of CD3⁺ and CD2⁺ CAR-T cells without a requirement for magnetic depletion of CD3+ cells. Representative FACS plots show FITC-staining for CD3 (y-axis) and CD2 (x-axis). Clones 5 (top) and 6 (bottom) shown.

[0011] FIG. 6 shows the purity of CAR-T product without mechanical depletion of CD3+ or CD2+ CAR-T cells. As shown through FACS analysis, there is a high purity of CD3⁺ and CD2⁺ CAR-T cells without a requirement for magnetic depletion selection of CD3+ cells. Representative FACS plots show FITC-staining for CD3 (y-axis) and CD2 (x-axis). Clones 7 (top) and 8 (bottom) shown.

[0012] **FIG. 7** shows the purity of CAR-T product without mechanical depletion of CD3+ or CD2+ CAR-T cells. As shown through FACS analysis, there is a high purity of CD3- and CD2- CAR-T cells without a requirement for magnetic depletion selection of CD3+ cells. Representative FACS plots show FITC-staining for CD3 (y-axis) and CD2 (x-axis). Clones 13 (top) and 14 (bottom) shown.

[0013] **FIG. 8** shows the purity of CAR-T product without mechanical depletion of CD3+ or CD2+ CAR-T cells. As shown through FACS analysis, there is a high purity of CD3- and CD2- CAR-T cells without a requirement for magnetic depletion selection of CD3+ cells. Representative FACS plots show FITC-staining for CD3 (y-axis) and CD2 (x-axis). Clones 15 (top) and 16 (bottom) shown.

[0014] **FIG. 9A** shows tumor cell killing of tandem CD2-CD3 CAR-T Clones 5 (top) and 6 (bottom); the legend shows ratio of effector to target cells (E:T ratio).

[0015] **FIG. 9B** shows tumor cell killing of tandem CD2-CD3 CAR-T Clones 7 (top) and 8 (bottom); the legend shows ratio of effector to target cells (E:T ratio).

[0016] **FIG. 9C** shows tumor cell killing of tandem CD2-CD3 CAR-T Clones 13 (top) and 14 (bottom); the legend shows ratio of effector to target cells (E:T ratio).

[0017] **FIG. 9D** shows tumor cell killing of tandem CD2-CD3 CAR-T Clones 15 (top) and 16 (bottom); the legend shows ratio of effector to target cells (E:T ratio).

[0018] **FIG. 10A** shows a schematic of a BCMA CAR construct to be transduced into T cells which will target BCMA.

[0019] **FIG. 10B** shows a tumor cell killing of BCMA-CAR-T cells in a ⁵¹Cr-release assay. Efficient killing of BCMA-CAR-T cells were observed at multiple Effector to Target (E:T) ratios. Non-transduced activated T cells and CD19-CAR-T cells were used as negative controls and did not induce killing of MM.1S-CG cells.

[0020] **FIG. 10C** shows *in vivo* efficacy of BCMA CAR-T cells. All seven mice treated with BCMA CAR-Ts lived to almost 150 days or more compared to controls which died around Day 50.

[0021] **FIG. 10D** shows serial bioluminescent imaging (BLI) measured in photo flux revealed showed a robust reduction of signal to background levels that never increased throughout the duration of the experiment in mice which received treatment with BCMA CAR-T cells.

[0022] **FIG. 11A.** shows a schematic of a CS1-CAR construct to be transduced into T cells which will target CS1.

[0023] **FIG. 11B** shows *in vivo* efficacy of CS1-CAR-T cells. Mice were engrafted with MM.1S-CG cells and MM.1S-CG cells lacking CS1 (using CAS9/CRISPR technology; MM.1S-CGΔCS1) as a method to test the specificity of CS1-CAR-T cells. All mice treated with CS1-CAR-T cells (n=10) lived >90 days while median survival of CD19-control mice (n=8) was 43 days.

[0024] **FIG. 11C** shows serial bioluminescent imaging (BLI) showed mice treated with CS1-CAR-T cells had a three log decrease in photon flux and clearance of marrow tumor (Experiment 1 through Experiment 3).

[0025] **FIG 12A** shows schematics of mono (CD19, CS1) and tandem (BCMA-CS1) constructs.

[0026] **FIG. 12B** shows FACS analysis of Jurkat cells expressing CD19 CAR did not bind to either BCMA or CS1 protein (lower left quadrant of each plot). Jurkat cells expressing BCMA CAR protein bound BCMA protein (upper left quadrant of each plot). Jurkat cells expressing CS1 CAR protein bound CS1 protein (lower right quadrant of each plot). Jurkat cells expressing the tandem BCMA-CS1 CAR protein bound to both recombinant proteins (upper right quadrant of each plot), suggesting expression of both scFvs.

[0027] **FIG. 12C** shows *in vitro* efficacy of single and tandem CAR-T cells using standard four-hour chromium release (⁵¹Cr) assays. BCMA-CS1 tCAR-T cells killed MM.1S-CG cells with similar efficacy of both single-antigen targeted BCMA and CS1 CAR-T cells.

[0028] **FIG. 13** shows testing efficacy of CD2*CD3Δ-dCARTΔCD2ΔCD3ε in a xenogeneic model of T-ALL.

DETAILED DESCRIPTION

[0029] The following disclosure will detail embodiments, alternatives, and uses engineered cells and the use sch cells in, for example, immunotherapy and adoptive cell transfer for the treatment of diseases. Accordingly, provided herein are the following embodiments.

[0030] **Embodiment 1.** A CAR-T cell, which comprises one or more chimeric antigen receptors (CARs) targeting one or more antigens, wherein the CAR-T cell is deficient in a

subunit of the T cell receptor complex and / or is deficient in at least one or more antigens to which the one or more CARs specifically binds.

[0031] Embodiment 2. A CAR-T cell, which comprises one or more chimeric antigen receptors (CARs) targeting one or more antigens, wherein the CAR-T cell is deficient in one or more antigens to which the one or more CARs specifically binds.

[0032] Embodiment 3. The CAR-T cell as recited in Embodiment 1, wherein the subunit of the T cell receptor complex is chosen from TCR α , TCR β , TCR δ , TCR γ , CD3 ϵ , CD3 γ , CD3 δ , and CD3 ζ .

[0033] Embodiment 4. The CAR-T cell as recited in any of Embodiments 1-2, wherein the chimeric antigen receptor (CAR) specifically binds one or more antigens expressed on a malignant T cell or myeloma cell.

[0034] Embodiment 5. The CAR-T cell as recited in any of Embodiments 1-4, wherein the chimeric antigen receptor (CAR) displays at least 95% sequence identity to an amino acid sequence chosen from SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:39.

[0035] Embodiment 6. The CAR-T cell as recited in any of Embodiments 1-4, wherein the chimeric antigen receptor (CAR) displays at least 98% sequence identity to an amino acid sequence chosen from SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:39.

[0036] Embodiment 7. The CAR-T cell as recited in any of Embodiments 1-4, wherein the chimeric antigen receptor (CAR) is an amino acid sequence chosen from SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:39.

[0037] Embodiment 8. The CAR-T cell as recited in any of Embodiments 1-4, wherein the chimeric antigen receptor(s) specifically binds one or more antigen(s) chosen from BCMA, CS1, CD38, CD138, CD19, CD33, CD123, CD371, CD117, CD135, Tim-3, CD5, CD7, CD2, CD4, CD3, CD79A, CD79B, APRIL, CD56, and CD1a.

[0038] Embodiment 9. The CAR-T cell as recited in any of Embodiments 1-5, wherein the chimeric antigen receptor(s) specifically binds at least one antigen expressed on a malignant T cell.

[0039] **Embodiment 10.** The CAR-T cell as recited in Embodiment 9, wherein the antigen expressed on a malignant T cell is chosen from CD2, CD3, CD4, CD5, CD7, TCRA, and TCR β .

[0040] **Embodiment 11.** The CAR-T cell as recited in any of Embodiments 1-5, wherein the chimeric antigen receptor specifically binds at least one antigen expressed on a malignant plasma cell.

[0041] **Embodiment 12.** The CAR-T cell as recited in Embodiment 11, wherein the antigen expressed on a malignant plasma cell is chosen from BCMA, CS1, CD38, CD79A, CD79B, CD138, and CD19.

[0042] **Embodiment 13.** The CAR-T cell as recited in any of Embodiments 1-5, wherein the chimeric antigen receptor(s) specifically binds at least one antigen expressed on a malignant B cell.

[0043] **Embodiment 14.** The CAR-T cell as recited in Embodiment 13, wherein the antigen expressed on a malignant B cell is chosen from CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD38, and CD45.

[0044] **Embodiment 15.** The CAR-T cell as recited in Embodiment 14, wherein the antigen expressed on a malignant B cell is chosen from CD19 and CD20.

[0045] **Embodiment 16.** The CAR-T cell as recited in any of Embodiments 1-15, wherein the CAR-T cell further comprises a suicide gene.

[0046] **Embodiment 17.** The CAR-T cell as recited in any of Embodiments 1-16, wherein endogenous T cell receptor mediated signaling is blocked in the CAR-T cell.

[0047] **Embodiment 18.** The CAR-T cell as recited in any of Embodiments 1-17, wherein the CAR-T cells do not induce alloreactivity or graft-versus-host disease.

[0048] **Embodiment 19.** The CAR-T cell as recited in any of Embodiments 1-18, wherein the CAR-T cells do not induce fratricide.

[0049] **Embodiment 20.** A dual or tandem CAR-T cell as recited in any of Embodiments 1-19.

[0050] **Embodiment 21.** The CAR-T cell as recited in Embodiment 20, wherein the wherein the CAR(s) specifically bind(s) two different targets chosen from: CD2xCD3 ϵ , CD2xCD4, CD2xCD5, CD2xCD7, CD3 ϵ xCD4, CD3 ϵ xCD5, CD3 ϵ xCD7, CD4xCD5, CD4xCD7, CD5xCD7, TRACxCD2, TRACxCD3 ϵ , TRACxCD4, TRACxCD5, TRACxCD7, TCR β xCD2, TCR β xCD3 ϵ , TCR β xCD4, TCR β xCD7, CD2xCD3 ϵ , CD2xCD4, CD2xCD5, CD2xCD7,

CD3 ϵ xCD4, CD3 ϵ xCD5, CD3 ϵ xCD7, CD4xCD5, CD4xCD7, CD5xCD7, TRACxCD2, TRACxCD3 ϵ , TRACxCD4, TRACxCD5, TRACxCD7, TCR β xCD2, TCR β xCD3 ϵ , TCR β xCD4, TCR β xCD5, TCR β xCD7, BCMAxCS1, BCMAxCD19, BCMAxCD38, CS1xCD19, CD19xCD38, APRILxCS1, APRILxBCMA, APRILxCD19, APRILxCD38, CS1xCD38, CD79AxBCMA, CD79AxCs1, CD79AxCD19, CD79AxCD38, CD79AxCD38, CD79AxAPRIL, CD79AxCD79B, CD79BxBCMA, CD79BxCs1, CD79BxCD19, CD79BxCD38, CD79BxAPRIL, CD79BxCD79A, CD138xBCMA, CD138xCs1, CD138xCD19, CD138xCD38, CD138xAPRIL, CD138xCD79A, CD138xCD79B, CD138xBCMA, and CD138xCs1.

[0051] Embodiment 22. The CAR-T cell as recited in Embodiment 21, wherein the CAR(s) specifically bind(s) two different targets chosen from: CD2xCD3 ϵ , CD2xCD4, CD2xCD5, CD2xCD7, CD3 ϵ xCD4, CD3 ϵ xCD5, CD3 ϵ xCD7, CD4xCD5, CD4xCD7, CD5xCD7, TRACxCD2, TRACxCD3 ϵ , TRACxCD4, TRACxCD5, TRACxCD7, TCR β xCD2, TCR β xCD3 ϵ , TCR β xCD4, TCR β xCD7, CD2xCD3 ϵ , CD2xCD4, CD2xCD5, CD2xCD7, CD3 ϵ xCD4, CD3 ϵ xCD5, CD3 ϵ xCD7, CD4xCD5, CD4xCD7, CD5xCD7, TRACxCD2, TRACxCD3 ϵ , TRACxCD4, TRACxCD5, TRACxCD7, TCR β xCD2, TCR β xCD3 ϵ , TCR β xCD4, TCR β xCD5, and TCR β xCD7.

[0052] Embodiment 23. The CAR-T cell as recited in Embodiment 21, wherein the CAR(s) specifically bind(s) two different targets chosen from: BCMAxCS1, BCMAxCD19, BCMAxCD38, CS1xCD19, CD19xCD38, APRILxCS1, APRILxBCMA, APRILxCD19, APRILxCD38, CS1xCD38, CD79AxBCMA, CD79AxCs1, CD79AxCD19, CD79AxCD38, CD79AxCD38, CD79AxAPRIL, CD79AxCD79B, CD79BxBCMA, CD79BxCs1, CD79BxCD19, CD79BxCD38, CD79BxAPRIL, CD79BxCD79A, CD138xBCMA, CD138xCs1, CD138xCD19, CD138xCD38, CD138xAPRIL, CD138xCD79A, CD138xCD79B, CD138xBCMA, and CD138xCs1.

[0053] Embodiment 24. The CAR-T cell as recited in Embodiment 21, wherein the CAR(s) specifically bind(s) two different targets chosen from: CD123xCD371, CD123xCLEC12A, CD123xCD117, CD123xFLT3, CD123xCD7, CD123xTim3, CD371xCLEC12A, CD371xCD117, CD371xFLT3, CD371xCD7, CD371xTim3, CLEC12AxCD117, CLEC12AxFLT3, CLEC12AxCD7, CLEC12AxTim3, CD117xFLT3, CD117xCD7, CD117xTim3, FLT3xCD7, FLT3xTim3, and CD7xTim3.

- [0054] **Embodiment 25.** A dual CAR-T cell as recited in any of Embodiments 21-24.
- [0055] **Embodiment 26.** A tandem CAR-T cell as recited in any of Embodiments 21-34.
- [0056] **Embodiment 27.** The CAR-T cell as recited in any of Embodiments 1-26, wherein the CAR-T cell further comprises a suicide gene.
- [0057] **Embodiment 28.** The CAR-T cell as recited in any of Embodiments 1-26, wherein endogenous T cell receptor mediated signaling is blocked in the CAR-T cell.
- [0058] **Embodiment 29.** The CAR-T cell as recited in any of Embodiments 1-26, wherein the CAR-T cells do not induce alloreactivity or graft-versus-host disease.
- [0059] **Embodiment 30.** The CAR-T cell as recited in any of Embodiments 1-26, wherein the CAR-T cells do not induce fratricide.
- [0060] **Embodiment 31.** A dual or tandem chimeric antigen receptor (dCAR or tCAR) targeting two or more plasma cell antigens.
- [0061] **Embodiment 32.** The CAR as recited in Embodiment 31, wherein the plasma cell antigen(s) is/are chosen from BCMA, CS1, CD38, CD79A, CD79B, CD138, and CD19.
- [0062] **Embodiment 33.** The CAR as recited in Embodiment 32, wherein the CAR(s) specifically bind(s) two different targets chosen from: BCMAxCS1, BCMAxCD19, BCMAxCD38, CS1xCD19, CD19xCD38, APRILxCS1, APRILxBCMA, APRILxCD19, APRILxCD38, CS1xCD38, CD79AxBCMA, CD79AxCs1, CD79AxCD19, CD79AxCD38, CD79AxCD38, CD79AxAPRIL, CD79AxCD79B, CD79BxBCMA, CD79BxCs1, CD79BxCD19, CD79BxCD38, CD79BxAPRIL, CD79BxCD79A, CD138xBCMA, CD138xCs1, CD138xCD19, CD138xCD38, CD138xAPRIL, CD138xCD79A, CD138xCD79B, CD138xBCMA, and CD138xCs1.
- [0063] **Embodiment 34.** The CAR as recited in any of Embodiments 31-33, wherein the CAR is a dCAR.
- [0064] **Embodiment 35.** The CAR as recited in any of Embodiments 31-33, wherein the CAR is a tCAR.
- [0065] **Embodiment 36.** A dual or tandem chimeric antigen receptor (dCAR or tCAR) targeting two or more leukemia cell antigens.
- [0066] **Embodiment 37.** The CAR as recited in Embodiment 36, wherein the plasma cell antigen(s) is/are chosen from CD123, CLEC12A, CD117, FLT3, CD7 and Tim3.

[0067] Embodiment 38. The CAR as recited in Embodiment 37, wherein the CAR(s) specifically bind(s) two different targets chosen from: CD123xCD371, CD123xCLEC12A, CD123xCD117, CD123xFLT3, CD123xCD7, CD123xTim3, CD371xCLEC12A, CD371xCD117, CD371xFLT3, CD371xCD7, CD371xTim3, CLEC12AxCD117, CLEC12AxFLT3, CLEC12AxCD7, CLEC12AxTim3, CD117xFLT3, CD117xCD7, CD117xTim3, FLT3xCD7, FLT3xTim3, and CD7xTim3.

[0068] Embodiment 39. The CAR as recited in any of Embodiments 36-38, wherein the CAR is a dCAR.

[0069] Embodiment 40. The CAR as recited in any of Embodiments 36-38, wherein the CAR is a tCAR.

[0070] Embodiment 41. A tandem chimeric antigen receptor (tCAR) targeting two or more T-cell antigens.

[0071] Embodiment 42. The tCAR as recited in Embodiment 41, wherein the T-cell antigens chosen from CD5, CD7, CD2, CD4, and CD3.

[0072] Embodiment 43. The tCAR as recited in Embodiment 42, targeting a pair of (i.e., two) antigens.

[0073] Embodiment 44. The tCAR as recited in Embodiment 43, wherein the antigen pair is chosen from CD2xCD3 ϵ , CD2xCD4, CD2xCD5, CD2xCD7, CD3 ϵ xCD4, CD3 ϵ xCD5, CD3 ϵ xCD7, CD4xCD5, CD4xCD7, CD5xCD7, TRACxCD2, TRACxCD3 ϵ , TRACxCD4, TRACxCD5, TRACxCD7, TCR β xCD2, TCR β xCD4, TCR β xCD7, CD2xCD3 ϵ , CD2xCD4, CD2xCD5, CD2xCD7, CD3 ϵ xCD4, CD3 ϵ xCD5, CD4xCD5, CD4xCD7, CD5xCD7, TRACxCD2, TRACxCD3 ϵ , TRACxCD4, TRACxCD5, TRACxCD7, TCR β xCD2, TCR β xCD3 ϵ , TCR β xCD4, TCR β xCD5, and TCR β xCD7.

[0074] Embodiment 45. The tCAR as recited in Embodiment 43, wherein the antigen pair is chosen from CD2xCD3 ϵ , CD2xCD4, CD2xCD5, CD2xCD7, CD3 ϵ xCD4, CD3 ϵ xCD5, CD3 ϵ xCD7, CD4xCD5, CD4xCD7, and CD5xCD7.

[0075] Embodiment 46. The tCAR as recited in any of Embodiments 35 and 40-45, wherein the CAR construct is a linear tCAR construct.

[0076] Embodiment 47. The tCAR as recited in Embodiment 46, wherein the linear tCAR construct comprises a first heavy (V_H) chain variable fragment and a first light (V_L) chain variable fragment, designated V_H1 and V_L1, joined by a (GGGGS)₂₋₆ linker to a second light

(V_L) chain variable fragment and a first heavy (V_H) chain variable fragment, designated V_L2 and V_H2.

[0077] Embodiment 48. The tCAR as recited in Embodiment 46, wherein the linear tCAR construct comprises a first heavy (V_H) chain variable fragment and a first light (V_L) chain variable fragment, designated V_H2 and V_L2, joined by a (GGGGS)₂₋₆ linker to a second light (V_L) chain variable fragment and a first heavy (V_H) chain variable fragment, designated V_H1 and V_L1.

[0078] Embodiment 49. The tCAR as recited in Embodiment 46, wherein the linear tCAR construct comprises a first light (V_L) chain variable fragment and a first heavy (V_H) chain variable fragment, designated V_L1 and V_H1, joined by a (GGGGS)₂₋₆ linker to a second heavy (V_H) chain variable fragment and a first light (V_L) chain variable fragment, designated V_H2 and V_L2.

[0079] Embodiment 50. The tCAR as recited in Embodiment 46, wherein the linear tCAR construct comprises a first light (V_L) chain variable fragment and a first heavy (V_H) chain variable fragment, designated V_L2 and V_H2, joined by a (GGGGS)₂₋₆ linker to a second heavy (V_H) chain variable fragment and a first light (V_L) chain variable fragment, designated V_H1 and V_L1.

[0080] Embodiment 51. The tCAR as recited in Embodiment 46, wherein the linear tCAR construct comprises a structure chosen from 7-I to 7-XXXII.

[0081] Embodiment 52. The tCAR as recited in any of Embodiments 35 and 40-45, wherein the CAR construct is a hairpin tCAR construct.

[0082] Embodiment 53. The tCAR as recited in Embodiment 52, wherein the hairpin tCAR construct comprises a first heavy (V_H) chain variable fragment derived from a first scFv, and a second heavy (V_H) chain variable fragment derived from a second scFv, designated V_H1 and V_H2, joined by a (GGGGS)₂₋₆ linker to a first light (V_L) chain variable fragment derived from the second scFv, and a second light (V_L) chain variable fragment derived from the first scFv, designated V_L2 and V_L1.

[0083] Embodiment 54. The tCAR as recited in Embodiment 52, wherein the hairpin tCAR construct comprises a second heavy (V_H) chain variable fragment derived from a second scFv, and a first heavy (V_H) chain variable fragment derived from a first scFv, designated V_H2 and V_H1, joined by a (GGGGS)₂₋₆ linker to a first light (V_L) chain variable fragment derived from

the first scFv, and a second light (V_L) chain variable fragment derived from the second scFv, designated V_{L1} and V_{L2} .

[0084] Embodiment 55. The tCAR as recited in Embodiment 52, wherein the hairpin tCAR construct comprises a first light (V_L) chain variable fragment derived from a first scFv, and a second light (V_L) chain variable fragment derived from a second scFv, designated V_{L1} and V_{L2} , joined by a (GGGGS)₂₋₆ linker to a first heavy (V_H) chain variable fragment derived from the first scFv, and a second heavy (V_H) chain variable fragment derived from the second scFv, designated V_{H2} and V_{H1} .

[0085] Embodiment 56. The tCAR as recited in Embodiment 52, wherein the hairpin tCAR construct comprises a second light (V_L) chain variable fragment derived from a second scFv, and a first light (V_L) chain variable fragment derived from a first scFv, designated V_{L2} and V_{L1} , joined by a (GGGGS)₂₋₆ linker to a first heavy (V_H) chain variable fragment derived from the first scFv, and a second light heavy (V_H) variable fragment derived from the second scFv, designated V_{H1} and V_{H2} .

[0086] Embodiment 57. The tCAR as recited in Embodiment 52, wherein the hairpin tCAR construct comprises a structure chosen from 9-I to 9-XXXII.

[0087] Embodiment 58. The tCAR as recited in any of Embodiments 35 and 40-45, wherein the CAR construct is a hairpin DSB tCAR construct with a (Cys=Cys) Double-Stranded Bond (DSB) in the linker.

[0088] Embodiment 59. The tCAR as recited in Embodiment 58, wherein the hairpin tCAR construct comprises a first heavy (V_H) chain variable fragment derived from a first scFv, and a second heavy (V_H) chain variable fragment derived from a second scFv, designated V_{H1} and V_{H2} , joined by a (GGGGS)₀₋₁-(GGGGC)₁-(GGGGS)₁₋₂-(GGGGP)₁-(GGGGS)₂₋₃-(GGGGC)₁-(GGGGS)₀₋₁ linker to a first light (V_L) chain variable fragment derived from the second scFv, and a second light (V_L) chain variable fragment derived from the first scFv, designated V_{L2} and V_{L1} .

[0089] Embodiment 60. The tCAR as recited in Embodiment 58, wherein the hairpin tCAR construct comprises a second heavy (V_H) chain variable fragment derived from a second scFv, and a first heavy (V_H) chain variable fragment derived from a first scFv, designated V_{H2} and V_{H1} , joined by a (GGGGS)₀₋₁-(GGGGC)₁-(GGGGS)₁₋₂-(GGGGP)₁-(GGGGS)₂₋₃-(GGGGC)₁-(GGGGS)₀₋₁ linker to a first light (V_L) chain variable fragment derived from the first scFv, and a

second light (V_L) chain variable fragment derived from the second scFv, designated V_{L1} and V_{L2} .

[0090] Embodiment 61. The tCAR as recited in Embodiment 58, wherein the hairpin tCAR construct comprises a first light (V_L) chain variable fragment derived from a first scFv, and a second light (V_L) chain variable fragment derived from a second scFv, designated V_{L1} and V_{L2} , joined by a $(GGGGS)_{0-1}-(GGGGC)_1-(GGGGS)_{1-2}-(GGGGP)_1-(GGGGS)_{2-3}-(GGGGC)_1-(GGGGS)_{0-1}$ linker to a first heavy (V_H) chain variable fragment derived from the first scFv, and a second heavy (V_L) chain variable fragment derived from the second scFv, designated V_{H2} and V_{H1} .

[0091] Embodiment 62. The tCAR as recited in Embodiment 58, wherein the hairpin tCAR construct comprises a second light (V_L) chain variable fragment derived from a second scFv, and a first light (V_L) chain variable fragment derived from a first scFv, designated V_{L2} and V_{L1} , joined by a $(GGGGS)_{0-1}-(GGGGC)_1-(GGGGS)_{1-2}-(GGGGP)_1-(GGGGS)_{2-3}-(GGGGC)_1-(GGGGS)_{0-1}$ linker to a first heavy (V_H) chain variable fragment derived from the first scFv, and a second light heavy (V_H) variable fragment derived from the second scFv, designated V_{H1} and V_{H2} .

[0092] Embodiment 63. The tCAR as recited in Embodiment 58, wherein the hairpin DSB tCAR construct comprises a structure chosen from 11-I to 11-XXXII.

[0093] Embodiment 64. The tCAR as recited in any of Embodiments 41-63, wherein each of the V_H and V_L chains is derived from an scFv that recognizes a different antigen chosen from CD5, CD7, CD2, CD4, and CD3.

[0094] Embodiment 65. The tCAR as recited in Embodiment 64, wherein each of the V_H and V_L chains is different and displays at least 95% sequence identity to an amino acid sequence chosen from SEQ ID NO:12 to SEQ ID NO:31.

[0095] Embodiment 66. The tCAR as recited in Embodiment 64, wherein each of the V_H and V_L chains is different and displays at least 98% sequence identity to an amino acid sequence chosen from SEQ ID NO:12 to SEQ ID NO:31.

[0096] Embodiment 67. The tCAR as recited in Embodiment 64, wherein each of the V_H and V_L chains is different and is a sequence chosen from SEQ ID NO:12 to SEQ ID NO:31.

[0097] Embodiment 68. The tCAR as recited in any of Embodiments 35, 39, and 41-67, comprising at least one costimulatory domain chosen from CD28 and 4-1BB.

[0098] **Embodiment 69.** The tCAR as recited in Embodiment 68, wherein the costimulatory domain is CD28.

[0099] **Embodiment 70.** The tCAR as recited in any of Embodiments 35 and 40-69, comprising a CD3 ζ signaling domain.

[00100] **Embodiment 71.** The tCAR as recited in any of Embodiments 41-63 and 68-70, wherein the each of the V_H and V_L chains is derived from an scFv recognizing CD2 or an scFv recognizing CD3.

[00101] **Embodiment 72.** The tCAR as recited in Embodiment 64, wherein the tCAR construct is chosen from Clone 5, Clone 6, Clone 7, Clone 8, Clone 13, Clone 14, Clone 15, and Clone 16.

[00102] **Embodiment 73.** The tCAR as recited in Embodiment 64, wherein the tCAR construct displays at least 95% sequence identity to an amino acid sequence chosen from SEQ ID NO:41 to SEQ ID NO:46.

[00103] **Embodiment 74.** A tandem chimeric antigen receptor (CAR) T cell (tCAR-T cell), which comprises a tCAR targeting two or more T-cell antigens, as recited in any of Embodiments 35 and 40-73.

[00104] **Embodiment 75.** The tCAR-T cell as recited in Embodiment 74, wherein the cell is deficient in one or more antigens to which the one or more CARs specifically binds.

[00105] **Embodiment 76.** The tCAR-T cell as recited in either of Embodiments 74 and 75, wherein the tCAR-T cell is deficient in a subunit of the T cell receptor complex.

[00106] **Embodiment 77.** The tCAR-T cell as recited in Embodiment 76, wherein the subunit of the T cell receptor complex is chosen from TCR α (TRAC), TCR β , TCR δ , TCR γ , CD3 ϵ , CD3 γ , CD3 δ , and CD3 ζ .

[00107] **Embodiment 78.** The tCAR-T cell as recited in Embodiment 77, wherein the subunit of the T cell receptor complex is chosen from TCR α (TRAC) and CD3 ϵ .

[00108] **Embodiment 79.** The tCAR-T cell as recited in Embodiment 78, wherein the subunit of the T cell receptor complex is TRAC.

[00109] **Embodiment 80.** The tCAR-T cell as recited in any of Embodiments 35 and 40-79, wherein the CAR-T cell further comprises a suicide gene.

[00110] **Embodiment 81.** The tCAR-T cell as recited in any of Embodiments 35 and 40-80, wherein endogenous T cell receptor mediated signaling is blocked in the CAR-T cell.

[00111] Embodiment 82. The tCAR-T cell as recited in any of Embodiments 35 and 40-81, wherein the CAR-T cells do not induce alloreactivity or graft-versus-host disease.

[00112] Embodiment 83. The tCAR-T cell as recited in any of Embodiments 35 and 40-82, wherein the CAR-T cells do not induce fratricide.

[00113] Embodiment 84. A tandem CAR-T cell having a CAR targeting CD2 and CD3, wherein the CAR-T cell is deficient in a subunit of the T cell receptor complex and is deficient in CD2.

[00114] Embodiment 85. The CAR-T cell as recited in Embodiment 85, wherein the CAR displays at least 95% sequence identity to an amino acid sequence chosen from SEQ ID NO:41 to SEQ ID NO:44.

[00115] Embodiment 86. The CAR-T cell as recited in Embodiment 85, wherein the CAR displays at least 98% sequence identity to an amino acid sequence chosen from SEQ ID NO:41 to SEQ ID NO:44.

[00116] Embodiment 87. The CAR-T cell as recited in Embodiment 85, wherein the CAR is an amino acid sequence chosen from SEQ ID NO:41 to SEQ ID NO:44.

[00117] Embodiment 88. A tandem CAR-T cell having a CAR targeting CD2 and CD7, wherein the CAR-T cell is deficient in a subunit of the T cell receptor complex and is deficient in CD2 and CD7.

[00118] Embodiment 89. The CAR-T cell as recited in Embodiment 88, wherein the CAR displays at least 95% sequence identity to an amino acid sequence chosen from SEQ ID NO:45 to SEQ ID NO:46.

[00119] Embodiment 90. The CAR-T cell as recited in Embodiment 88, wherein the CAR displays at least 98% sequence identity to an amino acid sequence chosen from SEQ ID NO:45 to SEQ ID NO:46.

[00120] Embodiment 91. The CAR-T cell as recited in Embodiment 88, wherein the CAR is an amino acid sequence chosen from SEQ ID NO:45 to SEQ ID NO:46.

[00121] Embodiment 92. A CAR-T cell, which comprises a chimeric antigen receptor (CAR) targeting CD7, wherein the CAR-T cell is deficient in TRAC and deficient in CD7, and comprises a CD28 costimulatory domain and a CD3 ζ signaling domain.

[00122] Embodiment 93. The CAR-T cell as recited in Embodiment 92, wherein the CAR displays at least 95% sequence identity to an amino acid sequence chosen from SEQ ID NO:32 to SEQ ID NO:39.

[00123] Embodiment 94. The CAR-T cell as recited in Embodiment 92, wherein the CAR displays at least 98% sequence identity to an amino acid sequence chosen from SEQ ID NO:32 to SEQ ID NO:39.

[00124] Embodiment 95. The CAR-T cell as recited in Embodiment 92, wherein the CAR is an amino acid sequence chosen from SEQ ID NO:32 to SEQ ID NO:39.

[00125] A therapeutic composition comprising a population of CAR-T cells as recited in any of any of Embodiments 1-30 and 74-95, or comprising a population of CAR-T cells comprising CAR(s) as recited in any of Embodiments 31-73, and at least one therapeutically acceptable carrier and/or adjuvant.

[00126] Embodiment 96. A method of treatment of cancer in a patient comprising administering genome-edited CAR-T cell, population of genome-edited CAR-T cells, dual CAR-T cells, or tandem CAR-T as recited in any of any of Embodiments 1-30 and 74-95, or comprising a population of CAR-T cells comprising CAR(s) as recited in any of Embodiments 31-73, to a patient in need thereof.

[00127] Embodiment 97. The method as recited in Embodiment 97, wherein the cancer is a hematologic malignancy.

[00128] Embodiment 98. The method as recited in Embodiment 98, wherein the hematologic malignancy is a T-cell malignancy.

[00129] Embodiment 99. The method as recited in Embodiment 99, wherein the T cell malignancy is T-cell acute lymphoblastic leukemia (T-ALL).

[00130] Embodiment 100. The method as recited in Embodiment 99, wherein the T cell malignancy is non-Hodgkin's lymphoma.

[00131] Embodiment 101. The method as recited in Embodiment 99, wherein the T cell malignancy is T-cell chronic lymphocytic leukemia (T-CLL).

[00132] Embodiment 102. The method as recited in Embodiment 98, wherein the hematologic malignancy is multiple myeloma.

[00133] Embodiment 103. The method as recited in Embodiment 98, wherein the hematologic malignancy is acute myeloid leukemia (AML).

[00134] Embodiment 104. A method of making a CAR-T cell as recited in any embodiment above or herein, using Cas9-CRISPR and a gRNA chosen from those disclosed herein.

[00135] Embodiment 105. A method of making a CAR-T cell as recited in any embodiment above or herein, using Cas9-CRISPR and a gRNA chosen from those disclosed Table 12 and Tables 15-47.

[00136] Embodiment 106. A method of making a CAR-T cell as recited in any embodiment above or herein, using Cas9-CRISPR and a gRNA chosen from those disclosed in Table 12 and those in boldface in Tables 15-47.

[00137] Embodiment 107. A method of making a CAR-T cell as recited in any embodiment above or herein, using Cas9-CRISPR and a gRNA chosen from those disclosed in Tables 12.

[00138] M

[00139] Disclosed herein is a genome-edited CAR-T cell, derived from a helper T cell, a cytotoxic T cell, a viral-specific cytotoxic T cell, a memory T cell, or a gamma delta ($\gamma\delta$) T cell, which comprise one or more chimeric antigen receptors (CARs) targeting one or more antigens, wherein the CAR-T cell is deficient in one or more antigens to which the one or more CARs specifically binds.

[00140] Also provided is a genome-edited CAR-T cell, derived from a helper T cell, a cytotoxic T cell, a viral-specific cytotoxic T cell, a memory T cell, or a gamma delta ($\gamma\delta$) T cell, which comprise one or more chimeric antigen receptors (CARs) targeting one or more antigens, wherein CAR-T cell is deficient in a subunit of the T cell receptor complex and one or more antigens to which the one or more CARs specifically binds.

[00141] Also provided is a CAR-T cell, derived from a helper T cell, a cytotoxic T cell, a viral-specific cytotoxic T cell, a memory T cell, or a gamma delta ($\gamma\delta$) T cell, in which the deficient subunit of the T cell receptor complex is selected from TCR α , TCR β , TCR δ , TCR γ , CD3 ϵ , CD3 γ , CD3 δ , and CD3 ζ .

[00142] In certain embodiments, the chimeric antigen receptor specifically binds at least one antigen expressed on a malignant T cell.

[00143] In certain embodiments, one or more antigens is selected from BCMA, CS1, CD38, CD138, CD19, CD33, CD123, CD371, CD117, CD135, Tim-3, CD5, CD7, CD2, CD4, CD3, CD79A, CD79B, APRIL, CD56, and CD1a.

[00144] In certain embodiments, CAR-T cell further comprises a suicide gene therapy system.

[00145] In certain embodiments, the endogenous T cell receptor-mediated signaling is blocked in the CAR-T cell.

[00146] In certain embodiments, the CAR-T cell does not induce alloreactivity or graft-versus-host disease.

[00147] In certain embodiments, the CAR-T cells do not induce fratricide.

[00148] Also provided is a dual or tandem CAR-T cell.

[00149] Also provided is a pharmaceutical composition comprising a population of CAR-T cells as disclosed herein, and at least one therapeutically acceptable carrier and/or adjuvant.

[00150] Also provided are methods for treating hematologic malignancies comprising administering a genome-edited CAR-T cell, a population of genome-edited CAR-T cells, wherein the population of genome-edited CAR-T cells are mono CAR-T cells, dual CAR-T cells, or tandem CAR-T cells as disclosed herein, or pharmaceutical compositions comprising them as disclosed herein to a patient in need thereof.

[00151] In certain embodiments, the hematologic malignancy is a T-cell malignancy.

[00152] In certain embodiments, the T cell malignancy is T-cell acute lymphoblastic leukemia (T-ALL).

[00153] In certain embodiments, the T cell malignancy is non-Hodgkin's lymphoma.

[00154] In certain embodiments, the T cell malignancy is T-cell chronic lymphocytic leukemia (T-CLL).

[00155] In certain embodiments, the hematologic malignancy is multiple myeloma.

[00156] In certain embodiments, the hematologic malignancy is acute myeloid leukemia (AML).

CAR-T Cells

[00157] The present disclosure provides chimeric antigen receptor-bearing T cells (CAR-T cells), pharmaceutical compositions comprising them, and methods of immunotherapy for the treatment of cancer, specifically hematologic malignancies.

[00158] A CAR-T cell is a T cell which expresses a chimeric antigen receptor. The T cell expressing a CAR molecule may be a helper T cell, a cytotoxic T cell, a viral-specific cytotoxic T cell, a memory T cell, or a gamma delta ($\gamma\delta$) T cell.

[00159] A chimeric antigen receptor (CAR), is a recombinant fusion protein comprising: 1) an extracellular ligand-binding domain, i.e., an antigen-recognition domain, 2) a transmembrane domain, and 3) a signaling transducing domain.

[00160] The extracellular ligand-binding domain is an oligo- or polypeptide that is capable of binding a ligand. Preferably, the extracellular ligand-binding domain will be capable of interacting with a cell surface molecule which may be an antigen, a receptor, a peptide ligand, a protein ligand of the target, or a polypeptide of the target. The extracellular ligand-binding domain can specifically bind to an antigen with an affinity constant or affinity of interaction (K_D) between about 0.1 pM to about 10 pM, to about 0.1 pM to about 1 pM, or more preferably to about 0.1 pM to about 100 nM. Methods for determining the affinity constant or affinity of interaction (K_D) are well-known in the art. In some instances, the extracellular ligand-binding domain is chosen to recognize a ligand that acts as a cell surface marker on target cells associated with particular disease states.

[00161] In one embodiment, the extracellular ligand-binding domain comprises a single chain antibody fragment (scFv) comprising the light (V_L) and the heavy (V_H) variable fragment joined by a linker (e.g., GGGGS₍₂₋₆₎) and confers specificity for either a T cell antigen or an antigen that is not specific to a T cell. In one embodiment, the chimeric antigen receptor of a CAR-T cell may bind to an T cell-specific antigen expressed or overexpressed on a malignant T cell for which a CAR-T cell is deficient in the antigen (e.g., a genome-edited CAR-T cell).

[00162] Non-limiting examples of CAR-targeted antigens expressed on malignant T cells include CD5, CD7, CD2, CD4, and CD3. In one embodiment, a CAR-T cell of the present disclosure comprises a chimeric antigen receptor with an extracellular ligand-binding domain that specifically binds to CD5.

[00163] In another embodiment, a CAR-T cell of the present disclosure comprises a chimeric antigen receptor with an extracellular ligand-binding domain that specifically binds to CD7. In another words, the CAR which specifically binds CD7, comprises an extracellular ligand-binding domain comprising a polypeptide sequence displaying at least 80%, 90%, 95%, 97%, or 99% identity with an amino acid sequence selected from SEQ ID NO:20 and SEQ ID NO:21, and linked together by a flexible linker comprising the sequence (GGGGS)₃₋₄.

[00164] In another embodiment, a CAR-T cell of the present disclosure comprises a chimeric antigen receptor with an extracellular ligand-binding domain that specifically binds to CD2. In

another words, the CAR which specifically binds CD2, comprises an extracellular ligand-binding domain comprising a polypeptide sequence displaying at least 80%, 90%, 95%, 97%, or 99% identity with an amino acid sequence selected from SEQ ID NO:12: and SEQ ID NO:13 or SEQ ID:14 and SEQ ID NO:15, and linked together by a flexible linker comprising the sequence (GGGGS)₃₋₄.

[00165] In yet another embodiment, a CAR-T cell of the present disclosure comprises a chimeric antigen receptor with an extracellular ligand-binding domain that specifically binds to CD4.

[00166] In still another embodiment, a CAR-T cell of the present disclosure comprises an extracellular ligand-binding domain of a chimeric antigen receptor that specifically binds to CD3. In another words, the CAR which specifically binds CD3, comprises an extracellular ligand-binding domain comprising a polypeptide sequence displaying at least 80%, 90%, 95%, 97%, or 99% identity with an amino acid sequence selected from SEQ ID NO:16: and SEQ ID NO:17 or SEQ ID:18 and SEQ ID NO:19, and linked together by a flexible linker comprising the sequence (GGGGS)₃₋₄.

[00167] Non-limiting examples of CAR-targeted antigens expressed on the surface of leukemia cells (e.g., abnormal myeloblasts, red blood cells, or platelets) include CD123 (IL3RA), CD371 (CLL-1; CLEC12A), CD117 (c-kit), and CD135 (FLT3), CD7, and Tim3. A CAR may be constructed with an extracellular ligand-binding domain to target these antigens for treatment of leukemia, i.e., acute myeloid leukemia (AML).

[00168] Non-limiting examples of CAR-targeted antigens expressed on the surface of a multiple myeloma cell (e.g., a malignant plasma cell) include BCMA, CS1, CD38, CD79A, CD79B, CD138, and CD19. A CAR may be constructed with an extracellular ligand-binding domain to target these antigens for treatment of multiple myeloma. In another embodiment, the CAR may be constructed with a portion of the APRIL protein, targeting the ligand for the B-Cell Maturation Antigen (BCMA) and Transmembrane Activator and CAML Interactor (TACI), effectively co-targeting both BCMA and TACI for the treatment of multiple myeloma. A signal peptide directs the transport of a secreted or transmembrane protein to the cell membrane and/or cell surface to allow for correct localization of the polypeptide. Particularly, the signal peptide of the present disclosure directs the appended polypeptide, i.e., the CAR receptor, to the cell membrane wherein the extracellular ligand-binding domain of the appended polypeptide is

displayed on the cell surface, the transmembrane domain of the appended polypeptide spans the cell membrane, and the signaling transducing domain of the appended polypeptide is in the cytoplasmic portion of the cell. In one embodiment, the signal peptide is the signal peptide from human CD8 α (SEQ ID NO:1). In one embodiment, the signal peptide is a functional fragment of the CD8 α signal peptide. A functional fragment is defined as a fragment of at least 10 amino acids of the CD8 α signal peptide that directs the appended polypeptide to the cell membrane and/or cell surface. Examples of functional fragments of the human CD8 α signal peptide include the amino acid sequences MALPVTALLLPLALLHAA, MALPVTALLLP, PVTALLLPLALL, and LLLPLALLLHAARP.

[00169] Typically, the extracellular ligand-binding domain is linked to the signaling transducing domain of the chimeric antigen receptor (CAR) by a transmembrane domain (Tm). The transmembrane domain traverses the cell membrane, anchors the CAR to the T cell surface, and connects the extracellular ligand-binding domain to the signaling transducing domain, impacting the expression of the CAR on the T cell surface.

[00170] The distinguishing feature of the transmembrane domain in the present disclosure is the ability to be expressed at the surface of an immune cell to direct an immune cell response against a pre-defined target cell. The transmembrane domain can be derived from natural or synthetic sources. Alternatively, the transmembrane domain of the present disclosure may be derived from any membrane-bound or transmembrane protein.

[00171] Non-limiting examples of transmembrane polypeptides of the present disclosure alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, *CDS*, *CDS*, CD9, CD16, CD22, CD33, CD37, CD64, *CDS0*, CD86, CD134, CD137 and CD154. Alternatively, the transmembrane domain can be synthetic and comprise predominantly hydrophobic amino acid residues (e.g., leucine and valine). In one embodiment, the transmembrane domain is derived from the T-cell surface glycoprotein CD8 alpha chain isoform 1 precursor (NP_001139345.1) (SEQ ID NO:4), and more preferably CD28 (SEQ ID NO:3). The transmembrane domain can further comprise a hinge region between extracellular ligand-binding domain and said transmembrane domain. The term "hinge region" generally means any oligo- or polypeptide that functions to link the transmembrane domain to the extracellular ligand-binding domain. In particular, hinge region is used to provide more flexibility and accessibility for the extracellular ligand-binding domain. A hinge region may comprise up to 300 amino

acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids. Hinge region may be derived from all or parts of naturally-occurring molecules such as CD28, 4-1BB (CD137), OX-40 (CD134), CD3 ζ , the T cell receptor α or β chain, CD45, CD4, CD5, CD8, CD8 α , CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, ICOS, CD154 or from all or parts of an antibody constant region. Alternatively, the hinge region may be a synthetic sequence that corresponds to a naturally-occurring hinge sequence or the hinge region may be an entirely synthetic hinge sequence. In one embodiment, the hinge domain comprises a part of human CD8 α (SEQ ID NO:2), Fc γ RIII α receptor, or IgG1, and have at least 80%, 90%, 95%, 97%, or 99% sequence identity thereto.

[00172] A chimeric antigen receptor (CAR) of the present disclosure comprises a signal transducing domain or intracellular signaling domain of a CAR which is responsible for intracellular signaling following the binding of the extracellular ligand binding domain to the target resulting in the activation of the immune cell and immune response. In other words, the signal transducing domain is responsible for the activation of at least one of the normal effector functions of the immune cell in which the CAR is expressed. For example, the effector function of a T cell can be a cytolytic activity or helper T cell activity, including the secretion of cytokines. Thus, the term "signal transducing domain" refers to the portion of a protein which transduces the effector signal function signal and directs the cell to perform a specialized function.

[00173] Examples of signal transducing domains for use in a CAR can be the cytoplasmic sequences of the T cell receptor and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivate or variant of these sequences and any synthetic sequence that has the same functional capability. Signal transduction domain comprises two distinct classes of cytoplasmic signaling sequence, those that initiate antigen-dependent primary activation, and those that act in an antigen-independent manner to provide a secondary or co- stimulatory signal. Primary cytoplasmic signaling sequence can comprise signaling motifs which are known as immunoreceptor tyrosine-based activation motifs of ITAMs. ITAMs are well defined signaling motifs found in the intracytoplasmic tail of a variety of receptors that serve as binding sites for syk/zap70 class tyrosine kinases. Non-limiting examples of ITAM that can be used in the present disclosure can include those derived from TCR ζ , FcR γ , FcR β , FcR ϵ , CD3 γ , CD3 δ , CD3 ϵ , CDS, CD22, CD79a, CD79b and CD66d. In one

embodiment, the signaling transducing domain of the CAR can comprise the CD3 ζ signaling domain with an amino acid sequence of at least 80%, 90%, 95%, 97%, or 99% sequence identity thereto.

[00174] In addition, the CAR-T cells of the present disclosure may further comprise one or more suicide gene therapy systems. Suitable suicide gene therapy systems known in the art include, but are not limited to, several herpes simplex virus thymidine kinase (HSVtk)/ganciclovir (GCV) or inducible caspase 9 proteins. In one embodiment, the suicide gene is a chimeric CD34/thymidine kinase.

[00175] T cells disclosed herein may be deficient in an antigen to which the chimeric antigen receptor specifically binds and are therefore fratricide-resistant. In some embodiments, the antigen of the T cell is modified such that the chimeric antigen receptor no longer specifically binds the modified antigen. For example, the epitope of the antigen recognized by the chimeric antigen receptor may be modified by one or more amino acid changes (e.g., substitutions or deletions) or the epitope may be deleted from the antigen. In other embodiments, expression of the antigen is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more. Methods for decreasing the expression of a protein are known in the art and include, but are not limited to, modifying or replacing the promoter operably linked to the nucleic acid sequence encoding the protein. In still other embodiments, the T cell is modified such that the antigen is not expressed, e.g., by deletion or disruption of the gene encoding the antigen. In each of the above embodiments, the T cell may be deficient in one or preferably all the antigens to which the chimeric antigen receptor specifically binds. Methods for genetically modifying a T cell to be deficient in an antigen are well known in art, and non-limiting examples are provided above. In an exemplary embodiment, CRISPR/cas9 gene editing can be used to modify a T cell to be deficient in an antigen, for example as described below. Alternatively, TALENs may be used to edit genes.

[00176] In an variation of the method above, a construct encoding one or more protein expression blocker (PEBL) may be transduced into the cell, either as the editing step or part of the editing step, or as part of CAR transduction. For example, an construct encoding an antibody-derived single-chain variable fragment specific for CD3 ϵ may be transduced, e.g. by a lentiviral vector. Once expressed, the PEBL colocalizes intracellularly with CD3 ϵ , blocking surface CD3 and TCR $\alpha\beta$ expression. Accordingly, PEBL blockade of surface CD3/TCR $\alpha\beta$

expression is an alternative method of preparing allogeneic CAR-T cells. Furthermore, PEBL and CAR expression can be combined in a single construct. Either of these methods may be achieved using the methods disclosed herein, and PEBLs may be produced for blockade of any of the targets of gene suppression disclosed herein.

[00177] The methods described above may be adapted to insert a CAR into a locus for a gene encoding an antigen, cell surface protein, or secretable protein, such as a cytokine. In this way, editing of the genome is effected by transfection of CAR. Thereafter, cells may be activated as described herein, removing separate genome editing step in certain embodiments. Ideally, such a step should be performed while cells are actively dividing. Such methods are also expected to result in robust expansion of engineered cells.

[00178] In certain circumstances, an T cell may be selected for deficiency in the antigen to which the chimeric antigen receptor specifically binds. Certain T cells will produce and display less of a given surface protein; instead if deleting or non-functionalizing the antigen that will be the target of the T-CAR, the T cell can be selected for deficiency in the antigen, and the population of antigen-deficient cells expanded for transduction of the CAR. Such a cell would also be fratricide-resistant.

[00179] Table 1. Amino acid sequences of different CAR components.

<u>Functional domains</u>	<u>SEQ ID</u> <u>NO:</u>	<u>Amino acid sequence</u>
CD8α signal peptide	SEQ ID NO:1	MALPVTALLPLALLHAARP
CD8α hinge	SEQ ID NO:2	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGA VHTRGLDFACD
CD28 Transmembrane (T_m) domain	SEQ ID NO:3	FWVLVVVGGVLACYSLLVTVAFIIFWV
Surface glycoprotein CD8 alpha chain isoform 1 precursor (NP_001139345.1)	SEQ ID NO:4	MALPVTALLPLALLHAARPSQFRVSPLDRT WNLGETVELKCQVLLSNPTSGCSWLFQPRGAA ASPTFLLYLSQNKPKAAEGLDTRFSGKRLGDT FVLTLSDFRRENEGYYFCSALSNSIMYFSHFVP VFLPAKPTTTAPRPPTPAPTIASQPLSLRPEACR PAAGGAVHTRGLDFACDIYIWAPLAGTCGVLL

		LSLVITLYCNHRNRRRVCKCPRPVVKSGDKPSL SARYV
4-1BB costimulatory domain	SEQ ID NO:5	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFP EEEEGGCEL
CD28 costimulatory domain	SEQ ID NO:6	RSKRSRLLHSDYMNMTPRRPGPTRKHYPYAP PRDFAAYRS
CD3 zeta (ζ)	SEQ ID NO:7	RVKFSRSADAPAYKQGQNQLYNELNLGRREEY DVLDKRRGRDPEMGGKPRRKNPQEGLYNELQ KDKMAEAYSEIGMKGERRRGKGHDGLYQGLS TATKDTYDALHMQALPPR
P2A peptide	SEQ ID NO:8	GSGATNFSLLKQAGDVEENPGP
(GGGG)₄ linker	SEQ ID NO:9	GGGGSGGGSGGGSGGGGS
hCD34	SEQ ID NO:10	MPRGWTALCLLSLLPSGFMSLDNNGTATPELP TQGTFSNVSTNVSYQETTPSTLGSTSLHPVSQ HGNEATTNITETTVKFTSTSVITSVYGNTNSSVQ SQTSVISTVFTTPANVSTPETTLKPSLSPGNVSD LSTTSTSLATSPTKPYTSSSPILSDIKAEIKCSGIR EVKLTQGICLEQNKTSSCAEFKKDRGEGLARV LCGEEQADADAGAQCSSLQAQSEVRPQCLLL VLANRTEISSKLQLMKKHQSDLKKLGILDFTEQ DVASHQSYSQKTLIALVTSGALLAVLGITGYFL MNRRSWSPI
Human-Herpes Simplex Virus-1 (HSV) - thymidine kinase (TK)	SEQ ID NO:11	MPRGWTALCLLSLLPSGFMSLDNNGTATPELP TQGTFSNVSTNVSYQETTPSTLGSTSLHPVSQ HGNEATTNITETTVKFTSTSVITSVYGNTNSSVQ SQTSVISTVFTTPANVSTPETTLKPSLSPGNVSD LSTTSTSLATSPTKPYTSSSPILSDIKAEIKCSGIR EVKLTQGICLEQNKTSSCAEFKKDRGEGLARV LCGEEQADADAGAQCSSLQAQSEVRPQCLLL

		VLANRTEISSKLQLMKKHQSDDLKKGILDFTEQ DVASHQSYSQKTLIALVTSGALLAVLGITGYFL MNRRSWSPTGEGGGGGDLGGVKLPHLFGKRL VEARMASYPCHQHASAFDQAARSRGHSNRRT ALRPRRQQEATEVRLEQKMPTLLRVYIDGPHG MGKTTTTQLLVALGSRDDIVYVPEPMTYWQV LGASETIANIYTTQHRLDQGEISAGDAAVVMTS AQITMGMPYAVTDAVLAPHVGGGEAGSSHAPPP ALTLLDRHPIAVMLCYPARYLMGSMTPQAV LAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQ RPGERLDLAMLAAIRRVYGLLANTVRYLQGGG SWWEDWGQLSGTAVPPQGAEPQSNAGPRPHIG DTLFTLFRAPPELLAPNGDLYNVFAWALDVLAK RLRPMHVFILDYDQSPAGCRDALLQLTSGMVQ THVTTGPSIPTICDLARTFAREMGEAN
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[00180] Table 2. Amino acid sequences of the variable heavy (V_H) and variable light (V_L) chains of the scFvs.

<u>ScFv sequences</u>	<u>SEQ ID</u> <u>NO:</u>	<u>Amino acid sequence</u>
CD2 heavy chain variable region (35.1 ATCC[®]HB-222[™])	SEQ ID NO:12	EVKLEESGAELVKPGASVKLSCRTSGFNIKDTI HWVKQRPEQGLKWIGRIDPANGNTKYDPKFQ DKATVTADTSSNTAYLQLSSLTSEDNAVYYCV TYAYDGNWYFDVWGAGTAVTVSS
CD2 light chain variable region (35.1 ATCC[®]HB-222[™])	SEQ ID NO:13	DIKNITQSPSSMYVSLGERVTITCKASQDINSFL SWFQQKPGKSPKTLIYRANRLVDGVPSRFSGS GSGQDYSLTISSELEYEDMEIYYCLQYDEFPYTF GGGTKLEMKR
CD2 heavy chain variable region (OKT 11 ATCC[®]CRL-8027[™])	SEQ ID NO:14	EVQLEESGAELVRPGTSVKLSCKASGYTFTSY WMHWIKQRPEQGLEWIGRIDPYDSETHYNEK FKDKAILSVDKSSSTAYIQLSSLTSDSAVYYC

		SRRDAKYDGYALDYWGQGTSVTVSS
CD2 light chain variable region (OKT 11 ATCC®CRL-8027™)	SEQ ID NO:15	DJVMTQAAPSVPVTPGESVSISCRSSKTLHSN GNTYLYWFLQRPGQSPQVLIYRMSNLASGVP NRFSGSGSETTFTLRISRVEAEDVGIYYCMQHL EYPYTFGGGTKLEIER
CD3 heavy chain variable region (OKT 3)	SEQ ID NO:16	GSQVQLQQSGAELARPGASVKMSCKASGYTF TRYTMHWVKQRPGQGLEWIGYINPSRGYTNY NQKFQDKATLTDDKSSSTAYMQLSSLTSEDSA VYYCARYYDDHYCLDYWGQGTTTLTVSS
CD3 light chain variable region (OKT 3)	SEQ ID NO:17	QIVLTQSPAIMASAPGEKVTMTCSASSSVSYM NWKYQQKSGTSPKRWIYDTSKLGSGVPAHFRG SGSGTSYSLTISGMEAEDAATYYCQQWSSNPF TFSGGTKLEINR
CD3 heavy chain variable region (UCHT1)	SEQ ID NO:18	EVQLVESGGGLVQPGGSLRLSCAASGYSFTGY TMNWVRQAPGKCLEWVALINPYKGVSTYNQ KFKDRFTISVDKSKNTAYLQMNSLRAEDTAV YYCARSGYYGDSDWYFDVWGQGTTLTVSS
CD3 heavy chain variable region (UCHT1)	SEQ ID NO:19	DIQMTQSPSSLSASVGDRTITCRASQDIRNYL NWKYQQKPGKAPKLLIYYTSRLESGVPSRFGSGS GSGTDYTLTISSLQPEDFATYYCQQGNTLPWT FGCGTKVEIK
CD7 heavy chain variable region	SEQ ID NO:20	EVQLVESGGGLVKPGGSLKLSCAASGLTFSSY AMSWVRQTPEKRLEWVASISSGGFTYYPDSDV KGRFTISRDNARNILYLQMSSLRSEDAMYYC ARDEVRGYLDVWGAGTTVTVS
CD7 light chain variable region	SEQ ID NO:21	DIQMTQTTSSLSASLGDRVTISCSASQGISNYL NWKYQQKPDGTVKLLIYYTSSLHSGVPSRFGSGS GSGTDYSLTISNLEPEDIATYYCQQYSKLPYTF GGGTKLEIKR
FTL3 heavy chain variable region (EB10)	SEQ ID NO:22	EVQLVQSGAEVKKPGASVKVSCKASGYTFTS YYMHWVRQAPGQGLEWMGIINPSGGSTSYAQ

		KFQGRVTMTRDTSTSTVYMELSSLRSEDNAVY YCARGVGAHDAFDIWGQGTTVTVSS
FTL3 light chain variable region (EB10)	SEQ ID NO:23	DVVMTQSPLSLPVTGPGEPAISCRSSQSLLHSN GNNYLDWYLQKPGQSPQLLIYLGSNRASGVP DRFSGSGSDTDFTLQISRVEAEDVGVYYCMQG THPAISFGQGTRLEIK
FTL3 heavy chain variable region (NC7)	SEQ ID NO:24	EVQLVQSGAEVKKPGSSVKVSCKASGGTFSSY AISWVRQAPGQGLEWMGGIIPFGTANYAQKF QGRVTITADKSTSTAYMELSSLRSEDNAVYYC ATFALFGFREQAFDIWGQGTTVTVSS
FTL3 light chain variable region (NC7)	SEQ ID NO:25	DIQMTQSPSSLSASVGDRVTITCRASQSISSYLN WYQQKPGKAPKLLIYAASSLQSGVPSRFSGSG SGTDFTLTISLQPEDLATYYCQSYSTPFTFGP GTKVDIK
FTL3 heavy chain variable region (D3-D4)	SEQ ID NO:26	EVQLVQSGAEVKKPGASVKVSCKASGYTFTS YYMHWARQAPGQGLEWMGIINPSGGSTSYAQ KFQGRVTMTRDTSTSTVYMELSSLRSEDNAVY YCARVVAADVWGQGTTLTVSS
FTL3 light chain variable region (D3-D4)	SEQ ID NO:27	DVVMTQSPLSLPVTGPGEPAISCRSSQSLLHSN GNNYLDWYLQKPGQSPQLLIYLGSNRASGVP DRFSGSGSGTDFTLKISRVEAEDVGVYYCMQS LQTPFTFGPGTKVDIK
CS1 heavy chain variable region	SEQ ID NO:28	QVQLQQPGAELVRPGASVKLSCKASGYSFTTY WMNWVKQRPGQGLEWIGMIHPDSETRL NQKFKDKATLTVDKSSSTAYMQLSSPTSEDSA VYYCARSTMIATRAMDYWGQGTSVTVSS
CS1 light chain variable region	SEQ ID NO:29	DIVMTQSQKSMSTSVGDRVSITCKASQDVITG VAWYQQKPGQSPKLLIYSASYRYTGVPD RFTGSGSGTDFTFTISNVQAEDLAVYYCQQHY STPLTFGAGTKLELK
CD33 heavy chain	SEQ ID	QVQLQQPGAEEVVKPGASVKMSCKASGYTFTS

variable region	NO:30	YYIHWIKQTPGQGLEWVGVIYPGNDDISYNQK FQGKATLTADKSSTTAYMQLSSLTSEDSAVYY CAREVRLRYFDVWGQGTTTVTVSSSG
CD33 light chain variable region	SEQ ID NO:31	GSEIVLTQSPGSLAVSPGERVTMSCKSSQSVFF SSSQKNYLAWYQQIPGQSPRLLIYWASTRESG VPDRFTGSGSGTDFTLTISVQPEDLAIIYCHQ YLSSRTFGQGTKLEIKR

Mono CAR-T Cells (mCAR-T)

[00181] The CAR-T cells encompassed by the present disclosure are deficient in one or more antigens to which the chimeric antigen receptor specifically binds and are therefore fratricide-resistant. In some embodiments, the one or more antigens of the T cell is modified such the chimeric antigen receptor no longer specifically binds the one or more modified antigens. For example, the epitope of the one or more antigens recognized by the chimeric antigen receptor may be modified by one or more amino acid changes (e.g., substitutions or deletions) or the epitope may be deleted from the antigen. In other embodiments, expression of the one or more antigens is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more. Methods for decreasing the expression of a protein are known in the art and include, but are not limited to, modifying or replacing the promoter operably linked to the nucleic acid sequence encoding the protein. In still other embodiments, the T cell is modified such that the one or more antigens is not expressed, e.g., by deletion or disruption of the gene encoding the one or more antigens. In each of the above embodiments, the CAR-T cell may be deficient in one or preferably all the antigens to which the chimeric antigen receptor specifically binds. The methods to genetically modify a T cell to be deficient in one or more antigens are well known in art and non-limiting examples are provided herein. In embodiments described in Examples 1-6, the CRISPR-Cas9 system is used to modify a T cell to be deficient in one or more antigens.

[00182] CAR-T cells encompassed by the present disclosure may further be deficient in endogenous T cell receptor (TCR) signaling as a result of deleting a part of the T Cell Receptor (TCR)-CD3 complex. In various embodiments it may be desirable to eliminate or suppress endogenous TCR signaling in CAR-T cells disclosed herein. For example, decreasing or

eliminating endogenous TCR signaling in CAR-T cells may prevent or reduce graft versus host disease (GvHD) when allogeneic T cells are used to produce the CAR-T cells. Methods for eliminating or suppressing endogenous TCR signaling are known in the art and include, but are not limited to, deleting a part of the TCR-CD3 receptor complex, e.g., the TCR receptor alpha chain (TRAC), the TCR receptor beta chain (TCR β), TCR δ , TCR γ , CD3 ϵ , CD3 γ , and/or CD3 δ . Deleting a part of the TCR receptor complex may block TCR mediated signaling and may thus permit the safe use of allogeneic T cells as the source of CAR-T cells without inducing life-threatening GvHD.

[00183] In addition, the CAR-T cells encompassed by the present disclosure may further comprise one or more suicide genes as described herein.

[00184] In an embodiment, the disclosure provides a T cell comprising a chimeric antigen receptor that specifically binds CD5, wherein the T cell is deficient in CD5, e.g., CD5 Δ CART5 cell. In non-limiting examples the deficiency in CD5 resulted from (a) modification of CD5 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD5, (b) modification of the T cell such that expression of the antigen is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD5 is not expressed (e.g., by deletion or disruption of the gene encoding CD5). In further embodiments, the T cell comprises a suicide gene and/or a modification such that endogenous T cell receptor (TCR) mediated signaling is blocked in the T cell. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA is expressed in CD5 Δ CART5 cells.

[00185] In another embodiment, the disclosure provides a T cell comprising a chimeric antigen receptor that specifically binds CD7, wherein the T cell is deficient in CD7, e.g., CD7 Δ CART7 cell. In non-limiting examples the deficiency in CD7 resulted from (a) modification of CD7 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD7, (b) modification of the T cell such that expression of the antigen is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 is not expressed (e.g., by deletion or disruption of the gene encoding CD7). In further embodiments, the T cell comprises a suicide gene and/or a modification such that endogenous T cell receptor (TCR) mediated signaling is

blocked in the T cell. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA is expressed in CD7 Δ CART7 cells.

[00186] In another embodiment, the disclosure provides a T cell comprising a chimeric antigen receptor that specifically binds CD2, wherein the T cell is deficient in CD2, *e.g.*, CD2 Δ CART2 cell. In non-limiting examples the deficiency in CD2 resulted from (a) modification of CD2 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD2, (b) modification of the T cell such that expression of the antigen is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD2 is not expressed (*e.g.*, by deletion or disruption of the gene encoding CD2). In further embodiments, the T cell comprises a suicide gene and/or a modification such that endogenous T cell receptor (TCR) mediated signaling is blocked in the T cell. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA is expressed in CD2 Δ CART2 cells.

[00187] In another embodiment, the disclosure provides a T cell comprising a chimeric antigen receptor that specifically binds CD4, wherein the T cell is deficient in CD4, *e.g.*, CD4 Δ CART4 cell. In non-limiting examples the deficiency in CD4 resulted from (a) modification of CD4 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD4, (b) modification of the T cell such that expression of the antigen is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD4 is not expressed (*e.g.*, by deletion or disruption of the gene encoding CD4). In further embodiments, the T cell comprises a suicide gene and/or a modification such that endogenous T cell receptor (TCR) mediated signaling is blocked in the T cell. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA is expressed in the CD4 Δ CART4 cells.

[00188] In another embodiment, the disclosure provides a T cell comprising a chimeric antigen receptor that specifically binds CD3, wherein the T cell is deficient in CD3 ϵ , *e.g.*, CD3 Δ CART3 ϵ cell. In non-limiting examples the deficiency in CD3 resulted from (a) modification of CD3 expressed by the T cell such that the chimeric antigen receptor no longer

specifically binds the modified CD3, (b) modification of the T cell such that expression of the antigen is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD3 is not expressed (*e.g.*, by deletion or disruption of the gene encoding CD3 ϵ). In further embodiments, the T cell comprises a suicide gene and/or a modification such that endogenous T cell receptor (TCR) mediated signaling is blocked in the T cell. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA is expressed in the CD3 Δ CART3 ϵ cells.

[00189] Disclosed are embodiments of CAR amino acid sequences that can be expressed on the surface of a genome-edited CAR-T cell derived from a cytotoxic T cell, a memory T cell, or a gamma delta ($\gamma\delta$) T cell.

[00190] **Table 3. Amino Acid Sequences of Mono Chimeric Antigen Receptors (CARs).**

<u>Mono CAR</u> <u>Constructs</u>	<u>SEQ ID</u> <u>NO:</u>	<u>Amino acid sequence</u>
CD7-CAR-4- 1BB_CD34	SEQ ID NO:32	MALPVTALLLPLALLLHAARPDIQMTQTSSLSASLG DRVTISCSASQGISNYLNWYQQKPDGTVKLLIYTSS LHSGVPSRFSGSGSGTDYSLTISNLEPEDIATYYCQQY SKLPYTFGGGKLEIKRGGGSGGGGSGGGGSGGGG SEVQLVESGGGLVKPGGSLKLSAASGLTFSSYAMS WVRQTPEKRLEWVASISSGGFTYYPDSVKGRFTISR NARNILYLQMSSLRSEDAMYYCARDEVRYLDVW GAGTTVTVSPRASTTTPAPRPPTPAPTASQPLSLRPEA CRPAAGGAVHTRGLDFACDFWVLVVVGGVLACYSL LVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEED GCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQL YNELNLGRREEYDVLDRRRGRDPEMGGKPRRKNPQ EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDLGY QGLSTATKDTYDALHMQALPPRRTDGSATNFSLLK QAGDVEENPGPVSEAMPRGWTALCLLSLLPSGFMSL DNNGTATPELPTQGTFSNVSTNVSYQETTTSTLGSTS

		LHPVSQHGNEATTNITETTVKFTSTSVITSVYGNTNSS VQSQTSVISTVFTTPANVSTPETTLKPSLSPGNVSDLS TTSTSLATSPTKPYTSSSPILSDIKAEIKCSGIREVKLTQ GICLEQNKTS SCAEFKKDRGEGLARVLCGEEQADAD AGAQVCSLLLAQSEVRPQCLLLVLANRTEISSKLQLM KKHQSDLKKLGILDFTEQDVASHQSYSQKTLIALVTS GALLAVLGITGYFLMNRRSWSPI
CD7-CAR-4- 1BB_CD34_TK	SEQ ID NO:33	MALPVTALLLPLALLLHAARPDQMTQTTSSLSASLG DRVTISCSASQGISNYLNWYQQKPDGTVKLLIYYTSS LHSGVPSRFSGSGSGTDYSLTISNLEPEDIATYYCQQY SKLPYTFGGGKLEIKRGGGSGGGGSGGGGSGGGG SEVQLVESGGGLVKPGGSLKLSAASGLTFSSYAMS WVRQTPEKRLEWVASISSGGFTYYPDSVKGRFTISR NARNILYLQMSSLRSEDAMYYCARDEVRGYLDVW GAGTTVTVSPRASTTTPAPRPPTPAPTASQPLSLRPEA CRPAAGGAVHTRGLDFACDFWVLVVVGGLVACYS LVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEED GCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQL YNELNLGRREEYDVLDRGRDPEMGGKPRRKNPQ EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDLGY QGLSTATKDTYDALHMQALPPRRTDGSGATNFSLLK QAGDVEENPGPVSEAMPGRWTALCLLSLLPSGFMSL DNNGTATPELPTQGTFSNVSTNVSYQETTTPTLGGTS LHPVSQHGNEATTNITETTVKFTSTSVITSVYGNTNSS VQSQTSVISTVFTTPANVSTPETTLKPSLSPGNVSDLS TTSTSLATSPTKPYTSSSPILSDIKAEIKCSGIREVKLTQ GICLEQNKTS SCAEFKKDRGEGLARVLCGEEQADAD AGAQVCSLLLAQSEVRPQCLLLVLANRTEISSKLQLM KKHQSDLKKLGILDFTEQDVASHQSYSQKTLIALVTS GALLAVLGITGYFLMNRRSWSPTEGGGGGGDLGGV

		KLPHLFGKRLVEARMASYPCHQHASAFDQAARSRG HSNRRRTALRPRRQQEATEVRLEQKMPTLLRVYIDGP HGMGKTTTTQLLVALGSRDDIVYVPEPMTYWQVLG ASETIANIYTTQHRLDQGEISAGDAAVVM TSAQITMG MPYAVTDAVLAPHVGGEGSSHAPPPALTLLDRHPI AVMLCYP AARYLMGSMTPQAVLAFVALIPPTLPGTN IVLGALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVY GLLANTVRYLQGGGSWWEDWGQLSGTAVPPQGAEP QSNAGPRPHIGDTLFTLFRAPPELLAPNGDLYNVFAWA LDVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGM VQTHVTTPGSIPTICDLARTFAREMGEAN
CD7-CAR- CD28_CD34	SEQ ID NO:34	MALPVTALLPLALLHAARPDIQMTQTTSSLSASLG DRVTISCSASQGISNYLNWYQQKPDGTVKLLIYTTSS LHSQVPSRFSGSGSGTDYSLTISNLEPEDIATYYCQQY SKLPYTFGGGKLEIKRGGGGSGGGGSGGGGSGGGG SEVQLVESGGGLVKPGGSLKLSAASGLTFSSYAMS WVRQTPEKRLEWVASISSGGFTYYPDSVKGRFTISR NARNILYLQMSSLRSED TAMY YCARDEV RGYLDVW GAGTTTVTVSPRASTTTTPAPRPPTPAPTIASQPLSLRPEA CRPAAGGAVHTRGLDFACDFWVLVVVGGVLACYSL LVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKH YQPYAPPRDFAAYRSRVKFSRSADAPAYKQGQNQLY NELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPRRTDGSGATNFSLLKQ AGDVEENPGPVSEAMPRGWTALCLLSLLPSGFMSLD NNGTATPELPTQGTF SNVSTNVSYQETTTPTSLGSTSL HPVSQHGNEATTNITETTVKFTSTSVITSVYGNTNSSV QSQTSVISTVFTTPANVSTPETTLKPSLSPGNVSDLSTT STSLATSPTKPYTSSSPILSDIKAEIKCSGIREVKLTQGI

		CLEQNKTSSCAEFKKDRGEGLARVLCGEEQADADAG AQVCSLLLAQSEVRPQCCLLVLANRTEISSKLQLMKK HQSDLKKLGILDFTEQDVASHQSYSQKTLIALVTSGA LLAVLGITGYFLMNRRSWSPI
CD7-CAR- CD28_CD34_T K	SEQ ID NO:35	MALPVTALLPLALLLHAARPDQMTQTTSSLSASLG DRVTISCSASQGISNYLNWYQQKPDGTVKLLIYTSS LHSGVPSRFSGSGSGTDYSLTISNLEPEDIATYYCQQY SKLPYTFGGGKLEIKRGGGSGGGGSGGGGSGGGG SEVQLVESGGGLVKPGGSLKLSAASGLTFSSYAMS WVRQTPEKRLEWVASISSGGFTYYPDSVKGRFTISR NARNILYLQMSSLRSEDAMYYCARDEVRYLDVW GAGTTVTVSPRASTTTPAPRPPTPAPTASQPLSLRPEA CRPAAGGAVHTRGLDFACDFWVLVVVGGVLAACYSL LVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKH YQPYAPPRDFAAYRSRVKFSRSADAPAYKQGQNLQY NELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPRRTDGSGATNFSLLKQ AGDVEENPGPVSEAMPRGWTALCLLSLLPSGFMSLD NNGTATPELPTQGTFNSVSTNVSYQETTPSTLGSTSL HPVSQHGNEATTNITETTVKFTSTSVITSVYGNTNSSV QSQTSVISTVFTTPANVSTPETTLKPSLSPGNVSDLSTT STSLATSPTKPYTSSSPILSDIKAEIKCSGIREVKLTQGI CLEQNKTSSCAEFKKDRGEGLARVLCGEEQADADAG AQVCSLLLAQSEVRPQCCLLVLANRTEISSKLQLMKK HQSDLKKLGILDFTEQDVASHQSYSQKTLIALVTSGA LLAVLGITGYFLMNRRSWSPTEGGGGGGDLGGVKLP HLFGKRLVEARMASYPCHQHASAFDQAARSRGHSN RRTALRPRRQQEATEVRLEQKMPTLLRVYIDGPHGM GKTTTTQLLVALGSRDDIVYVPEPMTYWQVLGASET

		<p> IANIYTTQHRLDQGEISAGDAAVVM TSAQITMGMPY AVTDAVLAPHVGGGEAGSSHAPPPAL TLLDRHPIAV MLCYPAARYLMGSMTPQAVLAFVALIPPTLP GTNIVL GALPEDRHIDRLAKRQRPGERLDLAMLAAIRRVYGL LANTVRYLQGGGSWWEDWGQLSGTAVPPQGAEPQS NAGPRPHIGDTLFTLFRAPPELLAPNGDLYNVFAWAL DVLAKRLRPMHVFILDYDQSPAGCRDALLQLTSGMV QTHVTTPGSIPTICDLARTFAREMGEAN </p>
CD79B-CAR- CD28_CD34	SEQ ID NO:36	<p> MALPVTALLPLALLLHAARPGSDIQLTQSPSSLSASV GDRV TITCKASQSVDYEGDSFLN WYQQKPGKAPKLL IYAASNLESGVPSRFSGSGSGTDFLTISLQPEDFATY YCQQSNEDPLTFGQGTKVEIKRGGGGSGGGGSGGGG SGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAAS GYTFSSYWIEWVRQAPGKGLEWIGEILPGGGDTNYN EIFKGRATFSADTSKNTAYLQMNSLRAEDTAVYYCT RRVPIRLDYWGQGT LVTVSSPRASTTTPAPRPPTPAPT IASQPLSLRPEACRPAAGGAVHTRGLDFACDFWVLV VVGGLVACYSLLVTVAFIIFWVR SKRSRLHSDYMN MTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSAD APAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPE MGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE RRRGKGHDGLYQGLSTATKDTYDALHMQALPPRRT DSGGATNFSLLKQAGDVEENPGPVSEAMP RGWTALC LLSLLPSGFMSLDNNGTATPELPTQGTFSNVSTNVSY QETTPSTLGSTSLHPVSQHGNEATTNITETTVKFTST SVITSVYGNTNSSVQSQTSVISTVFTTPANVSTPETTL KPSLSPGNVSDLSTTSTSLATSPTKPYTSSSPILSDIAE IKCSGIREVKLTQGICLEQNKTSSCAEFKKDRGEGLA RVL CGEEQADADAGAQC SLLLAQSEVRPQC LLLVL ANRTEISSKLQLMKKHQSDLKKLGILDFTEQDVASHQ SYSQKTLIALVTSGALLAVLGITGYFLMNRRSWSPTG </p>

		EGGGGGFKRDLGGVKLPHLFGKRLVEARMASYPCH QHASAFDQAARSRGHSNRRTALRPRRQQEATEVRLE QKMPTLLRVYIDGPHGMGKTTTTQLLVALGSRDDIV YVPEPMTYWQVLGASETIANIYTTQHRLDQGEISAGD AAVVMTSAQITMGMPYAVTDAVLAPHVGGGEAGSSH APPPALTLLLDRHPIAVMLCYPAARYLMGSMTPQAV LAFVALIPPTLPGTNIVLGALPEDRHIDRLAKRQRPGE RLDLAMLAAIRRVYGLLANTVRYLQGGGSWWEDW GQLSGTAVPPQGAEPQSNAGPRPHIGDTLFTLFRAPE LLAPNGDLYNVFAWALDVLAKRLRPMHVFILDYDQ SPAGCRDALLQLTSGMVQTHVTTPGSIPTICDLARTF AREMGEAN
CD2-CAR- CD28_CD34	SEQ ID NO:37	MALPVTALLPLALLHAARPDIVMTQAAPSVPVTPG ESVSISCRSSKTLHNSNGNTYLYWFLQRPQGSPQVLIY RMSNLASGVPNRFSGSGSETTFTLRISRVEAEDVGIIYY CMQHLEYPYTFGGGTKLEIERGGGGSGGGGSGGGGS GGGGSEVQLEESGAELVRPGTSVKLSCKASGYTFTSY WMHWIKQRPEQGLEWIGRIDPYDSETHYNEKFKDKA ILSVDKSSSTAYIQLSSLTSDDSAVYYCSRRDAKYDG YALDYWGQGTSVTVSSPRASSTTPAPRPPTPAPTAS QPLSLRPEACRPAAGGAVHTRGLDFACDFWVLVVV GGVLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMT PRRPGPTRKHYPYAPPRDFAAYRSRVKFSRSADAPA YKQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRR GKGDGLYQGLSTATKDTYDALHMQALPPRRTDGS GATNFSLLKQAGDVEENPGPVSEAMPRGWTALCLLS LLPSGFMSLDNNGTATPELPTQGTFSNVSTNVSYQET TTPSTLGSTSLHPVSQHGNEATTNITETTVKFTSTSVIT SVYGNTNSSVQSQTSVISTVFTTPANVSTPETTLKPSL SPGNVSDLSTTSTSLATSPTKPYTSSSPILSDIKAEIKCS

		GIREVKLTQGICLEQNKTSSCAEFKKDRGEGLARVLC GEEQADADAGAQVCSLLLAQSEVRPQCLLLVLANRT EISSKLQLMKKHQSDLKKLGILDFTEQDVASHQSYSQ KTLIALVTSGALLAVLGITGYFLMNRRSWSPI
CD2-CAR-4- 1BB_CD34	SEQ ID NO:38	MALPVTALLPLALLHAARPDIVMTQAAPSVPVTPG ESVSISCRSSKTLHNSNGNTYLYWFLQRPQGSPQVLIY RMSNLAGVPNRFSGSGSETTFTLRISRVEAEDVGIYY CMQHLEYPYTFGGGTKLEIERGGGGSGGGGSGGGGS GGGGSEVQLEESGAELVRPGTSVKLSCKASGYTFTSY WMHWIKQRPEQGLEWIGRIDPYDSETHYNEKFKDKA ILSVDKSSSTAYIQLSSLTSDDSAVYYCSRRDAKYDG YALDYWGQGTSTVTVSSPRASTTTPAPRPPTPAPTAS QPLSLRPEACRPAAGGAVHTRGLDFACDFWVLVVV GGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMR PVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPA YKQGQNQLYNELNLGRREEYDVLDKRRGRDPENMG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRR GKGHDGLYQGLSTATKDTYDALHMQUALPPRRTDGS GATNFSLLKQAGDVEENPGPVSEAMPRGWTALCLLS LLPSGFMSLDNNGTATPELPTQGTFSNVSTNVSYQET TTPSTLGSTSLHPVSQHGNEATTNITETTVKFTSTSVIT SVYGNTNSSVQSQTSVISTVFTTPANVSTPETTLKPSL SPGNVSDLSTTSTSLATSPTKPYTSSSPILSDIKAEIKCS GIREVKLTQGICLEQNKTSSCAEFKKDRGEGLARVLC GEEQADADAGAQVCSLLLAQSEVRPQCLLLVLANRT EISSKLQLMKKHQSDLKKLGILDFTEQDVASHQSYSQ KTLIALVTSGALLAVLGITGYFLMNRRSWSPI
CD3-CD28- CD34	SEQ ID NO:39	MALPVTALLPLALLHAARPGSQVQLQQSGAELAR PGASVKMSCKASGYTFTRYTMHWVKQRPQGQGLEWI GYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLS SLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSS

		GGGGSGGGSGGGSGGGGSQIVLTQSPAIMSASPG EKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSK LASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQ QWSSNPFTFGSGTKLEINRPRASSTTPAPRPPTPAPTIA SQPLSLRPEACRPAAGGAVHTRGLDFACDFWVLVVV GGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMT PRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPA YKQGQNQLYNELNLGRREEYDVLDRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRR GKGHDGLYQGLSTATKDTYDALHMQUALPPRRTDGS GATNFSLLKQAGDVEENPGPVSEAMPRGWTALCLLS LLPSGFMSLDNNGTATPELPTQGTFSNVSTNVSYQET TTPSTLGSTSLHPVSQHGNEATTNITETTVKFTSTSVIT SVYGNTNSSVQSQTSVISTVFTTPANVSTPETTLKPSL SPGNVSDLSTTSTSLATSPTKPYTSSSPILSDIKAEIKCS GIREVKLTQGICLEQNKTSSCAEFKKDRGEGLARVLC GEEQADADAGAQVCSLLLAQSEVRPQCLLLVLANRT EISSKLQLMKKHQSDLKKLGILDFTEQDVASHQSYSQ KTLIALVTSGALLAVLGITGYFLMNRRSWSPI
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[00191] In a similar manner, other mono-CAR-T cells may be constructed and are given below in Table 4.

[00192] Table 4. Mono-CARs and CAR-Ts.

Example	Antigen Target of CAR-T cells	Antigen Deletion/ Suppression
M1	APRIL	-
M2	APRIL	APRIL
M3	APRIL	APRIL + TRAC
M4	APRIL	APRIL + CD3ε

Example	Antigen Target of CAR-T cells	Antigen Deletion/ Suppression
M5	APRIL	CD3ε
M6	BCMA	-
M7	CD117	-
M8	CD117	CD117
M9	CD123	-

Example	Antigen Target of CAR-T cells	Antigen Deletion/Suppression
M10	CD123	CD123
M11	CD135	-
M12	CD135	CD135
M13	CD138	-
M14	CD19	-
M15	CD1a	-
M16	CD1a	CD3 ϵ
M17	CD1a	TRAC
M18	CD1a	CD1a + TRAC
M19	CD1a	CD1a + CD3 ϵ
M20	CD2	-
M21	CD2	CD2
M22	CD2	CD2 + TRAC
M23	CD2	CD2 + CD3 ϵ
M24	CD20	
M25	CD21	
M26	CD22	
M27	CD23	
M28	CD3	-
M29	CD3	CD3 ϵ
M30	CD3	CD3 ϵ + TRAC
M31	CD33	-
M32	CD33	CD33
M33	CD371	-
M34	CD371	CD371
M35	CD38	-
M36	CD38	CD38
M37	CD4	-
M38	CD4	CD4

Dual CAR-T Cells (dCAR-T)

[00193] A dual CAR-T cell (dCAR-T) may be generated by cloning a protein encoding sequence of a first extracellular ligand-binding domain into a lentiviral vector containing one or more costimulatory domains and a signaling transducing domain and cloning a second protein

Example	Antigen Target of CAR-T cells	Antigen Deletion/Suppression
M39	CD4	CD4 + TRAC
M40	CD4	CD4 + CD3 ϵ
M41	CD5	-
M42	CD5	CD5
M43	CD5	CD5 + TRAC
M44	CD5	CD5 + CD3 ϵ
M45	CD56	-
M46	CD56	CD56
M47	CD56	CD56 + TRAC
M48	CD56	CD56 + CD3 ϵ
M49	CD56	CD3 ϵ
M50	CD56	TRAC
M51	CD7	-
M52	CD7	CD7
M53	CD7	CD7 + TRAC
M54	CD7	CD7 + CD3 ϵ
M55	CD79A	-
M56	CD79B	-
M57	CS1	-
M58	CS1	CS1
M59	Tim-3	-
M60	Tim-3	Tim-3
M61	Tim-3	Tim-3 + TRAC
M62	Tim-3	TRAC
M63	Tim-3	CD3 ϵ
M64	Tim-3	Tim-3 + CD3 ϵ

encoding sequence of a second extracellular ligand-binding domain into the same lentiviral vector containing an additional one or more costimulatory domains and a signaling transducing domain resulting in a plasmid from which the two CAR constructs are expressed from the same vector.

[00194] In one embodiment, the disclosure provides an engineered T cell comprising a dual Chimeric Antigen Receptor (dCAR), i.e., protein encoding sequence of two CARs expressed from a single lentivirus construct, that specifically binds CD5 and TCR receptor alpha chain (TRAC), wherein the T cell is deficient in CD5 and TRAC (e.g., **CD5*TRAC**-dCARTΔCD5ΔTRAC cell). In non-limiting examples the deficiency in CD5 and the TCR receptor alpha chain (TRAC) resulted from (a) modification of CD5 and the TCR receptor alpha chain (TRAC) expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD5 and the TCR receptor alpha chain (TRAC), (b) modification of the T cell such that expression of the CD5 and the TCR receptor alpha chain (TRAC) is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD5 and the TCR receptor alpha chain (TRAC) is not expressed (e.g., by deletion or disruption of the gene encoding CD5 and / or the TCR receptor alpha chain (TRAC)). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD5*TRAC**-CARTΔCD5ΔTRAC cells.

[00195] In a second embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD7 and TCR receptor alpha chain (TRAC), wherein the T cell is deficient in CD7 and TRAC, e.g., **CD7*TRAC**-dCARTΔCD7ΔTRAC cell. In non-limiting examples the deficiency in CD7 and the TCR receptor alpha chain (TRAC) resulted from (a) modification of CD5 and the TCR receptor alpha chain (TRAC) expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD7 and the TCR receptor alpha chain (TRAC), (b) modification of the T cell such that expression of the CD7 and the TCR receptor alpha chain (TRAC) is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 and the TCR receptor alpha chain (TRAC) is not expressed (e.g., by deletion or disruption of the gene encoding CD7 and / or the TCR receptor alpha chain (TRAC)). In further embodiments, the

T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the CD7*TRAC-dCARTΔCD7ΔTRAC cells.

[00196] In a third embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD2 and TCR receptor alpha chain (TRAC), wherein the T cell is deficient in CD2 and TRAC, e.g., **CD2*TRAC-dCARTΔCD2ΔTRAC** cell. In non-limiting examples the deficiency in CD2 and the TCR receptor alpha chain (TRAC) resulted from (a) modification of CD2 and the TCR receptor alpha chain (TRAC) expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD2 and the TCR receptor alpha chain (TRAC), (b) modification of the T cell such that expression of the CD7 and the TCR receptor alpha chain (TRAC) is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD2 and the TCR receptor alpha chain (TRAC) is not expressed (e.g., by deletion or disruption of the gene encoding CD2 and / or the TCR receptor alpha chain (TRAC)). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene fused is in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD2*TRAC-dCARTΔCD2ΔTRAC** cells.

[00197] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD4 and TCR receptor alpha chain (TRAC), wherein the T cell is deficient in CD4 and TRAC, e.g., **CD4*TRAC-dCARTΔCD4ΔTRAC** cell. In non-limiting examples the deficiency in CD4 and the TCR receptor alpha chain (TRAC) resulted from (a) modification of CD4 and the TCR receptor alpha chain (TRAC) expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD4 and the TCR receptor alpha chain (TRAC), (b) modification of the T cell such that expression of the CD7 and the TCR receptor alpha chain (TRAC) is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD4 and the TCR receptor alpha chain (TRAC) is not expressed (e.g., by deletion or disruption of the gene encoding CD4 and / or the TCR receptor alpha chain (TRAC)). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a

modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD4*TRAC-dCARTΔCD4ΔTRAC** cells.

[00198] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD3 and TCR receptor alpha chain (TRAC), wherein the T cell is deficient in CD3 and TRAC, e.g., **CD3*TRAC-dCARTΔCD3TRAC** cell. In non-limiting examples the deficiency in CD3 and the TCR receptor alpha chain (TRAC) resulted from (a) modification of CD3 and the TCR receptor alpha chain (TRAC) expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD3 and the TCR receptor alpha chain (TRAC), (b) modification of the T cell such that expression of the CD3 and the TCR receptor alpha chain (TRAC) is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD3 and the TCR receptor alpha chain (TRAC) is not expressed (e.g., by deletion or disruption of the gene encoding CD3 and / or the TCR receptor alpha chain (TRAC)). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD3*TRAC-dCARTΔCD3ΔTRAC** cells.

[00199] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD2 and the CD3 epsilon (ε) chain, wherein the T cell is deficient in CD2 and CD3 epsilon, e.g., **CD2*CD3ε-dCARTΔCD2ΔCD3ε** cell. In non-limiting examples the deficiency in CD2 and the CD3 epsilon (ε) chain resulted from (a) modification of CD2 and CD3 epsilon expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD2 and CD3 epsilon, (b) modification of the T cell such that expression of the CD2 and CD3 epsilon is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD2 and CD3 epsilon is not expressed (e.g., by deletion or disruption of the gene encoding CD2 and / or CD3 epsilon). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD2*CD3ε-dCARTΔCD2ΔCD3ε** cells.

[00200] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD4 and the CD3 epsilon (ϵ) chain, wherein the T cell is deficient in CD2 and CD3 ϵ , e.g., **CD4*CD3 ϵ -dCART Δ CD4 Δ CD3 ϵ** cell. In non-limiting examples the deficiency in CD4 and the CD3 ϵ chain resulted from (a) modification of CD4 and CD3 epsilon expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD4 and CD3 ϵ , (b) modification of the T cell such that expression of the CD4 and CD3 ϵ is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD4 and CD3 ϵ is not expressed (e.g., by deletion or disruption of the gene encoding CD4 and / or CD3 ϵ). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD4*CD3 ϵ -dCART Δ CD4 Δ CD3 ϵ** cells.

[00201] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD5 and the TCR beta (β) chain, wherein the T cell is deficient in CD5 and TCR β , e.g., **CD5*TCR β -dCART Δ CD5 Δ TCR β** cell. In non-limiting examples the deficiency in CD5 and the TCR β chain resulted from (a) modification of CD5 and TCR β expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD5 and TCR β , (b) modification of the T cell such that expression of the CD5 and TCR β is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD5 and TCR β is not expressed (e.g., by deletion or disruption of the gene encoding CD5 and / or TCR β). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA is expressed in **CD5*TCR β -dCART Δ CD5 Δ TCR β** cells.

[00202] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD7 and the TCR beta (β) chain, wherein the T cell is deficient in CD5 and TCR beta, e.g., **CD7*TCR β -dCART Δ CD7 Δ TCR β** cell. In non-limiting examples the deficiency in CD7 and the TCR β chain resulted from (a) modification of CD7 and TCR β

expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD7 and TCR β , (b) modification of the T cell such that expression of the CD7 and TCR β is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 and TCR β is not expressed (e.g., by deletion or disruption of the gene encoding CD7 and / or TCR β). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA is expressed in the **CD7*TCR β -dCART Δ CD7 Δ TCR β** cells.

[00203] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD2 and the TCR beta (β) chain, wherein the T cell is deficient in CD2 and TCR β , e.g., **CD2*TCR β -dCART Δ CD7 Δ TCR β** cell. In non-limiting examples the deficiency in CD2 and the TCR β chain resulted from (a) modification of CD2 and TCR β expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD2 and TCR β , (b) modification of the T cell such that expression of the CD2 and TCR β is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD2 and TCR β is not expressed (e.g., by deletion or disruption of the gene encoding CD2 and / or TCR β). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD2*TCR β -dCART Δ CD2 Δ TCR β** cells.

[00204] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD4 and the TCR beta (β) chain, wherein the T cell is deficient in CD2 and TCR β , e.g., **CD4*TCR β -dCART Δ CD4 Δ TCR β** cell. In non-limiting examples the deficiency in CD4 and the TCR β chain resulted from (a) modification of CD4 and TCR β expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD4 and TCR β , (b) modification of the T cell such that expression of the CD4 and TCR β is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD4 and TCR β is not expressed (e.g., by deletion or disruption of the gene encoding CD4 and / or TCR β). In further embodiments, the T

cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD4*TCR β -dCART Δ CD4 Δ TCR β** cells.

[00205] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD7 and CD2, wherein the T cell is deficient in CD7 and CD2, e.g., **CD7*CD2-dCART Δ CD7 Δ CD2** cell. In non-limiting examples the deficiency in CD7 and CD2 resulted from (a) modification of CD7 and CD2 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD7 and CD2, (b) modification of the T cell such that expression of the CD7 and CD2 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 and CD2 is not expressed (e.g., by deletion or disruption of the gene encoding CD7 and / or CD2). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD7*CD2-dCART Δ CD7 Δ CD2** cells.

[00206] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD7 and CD5, wherein the T cell is deficient in CD7 and CD5, e.g., **CD7*CD5-dCART Δ CD7 Δ CD5** cell. In non-limiting examples the deficiency in CD7 and CD5 resulted from (a) modification of CD7 and CD5 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD7 and CD5, (b) modification of the T cell such that expression of the CD7 and CD5 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 and CD5 is not expressed (e.g., by deletion or disruption of the gene encoding CD7 and / or CD5). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD7*CD5-dCART Δ CD7 Δ CD5** cells.

[00207] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD7 and CD4, wherein the T cell is deficient in CD7 and CD4 (e.g., **CD7*CD4-dCART Δ CD7 Δ CD4** cell). In non-limiting examples the deficiency in CD7 and

CD4 resulted from (a) modification of CD7 and CD4 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD7 and CD4, (b) modification of the T cell such that expression of the CD7 and CD4 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 and CD4 is not expressed (e.g., by deletion or disruption of the gene encoding CD7 and / or CD4. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples the suicide gene expressed in the **CD7*CD4-dCARTΔCD7ΔCD4** cells encodes a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD7*CD4-dCARTΔCD7ΔCD4** cells.

[00208] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD2 and CD5, wherein the T cell is deficient in CD2, CD5, and TRAC, e.g., **CD2*CD5-dCARTΔCD2ΔCD5ΔTRAC** cell. In non-limiting examples the deficiency in CD2 and CD5 resulted from (a) modification of CD2 and CD5 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD2 and CD5, (b) modification of the T cell such that expression of the CD2 and CD5 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD2 and CD5 is not expressed (e.g., by deletion or disruption of the gene encoding CD2 and / or CD5. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD2*CD5-dCARTΔCD2ΔCD5** cells.

[00209] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD2 and CD4, wherein the T cell is deficient in CD2, CD4, and TRAC, e.g., **CD2*CD4-dCARTΔCD2ΔCD4ΔTRAC** cell. In non-limiting examples the deficiency in CD2 and CD4 resulted from (a) modification of CD2 and CD4 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD2 and CD4, (b) modification of the T cell such that expression of the CD2 and CD4 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD2 and CD4 is not expressed (e.g., by deletion or

disruption of the gene encoding CD2 and / or CD4. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD2*CD4-dCARTΔCD2ΔCD4** cells.

[00210] In another embodiment, the disclosure provides an engineered T cell comprising a dCAR that specifically binds CD5 and CD4, wherein the T cell is deficient in CD5 and CD4, e.g., **CD5*CD4-dCARTΔCD5ΔCD4** cell. In non-limiting examples the deficiency in CD5 and CD4 resulted from (a) modification of CD5 and CD4 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD5 and CD4, (b) modification of the T cell such that expression of the CD5 and CD4 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD5 and CD4 is not expressed (e.g., by deletion or disruption of the gene encoding CD5 and / or CD4. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in **CD5*CD4-dCARTΔCD5ΔCD4** cells.

[00211] In one embodiment, a dual CAR-T cell comprises (i) a first chimeric antigen receptor (CAR) polypeptide comprising a first signal peptide, a first extracellular ligand-binding domain, , a first hinge region, a first transmembrane domain, one or more co-stimulatory domains, and a first signaling transducing domain; and (ii) a second chimeric antigen receptor polypeptide comprising a second signaling peptide, a second extracellular ligand-binding domain, a second hinge region, a second transmembrane domain, one or more co-stimulatory domains, and a second signaling transducing domain; wherein the first extracellular ligand-binding domain and the second extracellular ligand-binding domain have affinities for different cell surface molecules; and wherein the dual CAR-T cell possesses one or more genetic disruptions resulting in reduced expression of the cell surface molecule in the dual CAR-T cell.

[00212] In a second embodiment, the first signal peptide is a CD8α signal sequence.

[00213] In a third embodiment, the first extracellular ligand-binding domain is a fusion protein of the variable regions of immunoglobulin heavy and light chains, designated V_H1 and V_L1, and connected by a short linker peptide of 5 amino acids (GGGGS). In some embodiments,

this linker peptide is repeated 3 or 4 times. In some embodiments, the first antigen recognition domain can be selected from $V_H1 - (GGGGS)_{3-4} - V_L1$ or $V_L1 - (GGGGS)_{3-4} - V_H1$.

[00214] In another embodiment, the first hinge region comprises CD8 α .

[00215] In another embodiment, the first transmembrane domain is CD8 or CD28.

[00216] In some embodiments, the first co-stimulatory domain comprises 4-1BB, CD28, or a combination of both, in either order, i.e., 4-1BB-CD28 or CD28-4-1BB.

[00217] In some embodiments, the first signaling domain is CD3 ζ or a CD3 ζ bi-peptide, i.e. CD3 ζ – CD3 ζ .

[00218] In some embodiments, the second signal peptide is a CD8 α signal sequence of SEQ NO:1.

[00219] In some embodiments, the second extracellular ligand-binding domain is fusion protein of the variable regions of immunoglobulin heavy and light chains, designated V_H2 and V_L2 , and connected by a short linker peptide of 5 amino acids (GGGGS). In some embodiments, this linker peptide is repeated 3 or 4 times. In some embodiments, the second antigen recognition domain can be selected from $V_H2 - (GGGGS)_{3-4} - V_L2$ or $V_L2 - (GGGGS)_{3-4} - V_H2$.

[00220] In another embodiment, the second hinge region comprises CD8 α .

[00221] In another embodiment, the second transmembrane domain is CD8 or CD28.

[00222] In some embodiments, the second co-stimulatory domain comprises 4-1BB, CD28, or a combination of both, in either order, i.e. 4-1BB-CD28 or CD28-4-1BB.

[00223] In some embodiments, the second signaling domain is CD3 ζ or a CD3 ζ bi-peptide, i.e. CD3 ζ – CD3 ζ .

[00224] In some embodiments, the CAR polypeptide comprises a first extracellular ligand-binding domain fusion protein of $V_H1 - (GGGGS)_{3-4} - V_L1$ and a second extracellular ligand-binding domain fusion protein of $V_H2 - (GGGGS)_{3-4} - V_L2$.

[00225] In some embodiments, the CAR polypeptide comprises a first extracellular ligand-binding domain fusion protein of $V_L1 - (GGGGS)_{3-4} - V_H1$ and a second extracellular ligand-binding domain fusion protein of $V_L2 - (GGGGS)_{3-4} - V_H2$.

[00226] In some embodiments, the CAR polypeptide comprises a first extracellular ligand-binding domain fusion protein of $V_H2 - (GGGGS)_{3-4} - V_L2$ and a second extracellular ligand-binding domain fusion protein of $V_H1 - (GGGGS)_{3-4} - V_L1$.

[00227] In some embodiments, the CAR polypeptide comprises a first extracellular ligand-binding domain fusion protein of $V_L2 - (GGGGS)_{3-4} - V_H2$ and a second extracellular ligand-binding domain fusion protein of $V_L1 - (GGGGS)_{3-4} - V_H1$.

[00228] In some embodiments, the CAR polypeptide comprises a first extracellular ligand-binding domain fusion protein of $V_H1 - (GGGGS)_{3-4} - V_L1$ and a second extracellular ligand-binding domain fusion protein of $V_L2 - (GGGGS)_{3-4} - V_H2$.

[00229] In some embodiments, the CAR polypeptide comprises a first extracellular ligand-binding domain fusion protein of $V_L1 - (GGGGS)_{3-4} - V_H1$ and a second extracellular ligand-binding domain fusion protein of $V_H2 - (GGGGS)_{3-4} - V_L2$.

[00230] In some embodiments, the CAR polypeptide comprises a first extracellular ligand-binding domain fusion protein of $V_H2 - (GGGGS)_{3-4} - V_L2$ and a second extracellular ligand-binding domain fusion protein of $V_L1 - (GGGGS)_{3-4} - V_H1$.

[00231] In some embodiments, the CAR polypeptide comprises a first extracellular ligand-binding domain fusion protein of $V_L2 - (GGGGS)_{3-4} - V_H2$ and a second extracellular ligand-binding domain fusion protein of $V_H1 - (GGGGS)_{3-4} - V_L1$.

[00232] In some embodiments, the CAR polypeptide comprises at least one high efficiency cleavage site, wherein the high efficiency cleavage site is selected from P2A, T2A, E2A, and F2A.

[00233] In some embodiments, the CAR polypeptide comprises a suicide gene.

[00234] In some embodiments, the CAR polypeptide comprises a mutant cytokine receptor.

[00235] In some embodiments, the dual CAR-T cell targets two antigens selected from CD5, CD7, CD2, CD4, CD3, CD33, CD123 (IL3RA), CD371 (CLL-1; CLEC12A), CD117 (c-kit), CD135 (FLT3), BCMA, CS1, CD38, CD79A, CD79B, CD138, and CD19, APRIL, and TACI.

[00236] Additional examples of dual CARs are given below in Table 5.

[00237] **Table 5. Dual CARs and dCAR-Ts**

Example	Antigen Targets of CARs in dCAR-T cell	Antigen Deletion/ Suppression
D1	APRILxBCMA	-
D2	APRILxCD19	-
D3	APRILxCD38	-
D4	APRILxCD38	CD38
D5	APRILxCS1	-
D6	APRILxCS1	CS1

D7	BCMAxCD19	-
D8	BCMAxCD38	-
D9	BCMAxCD38	CD38
D10	BCMAxCS1	-
D11	BCMAxCS1	CS1
D12	CD138xAPRIL	
D13	CD138xBCMA	
D14	CD138xCD19	
D15	CD138xCD38	
D16	CD138xCD38	CD38
D17	CD138xCD79A	
D18	CD138xCD79B	
D19	CD138xCS1	
D20	CD138xCS1	CS1
D21	CD19xCD38	-
D22	CD19xCD38	CD38
D23	CD2xCD3 ϵ	-
D24	CD2xCD3 ϵ	CD2
D25	CD2xCD3 ϵ	CD3 ϵ
D26	CD2xCD3 ϵ	CD2 and CD3 ϵ
D27	CD2xCD4	-
D28	CD2xCD4	CD2
D29	CD2xCD4	CD4
D30	CD2xCD4	CD2 and CD4
D31	CD2xCD4	CD2 and TRAC
D32	CD2xCD4	CD4 and TRAC
D33	CD2xCD4	CD2 and CD4 and TRAC
D34	CD2xCD5	-
D35	CD2xCD5	CD2
D36	CD2xCD5	CD5
D37	CD2xCD5	CD2 and CD5
D38	CD2xCD5	CD2 and TRAC
D39	CD2xCD5	CD5 and TRAC
D40	CD2xCD5	CD2 and CD5 and TRAC
D41	CD2xCD7	-
D42	CD2xCD7	CD2
D43	CD2xCD7	CD7
D44	CD2xCD7	CD2 and CD7
D45	CD2xCD7	CD2 and TRAC

D46	CD2xCD7	CD7 and TRAC
D47	CD2xCD7	CD2 and CD7 and TRAC
D48	CD3 ϵ xCD4	-
D49	CD3 ϵ xCD4	CD3 ϵ
D50	CD3 ϵ xCD4	CD4
D51	CD3 ϵ xCD4	CD3 ϵ and CD4
D52	CD3 ϵ xCD5	-
D53	CD3 ϵ xCD5	CD3 ϵ
D54	CD3 ϵ xCD5	CD5
D55	CD3 ϵ xCD5	CD3 ϵ and CD5
D56	CD3 ϵ xCD7	-
D57	CD3 ϵ xCD7	CD3 ϵ
D58	CD3 ϵ xCD7	CD7
D59	CD3 ϵ xCD7	CD3 ϵ and CD7
D60	CD4xCD5	-
D61	CD4xCD5	CD4
D62	CD4xCD5	CD5
D63	CD4xCD5	CD4 and CD5
D64	CD4xCD5	CD4 and TRAC
D65	CD4xCD5	CD5 and TRAC
D66	CD4xCD5	CD4 and CD5 and TRAC
D67	CD4xCD7	-
D68	CD4xCD7	CD4
D69	CD4xCD7	CD7
D70	CD4xCD7	CD4 and CD7
D71	CD4xCD7	CD4 and TRAC
D72	CD4xCD7	CD7 and TRAC
D73	CD4xCD7	CD4 and CD7 and TRAC
D74	CD5xCD7	-
D75	CD5xCD7	CD5
D76	CD5xCD7	CD7
D77	CD5xCD7	CD5 and CD7
D78	CD5xCD7	CD5 and TRAC
D79	CD5xCD7	CD7 and TRAC
D80	CD5xCD7	CD5 and CD7 and TRAC
D81	CD79AxAPRIL	
D82	CD79AxBCMA	

D83	CD79A $\times$ CD19	
D84	CD79A $\times$ CD38	
D85	CD79A $\times$ CD38	CD38
D86	CD79A $\times$ CD79B	
D87	CD79A $\times$ CS1	
D88	CD79A $\times$ CS1	CS1
D89	CD79B $\times$ APRIL	
D90	CD79B $\times$ BCMA	
D91	CD79B $\times$ CD19	
D92	CD79B $\times$ CD38	
D93	CD79B $\times$ CD38	CD38
D94	CD79B $\times$ CD79A	
D95	CD79B $\times$ CS1	
D96	CD79B $\times$ CS1	CS1
D97	CS1 $\times$ CD19	-
D98	CS1 $\times$ CD19	CS1
D99	CS1 $\times$ CD38	-
D100	CS1 $\times$ CD38	CS1
D101	CS1 $\times$ CD38	CD38
D102	CS1 $\times$ CD38	CS1 and CD38
D103	TCR β $\times$ CD2	-
D104	TCR β $\times$ CD2	TCR β
D105	TCR β $\times$ CD2	CD2
D106	TCR β $\times$ CD2	TCR β and CD2
D107	TCR β $\times$ CD3 ϵ	-
D108	TCR β $\times$ CD3 ϵ	TCR β
D109	TCR β $\times$ CD3 ϵ	CD3 ϵ
D110	TCR β $\times$ CD3 ϵ	TCR β and CD3 ϵ
D111	TCR β $\times$ CD4	-
D112	TCR β $\times$ CD4	TCR β
D113	TCR β $\times$ CD4	CD4
D114	TCR β $\times$ CD4	TCR β and CD4
D115	TCR β $\times$ CD5	-
D116	TCR β $\times$ CD5	TCR β
D117	TCR β $\times$ CD5	CD5
D118	TCR β $\times$ CD5	TCR β and CD5
D119	TCR β $\times$ CD7	-
D120	TCR β $\times$ CD7	TCR β
D121	TCR β $\times$ CD7	CD7
D122	TCR β $\times$ CD7	TCR β and CD7
D123	TRAC $\times$ CD2	-

D124	TRACxCD2	TRAC
D125	TRACxCD2	CD2
D126	TRACxCD2	TRAC and CD2
D127	TRACxCD3 ϵ	-
D128	TRACxCD3 ϵ	TRAC
D129	TRACxCD3 ϵ	CD3 ϵ
D130	TRACxCD3 ϵ	TRAC and CD3 ϵ
D131	TRACxCD4	-
D132	TRACxCD4	TRAC
D133	TRACxCD4	CD4
D134	TRACxCD4	TRAC and CD4
D135	TRACxCD5	-
D136	TRACxCD5	TRAC
D137	TRACxCD5	CD5
D138	TRACxCD5	TRAC and CD5
D139	TRACxCD7	-
D140	TRACxCD7	TRAC
D141	TRACxCD7	CD7
D142	TRACxCD7	TRAC and CD7

Tandem CAR-T Cells (tCAR-T)

[00238] A tandem CAR-T cell (tCAR-T), is a T cell with a single chimeric antigen polypeptide comprising two distinct extracellular ligand-binding domains capable of interacting with two different cell surface molecules, wherein the extracellular ligand-binding domains are linked together by a flexible linker and share one or more costimulatory domains, wherein the binding of the first or the second extracellular ligand-binding domain will signal through one or more the costimulatory domains and a signaling transducing domain.

[00239] In one embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD5 and the second extracellular ligand-binding domain binds the TCR receptor alpha chain (TRAC), wherein the T cell is deficient in CD5 and TRAC, e.g., **CD5*TRAC-tCART Δ CD5 Δ TRAC** cell. In non-limiting examples the deficiency in CD5 and the TCR receptor alpha chain (TRAC) resulted from (a) modification of CD5 and the TCR receptor alpha chain (TRAC) expressed by the T cell such that the tCAR no longer specifically binds the modified CD5 and the TCR receptor alpha chain (TRAC), (b) modification of the T cell such that expression of the CD5 and the TCR receptor alpha chain (TRAC) is reduced in the T cell by at least 50%, at least 60%, at

least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD5 and the TCR receptor alpha chain (TRAC) is not expressed (e.g., by deletion or disruption of the gene encoding CD5 and / or the TCR receptor alpha chain (TRAC). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of human CD34 cDNA and is expressed in the **CD5*TRAC-tCARTΔCD5ΔTRAC** cells.

[00240] In a second embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD7 and the second extracellular ligand-binding domain binds the TCR receptor alpha chain (TRAC), wherein the T cell is deficient in CD7 and TRAC, e.g., **CD7*TRAC-tCARTΔCD7ΔTRAC** cell. In non-limiting examples the deficiency in CD7 and the TCR receptor alpha chain (TRAC) resulted from (a) modification of CD7 and the TCR receptor alpha chain (TRAC) expressed by the T cell such that the tCAR no longer specifically binds the modified CD7 and the TCR receptor alpha chain (TRAC), (b) modification of the T cell such that expression of the CD7 and the TCR receptor alpha chain (TRAC) is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 and the TCR receptor alpha chain (TRAC) is not expressed (e.g., by deletion or disruption of the gene encoding CD7 and / or the TCR receptor alpha chain (TRAC). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD7*TRAC-tCARTΔCD7ΔTRAC** cells.

[00241] In a third embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD2 and the second extracellular ligand-binding domain binds the TCR receptor alpha chain (TRAC), wherein the T cell is deficient in CD2 and TRAC, e.g., **CD2*TRAC-tCARTΔCD2ΔTRAC** cell. In non-limiting examples the deficiency in CD2 and the TCR receptor alpha chain (TRAC) resulted from (a) modification of CD2 and the TCR receptor alpha chain (TRAC) expressed by the T cell such that the tCAR no longer specifically binds the modified CD2 and the TCR receptor alpha chain (TRAC), (b) modification of the T cell such that expression of the CD2 and

the TCR receptor alpha chain (TRAC) is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD2 and the TCR receptor alpha chain (TRAC) is not expressed (e.g., by deletion or disruption of the gene encoding CD2 and / or the TCR receptor alpha chain (TRAC)). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA is expressed in the **CD2*TRAC-tCARTΔCD2ΔTRAC** cells.

[00242] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD4 and the second extracellular ligand-binding domain binds the TCR receptor alpha chain (TRAC), wherein the T cell is deficient in CD4 and TRAC, e.g., **CD4*TRAC-tCARTΔCD4ΔTRAC** cell. In non-limiting examples the deficiency in CD4 and the TCR receptor alpha chain (TRAC) resulted from (a) modification of CD4 and the TCR receptor alpha chain (TRAC) expressed by the T cell such that the tCAR no longer specifically binds the modified CD4 and the TCR receptor alpha chain (TRAC), (b) modification of the T cell such that expression of the CD4 and the TCR receptor alpha chain (TRAC) is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD4 and the TCR receptor alpha chain (TRAC) is not expressed (e.g., by deletion or disruption of the gene encoding CD4 and / or the TCR receptor alpha chain (TRAC)). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD4*TRAC-tCARTΔCD4ΔTRAC** cells.

[00243] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD3 epsilon (ε) chain and the second extracellular ligand-binding domain binds the TCR receptor alpha chain (TRAC), wherein the T cell is deficient in CD3ε and TRAC, e.g., a **CD3ε*TRAC-tCARTΔCD3εΔTRAC** cell. In non-limiting examples the deficiency in CD3ε and the TCR receptor alpha chain (TRAC) resulted from (a) modification of CD3ε and the TCR receptor alpha chain (TRAC) expressed by the T cell such that the tCAR no longer specifically binds the

modified CD3ε and the TCR receptor alpha chain (TRAC), (b) modification of the T cell such that expression of the CD3ε and the TCR receptor alpha chain (TRAC) is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD3ε and the TCR receptor alpha chain (TRAC) is not expressed (e.g., by deletion or disruption of the gene encoding CD3ε and / or the TCR receptor alpha chain (TRAC)). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD3ε*TRAC-tCARTΔCD3εΔTRAC** cells.

[00244] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD2 and the second extracellular ligand-binding domain binds the CD3 epsilon (ε) chain, wherein the T cell is deficient in CD2 and CD3ε, e.g., **CD2*CD3ε-tCARTΔCD2ΔCD3ε** cell. In non-limiting examples the deficiency in CD2 and the CD3ε resulted from (a) modification of CD2 and CD3ε expressed by the T cell such that the tCAR no longer specifically binds the modified CD2 and CD3ε, (b) modification of the T cell such that expression of the CD2 and CD3ε is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD2 and CD3ε is not expressed (e.g., by deletion or disruption of the gene encoding CD2 and / or CD3ε. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD2*CD3ε-tCARTΔCD2ΔCD3ε** cells.

[00245] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD4 and the second extracellular ligand-binding domain binds the CD3 epsilon (ε) chain, wherein the T cell is deficient in CD4 and CD3ε, e.g., **CD4*CD3ε-tCARTΔCD4ΔCD3ε** cell. In non-limiting examples the deficiency in CD4 and the CD3ε resulted from (a) modification of CD4 and CD3ε expressed by the T cell such that the tCAR no longer specifically binds the modified CD4 and CD3ε, (b) modification of the T cell such that expression of the CD4 and CD3ε is reduced in the

T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD4 and CD3 ϵ is not expressed (e.g., by deletion or disruption of the gene encoding CD4 and / or CD3 ϵ . In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD4*CD3 ϵ -tCART Δ CD4 Δ CD3 ϵ** cells.

[00246] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD5 and the second extracellular ligand-binding domain binds the TCR β chain, wherein the T cell is deficient in CD5 and TCR β chain, e.g., a **CD5*TCR β -tCART Δ CD5 Δ TCR β** cell. In non-limiting examples the deficiency in CD5 and the TCR β chain resulted from (a) modification of CD5 and TCR β expressed by the T cell such that the tCAR no longer specifically binds the modified CD5 and TCR β , (b) modification of the T cell such that expression of the CD5 and TCR β is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD5 and TCR β is not expressed (e.g., by deletion or disruption of the gene encoding CD5 and / or TCR β . In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD5*TCR β -tCART Δ CD5 Δ TCR β** cells.

[00247] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD7 and the second extracellular ligand-binding domain binds the TCR β chain, wherein the T cell is deficient in CD7 and TCR β chain, e.g., a **CD7*TCR β -tCART Δ CD7 Δ TCR β** cell. In non-limiting examples the deficiency in CD7 and the TCR β chain resulted from (a) modification of CD7 and TCR β expressed by the T cell such that the tCAR no longer specifically binds the modified CD7 and TCR β , (b) modification of the T cell such that expression of the CD7 and TCR β is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 and TCR β is not expressed (e.g., by deletion or disruption of the gene encoding CD7 and / or TCR β . In further embodiments, the T

cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD7*TCR β -tCART Δ CD7 Δ TCR β** cells.

[00248] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD2 and the second extracellular ligand-binding domain binds the TCR β chain, wherein the T cell is deficient in CD2 and TCR β chain, e.g., a **CD2*TCR β -tCART Δ CD7 Δ TCR β** cell. In non-limiting examples the deficiency in CD2 and the TCR β chain resulted from (a) modification of CD2 and TCR β expressed by the T cell such that the tCAR no longer specifically binds the modified CD2 and TCR β , (b) modification of the T cell such that expression of the CD2 and TCR β is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD2 and TCR β is not expressed (e.g., by deletion or disruption of the gene encoding CD2 and / or TCR β). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD2*TCR β -tCART Δ CD2 Δ TCR β** cells.

[00249] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD4 and the second extracellular ligand-binding domain binds the TCR β chain, wherein the T cell is deficient in CD4 and TCR β chain, e.g., a **CD4*TCR β -tCART Δ CD4 Δ TCR β** cell. In non-limiting examples the deficiency in CD4 and the TCR β chain resulted from (a) modification of CD4 and TCR β expressed by the T cell such that the tCAR no longer specifically binds the modified CD4 and TCR β , (b) modification of the T cell such that expression of the CD4 and TCR β is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD4 and TCR β is not expressed (e.g., by deletion or disruption of the gene encoding CD4 and / or TCR β). In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the

extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD4*TCR β -tCART Δ CD4 Δ TCR β** cells.

[00250] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD7 and the second extracellular ligand-binding domain binds CD2, wherein the T cell is deficient in CD7 and CD2, e.g., **CD7*CD2-tCART Δ CD7 Δ CD2** cell. In non-limiting examples the deficiency in CD7 and CD2 resulted from (a) modification of CD7 and CD2 expressed by the T cell such that the tCAR no longer specifically binds the modified CD7 and CD2, (b) modification of the T cell such that expression of the CD7 and CD2 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 and CD2 is not expressed (e.g., by deletion or disruption of the gene encoding CD7 and / or CD2. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD7*CD2-tCART Δ CD7 Δ CD2** cells.

[00251] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD7 and the second extracellular ligand-binding domain binds CD5, wherein the T cell is deficient in CD7 and CD5, e.g., **CD7*CD5-tCART Δ CD7 Δ CD5** cell. In non-limiting examples the deficiency in CD7 and CD5 resulted from (a) modification of CD7 and CD5 expressed by the T cell such that the tCAR no longer specifically binds the modified CD7 and CD5, (b) modification of the T cell such that expression of the CD7 and CD5 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 and CD5 is not expressed (e.g., by deletion or disruption of the gene encoding CD7 and / or CD5. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD7*CD5-tCART Δ CD7 Δ CD5** cells.

[00252] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD7 and the second extracellular ligand-binding domain binds CD4, wherein the T cell is deficient in

CD7 and CD4, e.g., **CD7*CD4-tCART Δ CD7 Δ CD4** cell. In non-limiting examples the deficiency in CD7 and CD4 resulted from (a) modification of CD7 and CD4 expressed by the T cell such that the tCAR no longer specifically binds the modified CD7 and CD4, (b) modification of the T cell such that expression of the CD7 and CD4 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD7 and CD4 is not expressed (e.g., by deletion or disruption of the gene encoding CD7 and / or CD4. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD7*CD4-tCART Δ CD7 Δ CD4** cells.

[00253] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD2 and the second extracellular ligand-binding domain binds CD5, wherein the T cell is deficient in CD2 and CD5, e.g., **CD2*CD5-tCART Δ CD2 Δ CD5** cell. In non-limiting examples the deficiency in CD2 and CD5 resulted from (a) modification of CD2 and CD5 expressed by the T cell such that the tCAR no longer specifically binds the modified CD2 and CD5, (b) modification of the T cell such that expression of the CD2 and CD5 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD2 and CD5 is not expressed (e.g., by deletion or disruption of the gene encoding CD2 and / or CD5. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD2*CD5-tCART Δ CD2 Δ CD5** cells.

[00254] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD2 and the second extracellular ligand-binding domain binds CD4, wherein the T cell is deficient in CD2 and CD4, e.g., **CD2*CD4-tCART Δ CD2 Δ CD4** cell. In non-limiting examples the deficiency in CD2 and CD4 resulted from (a) modification of CD2 and CD4 expressed by the T cell such that the tCAR no longer specifically binds the modified CD2 and CD4, (b) modification of the T cell such that expression of the CD2 and CD4 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such

that CD2 and CD4 is not expressed (e.g., by deletion or disruption of the gene encoding CD2 and / or CD4. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene is fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD2*CD4-tCARTΔCD2ΔCD4** cells.

[00255] In another embodiment, an engineered T cell comprises a tandem Chimeric Antigen Receptor (tCAR), wherein one extracellular ligand-binding domain specifically binds CD5 and the second extracellular ligand-binding domain binds CD4, wherein the T cell is deficient in CD5 and CD4, e.g., **CD5*CD4-tCARTΔCD5ΔCD4** cell. In non-limiting examples the deficiency in CD5 and CD4 resulted from (a) modification of CD5 and CD4 expressed by the T cell such that the chimeric antigen receptor no longer specifically binds the modified CD5 and CD4, (b) modification of the T cell such that expression of the CD5 and CD4 is reduced in the T cell by at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more, or (c) modification of the T cell such that CD5 and CD4 is not expressed (e.g., by deletion or disruption of the gene encoding CD5 and / or CD4. In further embodiments, the T cell comprises a suicide gene. In non-limiting examples, a protein-coding sequence of a modified Human-Herpes Simplex Virus-1-thymidine kinase (TK) gene fused in-frame to the extracellular and transmembrane domains of the human CD34 cDNA and is expressed in the **CD5*CD4-tCARTΔCD5ΔCD4** cells.

[00256] In another embodiment, a linear tandem CAR-T cell comprises a chimeric antigen receptor (CAR) polypeptide comprising a first signal peptide, a first extracellular ligand-binding domain, a second extracellular ligand-binding domain, a hinge region, a transmembrane domain, one or more co-stimulatory domains, and a signaling transducing domain, wherein the first extracellular ligand-binding antigen recognition domain and the second extracellular ligand-binding antigen recognition domain have affinities for different cell surface molecules, i.e., antigens on a cancer cell, for example, a malignant T cell, malignant B cell, or malignant plasma cell; and wherein the linear tandem CAR-T cell possesses one or more genetic modifications, deletions, or disruptions resulting in reduced expression of the cell surface molecules in the linear tandem CAR-T cell.

[00257] In another embodiment, the signal peptide is the signal peptide from human CD8α (SEQ ID NO:1).

[00258] In a third embodiment, the first extracellular ligand-binding domain comprises a single chain antibody fragment (scFv), comprising the light (V_L) and the heavy (V_H) variable fragment, designated V_{H1} and V_{L1} and joined by a linker (e.g., GGGGS). In some embodiments, this linker peptide is repeated 2, 3, 4, 5 or 6 times. In some embodiments, the first antigen recognition domain can be selected from: 1) V_{H1} - (GGGGS)₃₋₄ - V_{L1} or 2) V_{L1} - (GGGGS)₃₋₄ - V_{H1} .

[00259] In some embodiments, the second extracellular ligand-binding domain comprises a single chain antibody fragment (scFv), comprising the light (V_L) and the heavy (V_H) variable fragment, designated V_{H2} and V_{L2} and joined by a linker (e.g., GGGGS). In some embodiments, this linker peptide is repeated 2, 3, 4, 5 or 6 times. In some embodiments, the first antigen recognition domain can be selected from: 1) V_{H2} - (GGGGS)₃₋₄ - V_{L2} or 2) V_{L2} - (GGGGS)₃₋₄ - V_{H2} .

[00260] In further embodiments, the first antigen recognition domain and second antigen recognition domain are connected by a short linker peptide of 5 amino acids (GGGGS). In some embodiments, this linker peptide is repeated 2, 3, 4, 5 or 6 times.

Linear Tandem CAR Constructs

[00261] In one embodiment of a linear tandem CAR construct, the first extracellular ligand-binding domain comprises a single chain antibody fragment (scFv), comprising the heavy (V_H) and the light (V_L) variable fragment, designated V_{H1} and V_{L1} , and joined by a linker (e.g., GGGGS)₂₋₆. The second extracellular ligand-binding domain antigen recognition comprises a single chain antibody fragment (scFv), comprising the light (V_L) and the heavy (V_H) variable fragment, designated V_{L2} and V_{H2} , and joined by a linker (e.g., GGGGS)₂₋₆.

[00262] In a second embodiment of a linear tandem CAR construct, the first extracellular ligand-binding domain comprises a single chain antibody fragment (scFv), comprising the heavy (V_H) and the light (V_L) variable fragment, designated V_{H2} and V_{L2} , and joined by a linker (e.g., GGGGS)₂₋₆. The second extracellular ligand-binding domain antigen recognition comprises a single chain antibody fragment (scFv), comprising the light (V_L) and the heavy (V_H) variable fragment, designated V_{L1} and V_{H1} , and joined by a linker (e.g., GGGGS)₂₋₆.

[00263] In a third embodiment of a linear tandem CAR construct, the first extracellular ligand-binding domain comprises a single chain antibody fragment (scFv), comprising the heavy (V_H) and the light (V_L) variable fragment, designated V_{L1} and V_{H1} , and joined by a linker (e.g.,

GGGGS)₂₋₆. The second extracellular ligand-binding domain antigen recognition comprises a single chain antibody fragment (scFv), comprising the light (V_H) and the heavy (V_L) variable fragment, designated V_{H2} and V_{L2}, and joined by a linker (e.g., GGGGS)₂₋₆.

[00264] In a fourth embodiment of a linear tandem CAR construct, the first extracellular ligand-binding domain comprises a single chain antibody fragment (scFv), comprising the heavy (V_L) and the light (V_H) variable fragment, designated V_{L2} and V_{H2}, and joined by a linker (e.g., GGGGS)₂₋₆. The second extracellular ligand-binding domain antigen recognition comprises a single chain antibody fragment (scFv), comprising the light (V_H) and the heavy (V_L) variable fragment, designated V_{H1} and V_{L1}, and joined by a linker (e.g., GGGGS)₂₋₆.

[00265] For each of the linear tandem CAR construct embodiments, the first and second extracellular ligand-binding domains targets a surface molecule, i.e., an antigen expressed on a malignant T cell is selected from, but not limited to, BCMA, CS1, CD38, CD138, CD19, CD33, CD123, CD371, CD117, CD135, Tim-3, CD5, CD7, CD2, CD4, CD3, CD79A, CD79B, APRIL, CD56, and CD1a.

[00266] Further examples of linear tandem CARs are given below in Table 6.

Table 6. Tandem CARs and CAR-Ts (Linear or Hairpin).

Example	Antigen Target CAR-T cell	Antigen Deletion/ Suppression
T1	APRILxBCMA	-
T2	APRILxCD19	-
T3	APRILxCD38	-
T4	APRILxCD38	CD38
T5	APRILxCS1	-
T6	APRILxCS1	CS1
T7	BCMAxCD19	-
T8	BCMAxCD38	-
T9	BCMAxCD38	CD38
T10	BCMAxCS1	-
T11	BCMAxCS1	CS1
T12	CD138xAPRIL	
T13	CD138xBCMA	
T14	CD138xCD19	
T15	CD138xCD38	
T16	CD138xCD38	CD38
T17	CD138xCD79A	
T18	CD138xCD79B	

T19	CD138xCS1	
T20	CD138xCS1	CS1
T21	CD19xCD38	-
T22	CD19xCD38	CD38
T23	CD2xCD3 ϵ	-
T24	CD2xCD3 ϵ	CD2
T25	CD2xCD3 ϵ	CD3 ϵ
T26	CD2xCD3 ϵ	CD2 and CD3 ϵ
T27	CD2xCD4	-
T28	CD2xCD4	CD2
T29	CD2xCD4	CD4
T30	CD2xCD4	CD2 and CD4
T31	CD2xCD4	CD2 and TRAC
T32	CD2xCD4	CD4 and TRAC
T33	CD2xCD4	CD2 and CD4 and TRAC
T34	CD2xCD5	-
T35	CD2xCD5	CD2
T36	CD2xCD5	CD5
T37	CD2xCD5	CD2 and CD5
T38	CD2xCD5	CD2 and TRAC
T39	CD2xCD5	CD5 and TRAC
T40	CD2xCD5	CD2 and CD5 and TRAC
T41	CD2xCD7	-
T42	CD2xCD7	CD2
T43	CD2xCD7	CD7
T44	CD2xCD7	CD2 and CD7
T45	CD2xCD7	CD2 and TRAC
T46	CD2xCD7	CD7 and TRAC
T47	CD2xCD7	CD2 and CD7 and TRAC
T48	CD3 ϵ xCD4	-
T49	CD3 ϵ xCD4	CD3 ϵ
T50	CD3 ϵ xCD4	CD4
T51	CD3 ϵ xCD4	CD3 ϵ and CD4
T52	CD3 ϵ xCD5	-
T53	CD3 ϵ xCD5	CD3 ϵ
T54	CD3 ϵ xCD5	CD5
T55	CD3 ϵ xCD5	CD3 ϵ and CD5
T56	CD3 ϵ xCD7	-
T57	CD3 ϵ xCD7	CD3 ϵ
T58	CD3 ϵ xCD7	CD7

T59	CD3εxCD7	CD3ε and CD7
T60	CD4xCD5	-
T61	CD4xCD5	CD4
T62	CD4xCD5	CD5
T63	CD4xCD5	CD4 and CD5
T64	CD4xCD5	CD4 and TRAC
T65	CD4xCD5	CD5 and TRAC
T66	CD4xCD5	CD4 and CD5 and TRAC
T67	CD4xCD7	-
T68	CD4xCD7	CD4
T69	CD4xCD7	CD7
T70	CD4xCD7	CD4 and CD7
T71	CD4xCD7	CD4 and TRAC
T72	CD4xCD7	CD4 and TRAC
T73	CD4xCD7	CD4 and CD7 and TRAC
T74	CD5xCD7	-
T75	CD5xCD7	CD5
T76	CD5xCD7	CD7
T77	CD5xCD7	CD5 and CD7
T78	CD5xCD7	CD5 and TRAC
T79	CD5xCD7	CD7 and TRAC
T80	CD5xCD7	CD5 and CD7 and TRAC
T81	CD79AxA ₄ PRIL	
T82	CD79AxB ₆ MA	
T83	CD79AxC ₁₉	
T84	CD79AxD ₃₈	
T85	CD79AxE ₃₈	CD38
T86	CD79AxF _{79B}	
T87	CD79AxF _{CS1}	
T88	CD79AxF _{CS1}	CS1
T89	CD79BxA ₄ PRIL	
T90	CD79BxB ₆ MA	
T91	CD79BxC ₁₉	
T92	CD79BxD ₃₈	
T93	CD79BxE ₃₈	CD38
T94	CD79BxF _{79A}	
T95	CD79BxF _{CS1}	
T96	CD79BxF _{CS1}	CS1
T97	CS1x ₁₉	-
T98	CS1x ₁₉	CS1
T99	CS1x ₃₈	-

T100	CS1xCD38	CS1
T101	CS1xCD38	CD38
T102	CS1xCD38	CS1 and CD38
T103	TCR β xCD2	-
T104	TCR β xCD2	TCR β
T105	TCR β xCD2	CD2
T106	TCR β xCD2	TCR β and CD2
T107	TCR β xCD3 ϵ	-
T108	TCR β xCD3 ϵ	TCR β
T109	TCR β xCD3 ϵ	CD3 ϵ
T110	TCR β xCD3 ϵ	TCR β and CD3 ϵ
T111	TCR β xCD4	-
T112	TCR β xCD4	TCR β
T113	TCR β xCD4	CD4
T114	TCR β xCD4	TCR β and CD4
T115	TCR β xCD5	-
T116	TCR β xCD5	TCR β
T117	TCR β xCD5	CD5
T118	TCR β xCD5	TCR β and CD5
T119	TCR β xCD7	-
T120	TCR β xCD7	TCR β
T121	TCR β xCD7	CD7
T122	TCR β xCD7	TCR β and CD7
T123	TRACxCD2	-
T124	TRACxCD2	TRAC
T125	TRACxCD2	CD2
T126	TRACxCD2	TRAC and CD2
T127	TRACxCD3 ϵ	-
T128	TRACxCD3 ϵ	TRAC
T129	TRACxCD3 ϵ	CD3 ϵ
T130	TRACxCD3 ϵ	TRAC and CD3 ϵ
T131	TRACxCD4	-
T132	TRACxCD4	TRAC
T133	TRACxCD4	CD4
T134	TRACxCD4	TRAC and CD4
T135	TRACxCD5	-
T136	TRACxCD5	TRAC
T137	TRACxCD5	CD5
T138	TRACxCD5	TRAC and CD5
T139	TRACxCD7	-
T140	TRACxCD7	TRAC

T141	TRACxCD7	CD7
T142	TRACxCD7	TRAC and CD7

[00267] For example, provided herein are linear tandem CAR constructs which may incorporate the V_H and V_L domains of scFvs targeting any of the antigen pairs provided in Table 6 above.

Table 7. Linear Tandem CAR Constructs.

7-I	7-II	7-III	7-IV	7-V	7-VI	7-VII	7-VIII
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎
V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎
7-IX	7-X	7-XI	7-XII	7-XIII	7-XIV	7-XV	7-XVI
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎
V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1

GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎
V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎
7-XVII	7-XVIII	7-XIX	7-XX	7-XXI	7-XXII	7-XXIII	7-XIV
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎
V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎
7-XXV	7-XXVI	7-XXVII	7-XXVIII	7-XIX	7-XXX	7-XXXI	7-XXXII
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎

V _H 1	V _H 1	V _H 1	V _H 1	V _H 1	V _H 1	V _H 1	V _H 1
GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎
V _L 1	V _L 1	V _L 1	V _L 1	V _L 1	V _L 1	V _L 1	V _L 1
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎

Hairpin Tandem CAR Constructs

[00268] In one embodiment of a hairpin tandem CAR construct, the first extracellular ligand-binding domain comprises a single chain antibody fragment (scFv), comprising two heavy chain variable fragments, designated V_H1 and V_H2, and joined by a linker (e.g., GGGGS)₂₋₆. The second extracellular ligand-binding domain antigen recognition comprises a single chain antibody fragment (scFv), comprising two light chain variable fragments, designated V_L2 and V_L1, and joined by a linker (e.g., GGGGS)₂₋₆.

[00269] In a second embodiment of a hairpin tandem CAR construct, the first extracellular ligand-binding domain comprises a single chain antibody fragment (scFv), comprising two heavy chain variable fragments, designated V_H2 and V_H1, and joined by a linker (e.g., GGGGS)₂₋₆. The second extracellular ligand-binding domain antigen recognition comprises a single chain antibody fragment (scFv), comprising two light chain variable fragments, designated V_L1 and V_L2, and joined by a linker (e.g., GGGGS)₂₋₆.

[00270] In a third embodiment of a hairpin tandem CAR construct, the first extracellular ligand-binding domain comprises a single chain antibody fragment (scFv), comprising two light chain variable fragments, designated V_L1 and V_L2, and joined by a linker (e.g., GGGGS)₂₋₆. The second extracellular ligand-binding domain antigen recognition comprises a single chain antibody fragment (scFv), comprising two heavy chain variable fragments, designated V_H2 and V_H1, and joined by a linker (e.g., GGGGS)₂₋₆.

[00271] In a fourth embodiment of a hairpin tandem CAR construct, the first extracellular ligand-binding domain comprises a single chain antibody fragment (scFv), comprising two light chain variable fragments, designated V_L2 and V_L1, and joined by a linker (e.g., GGGGS)₂₋₆. The second extracellular ligand-binding domain antigen recognition comprises a single chain

antibody fragment (scFv), comprising two heavy chain variable fragments, designated V_H1 and V_H2, and joined by a linker (e.g., GGGGS)₂₋₆.

[00272] For each of the hairpin tandem CAR construct embodiments, the first and second extracellular ligand-binding domains targets a surface molecule, i.e., an antigen expressed on a malignant T cell is selected from, but not limited to, BCMA, CS1, CD38, CD138, CD19, CD33, CD123, CD371, CD117, CD135, Tim-3, CD5, CD7, CD2, CD4, CD3, CD79A, CD79B, APRIL, CD56, and CD1a.

[00273] Additional examples of hairpin tandem CARs are given above in Table 6.

[00274] Furthermore, provided herein are CAR constructs and CAR- T cells which may incorporate the V_H and V_L domains of scFvs targeting (1) CD2 and CD3; and (2) CD2 and CD7 and are provided below in Table 8.

Table 8. Amino Acid Sequences of Hairpin Tandem Chimeric Antigen Receptors (CARs).

<u>Hairpin Tandem CAR Constructs</u>	<u>Designation in Examples</u>	<u>SEQ ID NO:</u>	<u>Amino acid sequence</u>
OKT3 V_L – CD2 V_L – CD2 V_H – OKT3 V_H	WC5	SEQ ID NO:41	MALPVTALLLPLALLLHAARPQIVLTQSPAIM SASPGEKVTMTCSASSSVSYMNWYQQKSGTS PKRWIYDTSKLASGVPAHFRGSGSGTSYSLTI SGMEAEDAATYYCQQWSSNPFTFGSGTKLEI NRGGGGSGGGGSGGGGSGGGGSDIKNITQSP SSMYVSLGERVTITCKASQDINSFLSWFQQKP GKSPKTLIYRANRLVDGVPSRFSGSGSGQDYS LTISSLEYEDMEIYYCLQYDEFPTYTFGGGTKL EMKRGGGGSGGGGSGGGGSGGGGSGGGGS GGGGSGGGGSGGGGSGGGGSGGGGSEVKLE ESGAELVKPGASVKLSCRTSGFNIKDTIHWVK QRPEQGLKWIGRIDPANGNTKYDPKFQDKAT VTADTSSNTAYLQLSSLTSEDTAVYYCVTYA YDGNWYFDVWGAGTAVTVSSGGGGSGGGG SGGGGSGGGGSGSQVQLQQSGAELARPGAS VKMSCKASGYTFTRYTMHWVKQRPGQGLE

			<p>WIGYINPSRGYTNYNQKFKDKATLTDDKSSS TAYMQLSSLTSEDSAVYYCARYYDDHYCLD YWGQGTTTLTVSSPRASTTTTPAPRPPTPAPTAS QPLSLRPEACRPAAGGAVHTRGLDFACDFWV LVVVGGVLACYSLLVTVAFIIFWVRSKRSRLL HSDYMNMTPRRPGPTRKHYPYAPPRDFAA YRSRVKFSRSADAPAYKQGQNQLYNELNLG RREEYDVLDKRRGRDPEMGGKPRRKNPQEG LYNELQDKMAEAYSEIGMKGERRRGKGHD GLYQGLSTATKDTYDALHMQALPPRRTDGS GATNFSLLKQAGDVEENPGPVSEAMPGRWT ALCLLSLLPSGFMSLDNNGTATPELPTQGTF NVSTNVSYQETTTTPSTLGSTSLHPVSQHGNEA TTNITETTVKFTSTSVITSVYGNTNSSVQSQTS VISTVFTTPANVSTPETTLKPSLSPGNVSDLST TSTSLATSPTKPYTSSSPILSDIKAEIKCSGIREV KLTQGICLEQNKTSSCAEFKKDRGEGLARVL CGEEQADADAGAQVCSLLLAQSEVRPQCLLL VLNRTEISSKLQLMKKHQSDLKKLGILDFTE QDVASHQSYSQKTLIALVTSGALLAVLGITGY FLMNRRSWSPI</p>
CD3 V_L – CD2 V_L – CD2 – V_H – CD3 V_H	WC7	SEQ ID NO:42	<p>MALPVTALLPLALLLHAARPDIQMTQSPSSL SASVGDRVTITCRASQDIRNYLNWYQQKPGK APKLLIYYTSRLESGVPSRFSGSGSGTDYTLTI SSLQPEDFATYYCQQGNTLPWTFGCGTKVEI KGGGGSGGGGSGGGGSGGGGSDIKNITQSPS SMYVSLGERVTITCKASQDINSFLSWFQQKPG KSPKTLIYRANRLVDGVPSRFSGSGSGQDYSL TISSLEYEDMEIYYCLQYDEFPYTFGGGTKLE MKRGGGGSGGGGSGGGGSGGGGSGGGGSGG</p>

			GGGSGGGSGGGSGGGSGGGSGGGSEVKLEE SGAELVKPGASVKLSCRTSGFNIKDTIHVVK QRPEQGLKWIGRIDPANGNTKYDPKFQDKAT VTADTSSNTAYLQLSSLTSEDNAVYYCVTYA YDGNWYFDVWGAGTAVTVSSGGGGSGGGG SGGGSGGGGSEVQLVESGGGLVQPGGSLRL SCAASGYSFTGYTMNWVRQAPGKCLEWVAL INPYKGVSTYNQKFKDRFTISVDKSKNTAYL QMNSLRAEDTAVYYCARSGYYGDSDWYFD VWGQGTLLTVSSPRASTTTPAPRPPTAPTIA SQPLSLRPEACRPAAGGAVHTRGLDFACDFW VLVVVGVLACYSLLVTVAFIIFWVRSKRSR LLHSDYMNMTPRRPGPTRKHYQPYAPPRDFA AYRSRVKFSRSADAPAYKQGQNQLYNELNL GRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGH DGLYQGLSTATKDTYDALHMQALPPRRTDG SGATNFSLLKQAGDVEENPGPVSEAMPRGWT ALCLLSLLPSGFMSLDNNGTATPELPTQGTF NVSTNVSYQETTTTPSTLGSTSLHPVSQHGNEA TTNITETTVKFTSTSVITSVYGNTNSSVQSQTS VISTVFTTPANVSTPETTLKPSLSPGNVSDLST TSTSLATSPTKPYTSSSPILSDIKAEIKCSGIREV KLTQGICLEQNKTSSCAEFKKDRGEGLARVL CGEEQADADAGAQVCSLLLAQSEVRPQCLLL VLANRTEISSKLQLMKKHQSDDLKGLGILDFTE QDVASHQSYSQKTLIALVTSGALLAVLGITGY FLMNRRSWSPI
CD2 V_L – CD3 V_L – CD3 V_H – CD2 - V_H	WC15	SEQ ID NO:43	MALPVTALLPLALLHAARPDIKNITQSPSS MYVSLGERVTITCKASQDINSFLSWFQQKPG KSPKTLIYRANRLVDGVPSRFSGSGSGQDYSL

			TISSLEYEDMEIYYCLQYDEFPYTFGGGTKLE MKRGGGSGGGSGGGSGGGSGGGSDIQMTQS PSSLASVGDRVTTTCRASQDIRNYLNWYQQ KPGKAPKLLIYYTSRLESGVPSRFSGSGSGTD YTLTISSLQPEDFATYYCQQGNTLPWTFGCGT KVEIKGGGSGGGSGGGSGGGSGGGSGGGGS GGGSGGGSGGGSGGGSGGGSGGGGSEVQLV ESGGGLVQPGGSLRLSCAASGYSTGYTMN WVRQAPGKCLEWVALINPYKGVSTYNQKFK DRFTISVDKSKNTAYLQMNSLRAEDTAVYYC ARSGYYGSDSDWYFDVWGQGTTLTVSSGGG GSGGGSGGGSGGGSGGGSEVKLEESGAELVKP GASVKLSCRTSGFNIKDTIHVVKQRPEQGLK WIGRIDPANGNTKYDPKFQDKATVTADTSSN TAYLQLSSLTSEDVAVYYCVTYAYDGNWYF DVWGAGTAVTVSSPRASTTTPAPRPPTPPTI ASQPLSLRPEACRPAAGGAVHTRGLDFACDF WVLVVVGGLACYSLLVTVAFIIFWVRSKRS RLLHSDYMNMTPRRPGPTRKHYQPYAPPRDF AAYRSRVKFSRSADAPAYKQQQNQLYNELN LGRREEYDVLDKRRGRDPEMGGKPRRKNPQ EGLYNELQKDKMAEAYSEIGMKGERRRGKG HDGLYQGLSTATKDTYDALHMQALPPRRTD GSGATNFSLLKQAGDVEENPGPVSEAMPRG WTALCLLSLLPSGFMSLDNNGTATPELPTQG TFSNVSTNVSYQETTTPTLGSTSLHPVSQHG NEATTNITETTVKFTSTSVITSVYGNTNSSVQS QTSVISTVFTTPANVSTPETTLKPSLSPGNVSD LSTTSTSLATSPTKPYTSSSPILSDIKAEIKCSGI REVKLTQGICLEQNKTSSCAEFKKDRGEGLA RVLCGEEQADADAGAQCSSLQAQSEVRPQ
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			CLLLVLANRTEISSKLQLMKKHQSDLKKLGIL DFTEQDVASHQSYSQKTLIALVTSGALLAVL GITGYFLMNRRSWSPI
CD2 V_L – OKT3 V_L – OKT3 V_H – CD2 V_H	WC13	SEQ ID NO:44	MALPVTALLLPLALLLHAARPDIKNITQSPSS MYVSLGERVTITCKASQDINSFLSWFQQKPG KSPKTLIYRANRLVDGVPSRFSGSGSGQDYSL TISSLEYEDMEIYYCLQYDEFPYTFGGGTKLE MKRGGGGSGGGGSGGGGSGGGGSGQIVLTQS PAIMSASPGEKVTMTCSASSSVSYMNWYQQ KSGTSPKRWIYDTSKLASGVPAHFRGSGSGTS YSLTISGMEAEDAATYYCQQWSSNPFTFGSG TKLEINRGGGGSGGGGSGGGGSGGGGSGGG GSGGGGSGGGGSGGGGSGGGGSGGGGSGSQ VQLQQSGAELARPGASVKMSCKASGYTFTR YTMHWVKQRPQGQLEWIGYINPSRGYTNYN QKFKDKATLTDDKSSSTAYMQLSSLTSEDSA VYYCARYYDDHYCLDYWGQGTTLTVSSGG GGSGGGGSGGGGSGGGGSEVKLEESGAELV KPGASVKLSCRTSGFNIKDTIHWVKQRPEQGL KWIGRIDPANGNTKYDPKFQDKATVTADTSS NTAYLQLSSLTSEDNAVYYCVTYAYDGNWY FDVWGAGTAVTVSSPRASTTTPAPRPPTPAPT IASQPLSLRPEACRPAAGGAVHTRGLDFACDF WVLVVVGGLACYSLLVTVAFIIFWVRSKRS RLLHSDYMNMTPRRPGPTRKHYQPYAPPRDF AAYRSRVKFSRSADAPAYKQGQNQLYNELN LGRREEYDVLDKRRGRDPEMGGKPRRKNPQ EGLYNELQKDKMAEAYSEIGMKGERRRGKG HDGLYQGLSTATKDTYDALHMQALPPRRTD GSGATNFSLLKQAGDVEENPGPVSEAMPRG WTALCLLSLLPSGFMSLDNNGTATPELPTQG

			<p>TFSNVSTNVSYQETTTSTLGSTSLHPVSQHG NEATTNITETTVKFTSTSVITSVYGNTNSSVQS QTSVISTVFTTPANVSTPETTLKPSLSPGNVSD LSTTSTSLATSPTKPYTSSSPILSDIKAEIKCSGI REVKLTQGICLEQNKTSSCAEFKKDRGEGLA RVLCGEEQADADAGAQCSSLQAQSEVRPQ CLLLVLANRTEISSKLQLMKKHQSCLKKLGIL DFTEQDVASHQSYSQKTLIALVTSGALLAVL GITGYFLMNRRSWSPI</p>
<p>CD7 V_L – CD2 V_L – CD2 V_H – CD7 V_H</p>		<p>SEQ ID NO:45</p>	<p>MALPVTALLPLALLHAARPDIQMTQTSSL SASLGDRVTISCSASQGISNYLNWYQQKPDG TVKLLIYYTSSLHSGVPSRFSGSGSGTDYSLTI SNLEPEDIATYYCQQYSKLPYTFGGGTKLEIK RGGGSGGGGSGGGGSGGGGSDIKNITQSPS SMYVSLGERVTITCKASQDINSFLSWFQQKPG KSPKTLYRANRLVDGVPSRFSGSGSGQDYSL TISSLEYEDMEIYYCLQYDEFPYTFGGGTKLE MKRGGGSGGGGSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSGGGGSGGGGSEVKLEE SGAELVKPGASVKLSCRTSGFNKDTIHWVK QRPEQGLKWIGRIDPANGNTKYDPKFQDKAT VTADTSSNTAYLQLSSLTSEDVAVYYCVTYA YDGNWYFDVWGAGTAVTVSSGGGSGGGG SGGGSGGGGSEVQLVESGGGLVKPGGSLKL SCAASGLTFSSYAMSWVRQTPEKRLEWVASI SSGGFTYYPDVKGRFTISRDNARNILYLQMS SLRSEDAMYYCARDEVRYLDVWGAGTTV TVSPRASTTTTPAPRPPTPAPTASQPLSLRPEAC RPAAGGAVHTRGLDFACDFWVLVVVGGLA CYSLLVTVAFIIFWVRSKRSRLHSDYMNMT</p>

			PRRPGPTRKHYPYAPPRDFAAYRSRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDK RRRGRDPEMGGKPRRKNPQEGLYNELQDKM AEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDALHMQALPPRRTDGSGATNFSLLKQ AGDVEENPGPVSEAMPRGWTALCLLSLLPSG FMSLDNNGTATPELPTQGTFNSVSTNVSYQE TTTPTSLGSTSLHPVSQHGNEATTNITETTVKF TSTSVITSVYGNTNSSVQSQTSVISTVFTTPAN VSTPETTLKPSLSPGNVSDLSTTSTSLATSPTK PYTSSSPILSDIKAEIKCSGIREVKLTQGICLEQ NKTSSCAEFKKDRGEGLARVLCGEEQADAD AGAQVCSLLLAQSEVRPQCLLLVLNRTEISS KLQLMKKHQSDLKKLGLDFTEQDVASHQSY SQKTLIALVTSGALLAVLGITGYFLMNRRSWS PI
CD2 V_L – CD7 V_L – CD7 V_H – CD2 V_H		SEQ ID NO:46	MALPVTALLPLALLHAARPDIKNITQSPSS MYVSLGERVTITCKASQDINSFLSWFQQKPG KSPKTLIYRANRLVDGVPSRFSGSGSGQDYSL TISSLEYEDMEIYYCLQYDEFPYTFGGGTKLE MKRGGGGSGGGGSGGGGSGGGGSDIQMTQT TSSLSASLGDRVTISCSASQGISNYLNWYQQK PDGTVKLLIYYTSSLHSGVPSRFSGSGSGTDY SLTISNLEPEDIATYYCQQYSKLPYTFGGGTK LEIKRGGGGSGGGGSGGGGSGGGGSGGGGS GGGGSGGGGSGGGGSGGGGSGGGGSEVQLV ESGGGLVKPGSLKLSCAASGLTFSSYAMSW VRQTPEKRLEWVASISSGGFTYYPDSVKGRFT ISRDNARNILYLQMSSLRSEDAMYYCARDE VRGYLDVWGAGTTVTVSGGGGSGGGGSGG GGGGGGGSEVKLEESGAELVKPGASVKLSCR

			TSGFNIKDTIHWVKQRPEQGLKWIGRIDPANG NTKYDPKFQDKATVTADTSSNTAYLQLSSLT SEDTAVYYCVTYAYDGNWYFDVWGAGTAV TVSSPRASTTTTPAPRPPTPAPTIASQPLSLRPEA CRPAAGGAVHTRGLDFACDFWVLVVGGVL ACYSLLVTVAFIIFWVRSKRSRLLHSDYMN TPRRPGPTRKHYQPYAPPRDFAAYRSRVKFS RSADAPAYKQGQNQLYNELNLGRREEYDVL DKRRGRDPEMGGKPRRKNPQEGLYNELQKD KMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPRRTDGSGATNFSLLK QAGDVEENPGPVSEAMPRGWTALCLLSLLPS GFMSLDNNGTATPELPTQGTFNSVSTNVSYQ ETTTPSTLGSTSLHPVSQHGNEATTNITETTVK FTSTSVITSVYGNTNSSVQSQTSVISTVFTTPA NVSTPETTLKPSLSPGNVSDLSTTSTSLATSPT KPYTSSSPILSDIAEIKCSGIREVKLTQGICLE QNKTSSCAEFKKDRGEGLARVLCGEEQADA DAGAQVCSLLLAQSEVRPQCCLLVLANRTEIS SKLQLMKKHQSDLKKLGILDFTEQDVASHQS YSQKTLIALVTSGALLAVLGITGYFLMNRRS WSPI
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[00275] Additionally, provided herein are hairpin tandem CAR constructs which may incorporate the V_H and V_L domains of scFvs targeting any of the antigen pairs provided in Table 6.

Table 9. Hairpin Tandem CAR Constructs.

9-I	9-II	9-III	9-IV	9-V	9-VI	9-VII	9-VIII
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V _H 1	V _H 1	V _H 1	V _H 1	V _H 1	V _H 1	V _H 1	V _H 1
GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋

6)	6)	6)	6)	6)	6)	6)	6)
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋
6)	6)	6)	6)	6)	6)	6)	6)
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋
4)	4)	4)	4)	4)	4)	4)	4)
V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎
9-IX	9-X	9-XI	9-XII	9-XIII	9-XIV	9-XV	9-XVI
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋
6)	6)	6)	6)	6)	6)	6)	6)
V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1
GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋	GGGGS ₍₂₋
6)	6)	6)	6)	6)	6)	6)	6)
V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1
GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋	GGGGS ₍₃₋
4)	4)	4)	4)	4)	4)	4)	4)
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎	CD3 ζ ₍₁₋₂₎
9-XVII	9-XVIII	9-XIX	9-XX	9-XXI	9-XXII	9-XXIII	9-XIV
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1

GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)
V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎
9-XXV	9-XXVI	9-XXVII	9-XXVIII	9-XIX	9-XXX	9-XXXI	9-XXXII
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)
V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1
GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)
V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1
GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎

[00276] For example, provided herein in Table 10 are hairpin tandem CAR constructs which incorporate the V_H and V_L domains of CD2 and CD3 scFvs.

[00277] Table 10. Hairpin Tandem CAR Constructs Targeting CD2 and CD3.

Clone 5	Clone 6	Clone 7	Clone 8	Clone 13	Clone 14	Clone 15	Clone 16
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
CD3-V_L	CD3-V_L	CD3-V_L	CD3-V_L	CD2-V_L	CD2-V_L	CD3-V_L	CD3-V_L
GGGGS ₄	GGGGS ₄	GGGGS ₄	GGGGS ₄	GGGGS ₄	GGGGS ₄	GGGGS ₄	GGGGS ₄
CD2-V_L	CD2-V_L	CD2-V_L	CD2-V_L	CD3-V_L	CD3-V_L	CD2-V_L	CD2-V_L
(GGGGS) ₁₀	(GGGGS) ₄ GGGGP(GGGGS) ₄	(GGGGS) ₁₀	(GGGGS) ₄ GGGGP(GGGGS) ₄	(GGGGS) ₁₀	(GGGGS) ₄ GGGGP(GGGGS) ₄	(GGGGS) ₁₀	(GGGGS) ₄ GGGGP(GGGGS) ₄
CD2-V_H	CD2-V_H	CD2-V_H	CD2-V_H	CD3-V_H	CD3-V_H	CD2-V_H	CD2-V_H
GGGGS ₄	GGGGS ₄	GGGGS ₄	GGGGS ₄	GGGGS ₄	GGGGS ₄	GGGGS ₄	GGGGS ₄
CD3-V_H	CD3-V_H	CD3-V_H	CD3-V_H	CD2-V_H	CD2-V_H	CD3-V_H	CD3-V_H
CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
CD28	CD28	CD28	CD28	CD28	CD28	CD28	CD28
CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎
P2A	P2A	P2A	P2A	P2A	P2A	P2A	P2A
CD34	CD34	CD34	CD34	CD34	CD34	CD34	CD34

[00278] Also provided herein in Table 11 are hairpin tandem CAR constructs with a (Cys = Cys) double-stranded bond (DSB) which may incorporate the V_H and V_L domains of scFvs targeting any of the antigen pairs provided in Table 6.

Table 11. Hairpin Tandem DSB CAR Constructs with a (Cys=Cys) Double-Stranded Bond (DSB).

11-I	11-II	11-III	11-IV	11-V	11-VI	11-VII	11-VIII
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₎

1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍
2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍
0-1) V_L2	0-1) V_L2	0-1) V_L2	0-1) V_L2	0-1) V_L2	0-1) V_L2	0-1) V_L2	0-1) V_L2
GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)	GGGGS ₍₃₋ 4)
V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3 $\zeta_{(1-2)}$	CD3 $\zeta_{(1-2)}$	CD3 $\zeta_{(1-2)}$	CD3 $\zeta_{(1-2)}$	CD3 $\zeta_{(1-2)}$	CD3 $\zeta_{(1-2)}$	CD3 $\zeta_{(1-2)}$	CD3 $\zeta_{(1-2)}$
11-IX	11-X	11-XI	11-XII	11-XIII	11-XIV	11-XV	11-XVI
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)	GGGGS ₍₂₋ 6)
V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1
GGGGS ₍₀₋ 1)GGGGC (1)GGGGS ₍	GGGGS ₍₀₋ 1)GGGGC (1)GGGGS ₍	GGGGS ₍₀₋ 1)GGGGC (1)GGGGS ₍	GGGGS ₍₀₋ 1)GGGGC (1)GGGGS ₍	GGGGS ₍₀₋ 1)GGGGC (1)GGGGS ₍	GGGGS ₍₀₋ 1)GGGGC (1)GGGGS ₍	GGGGS ₍₀₋ 1)GGGGC (1)GGGGS ₍	GGGGS ₍₀₋ 1)GGGGC (1)GGGGS ₍
1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍	1-2) GGGGP (1)GGGGS ₍
2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍	2-3) GGGGC (1)GGGGS ₍
0-1) V_L1	0-1) V_L1	0-1) V_L1	0-1) V_L1	0-1) V_L1	0-1) V_L1	0-1) V_L1	0-1) V_L1

GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎
11-XVII	11-XVIII	11-XIX	11-XX	11-XXI	11-XXII	11-XXIII	11-XXIV
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₋₂₎ GGGGP ₍₁₎ GGGGS ₍₂₋₃₎ GGGGC ₍₁₎ GGGGS ₍₀₋₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₋₂₎ GGGGP ₍₁₎ GGGGS ₍₂₋₃₎ GGGGC ₍₁₎ GGGGS ₍₀₋₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₋₂₎ GGGGP ₍₁₎ GGGGS ₍₂₋₃₎ GGGGC ₍₁₎ GGGGS ₍₀₋₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₋₂₎ GGGGP ₍₁₎ GGGGS ₍₂₋₃₎ GGGGC ₍₁₎ GGGGS ₍₀₋₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₋₂₎ GGGGP ₍₁₎ GGGGS ₍₂₋₃₎ GGGGC ₍₁₎ GGGGS ₍₀₋₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₋₂₎ GGGGP ₍₁₎ GGGGS ₍₂₋₃₎ GGGGC ₍₁₎ GGGGS ₍₀₋₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₋₂₎ GGGGP ₍₁₎ GGGGS ₍₂₋₃₎ GGGGC ₍₁₎ GGGGS ₍₀₋₁₎	GGGGS ₍₀₋₁₎ GGGGC ₍₁₎ GGGGS ₍₁₋₂₎ GGGGP ₍₁₎ GGGGS ₍₂₋₃₎ GGGGC ₍₁₎ GGGGS ₍₀₋₁₎
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎
V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎	CD3ζ ₍₁₋₂₎

11-XXV	11-XXVI	11-XXVII	11-XXVIII	11-XXIX	11-XXX	11-XXXI	11-XXXII
CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a	CD8a
V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2	V_L2
GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎	GGGGS ₍₂₋₆₎
V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1	V_L1
GGGGS ₍₀₋₁₎ GGGGC _{(1)GGGGS₍₁₋₂₎}	GGGGS ₍₀₋₁₎ GGGGC _{(1)GGGGS₍₁₋₂₎}	GGGGS ₍₀₋₁₎ GGGGC _{(1)GGGGS₍₁₋₂₎}	GGGGS ₍₀₋₁₎ GGGGC _{(1)GGGGS₍₁₋₂₎}	GGGGS ₍₀₋₁₎ GGGGC _{(1)GGGGS₍₁₋₂₎}	GGGGS ₍₀₋₁₎ GGGGC _{(1)GGGGS₍₁₋₂₎}	GGGGS ₍₀₋₁₎ GGGGC _{(1)GGGGS₍₁₋₂₎}	GGGGS ₍₀₋₁₎ GGGGC _{(1)GGGGS₍₁₋₂₎}
GGGGP _{(1)GGGGS₍₂₋₃₎}	GGGGP _{(1)GGGGS₍₂₋₃₎}	GGGGP _{(1)GGGGS₍₂₋₃₎}	GGGGP _{(1)GGGGS₍₂₋₃₎}	GGGGP _{(1)GGGGS₍₂₋₃₎}	GGGGP _{(1)GGGGS₍₂₋₃₎}	GGGGP _{(1)GGGGS₍₂₋₃₎}	GGGGP _{(1)GGGGS₍₂₋₃₎}
GGGGC _{(1)GGGGS₍₀₋₁₎}	GGGGC _{(1)GGGGS₍₀₋₁₎}	GGGGC _{(1)GGGGS₍₀₋₁₎}	GGGGC _{(1)GGGGS₍₀₋₁₎}	GGGGC _{(1)GGGGS₍₀₋₁₎}	GGGGC _{(1)GGGGS₍₀₋₁₎}	GGGGC _{(1)GGGGS₍₀₋₁₎}	GGGGC _{(1)GGGGS₍₀₋₁₎}
V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1	V_H1
GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎	GGGGS ₍₃₋₄₎
V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2	V_H2
CD8 Tm	CD8 Tm	CD8 Tm	CD8 Tm	CD28 Tm	CD28 Tm	CD28 Tm	CD28 Tm
41BB	CD28	41BB - CD28	CD28 - 41BB	41BB	CD28	41BB - CD28	CD28 - 41BB
CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎	CD3z ₍₁₋₂₎

Methods for Engineering CARs in a Dual or Tandem Construction with Gene Editing

[00279] In a further aspect, a CAR-T cell control may be created. For example, the control CAR-T cell may include an extracellular domain that binds to an antigen not expressed on a malignant T-cell. For example, if the therapeutic CAR-T cell targets a T-cell antigen such as CD7, or multiple T cell antigens, such as CD2 and CD3, the antigen the control CAR-T cell binds to may be CD19. CD19 is an antigen expressed on B cells but not on T cells, so a CAR-T cell with an extracellular domain adapted to bind to CD19 will not bind to T cells. These CAR-T

cells may be used as controls to analyze the binding efficiencies and non-specific binding of CAR-T cells targeted to the cancer of interest and/or recognizing the antigen of interest.

[00280] CARs may be further designed as disclosed in WO2018027036A1, optionally employing variations which will be known to those of skill in the art. Lentiviral vectors and cell lines can be obtained, and guide RNAs designed, validated, and synthesized, as disclosed therein as well as by methods known in the art and from commercial sources.

[00281] Engineered CARs may be introduced into T cells using retroviruses, which efficiently and stably integrate a nucleic acid sequence encoding the chimeric antigen receptor into the target cell genome. Other methods known in the art include, but are not limited to, lentiviral transduction, transposon-based systems, direct RNA transfection, and CRISPR/Cas systems (e.g., type I, type II, or type III systems using a suitable Cas protein such as Cas3, Cas4, Cas5, Cas5e (or CasD), Cas6, Cas6e, Cas6f, Cas7, Cas8a1, Cas8a2, Cas8b, Cas8c, Cas9, Cas10, Cas10d, CasF, CasG, CasH, Csy1, Csy2, Csy3, Cse1 (or CasA), Cse2 (or CasB), Cse3 (or CasE), Cse4 (or CasC), Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csz1, Csx15, Csf1, Csf2, Csf3, Csf4, and Cu1966, etc.). Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) may also be used. See, e.g., Shearer RF and Saunders DN, "Experimental design for stable genetic manipulation in mammalian cell lines: lentivirus and alternatives," *Genes Cells* 2015 Jan; 20(1):1-10.

[00282] Manipulation of PI3K signaling can be used to prevent altered CAR-T cell differentiation due to constitutive CAR self-signaling and foster long-lived memory T cell development. pharmacologic blockade of PI3K during CAR-T manufacture and ex vivo expansion can abrogate preferential effector T cell development and restore CAR-T effector/memory ratio to that observed in empty vector transduced T cells, which can improve *in vivo* T cell persistence and therapeutic activity. Inhibition of p110 δ PI3K can enhance efficacy and memory in tumor-specific therapeutic CD8 T cells, while inhibition of p110 α PI3K can increase cytokine production and antitumor response.

This is proposed to be because the presence of a CAR on a T cell's surface can alter its activation and differentiation, even in the absence of ligand. Constitutive self-signaling through CAR, related to both the scFv framework and the signaling domains, can lead to aberrant T cell behavior, including altered differentiation and decreased survival. This is significant as the

effectiveness of CAR-T cells in patients is directly associated with their in vivo longevity. The presence of the CD28 costimulatory domain increased CAR-T cell exhaustion induced by persistent CAR self-signaling; the 4-1BB costimulatory domain had a lesser effect. Furthermore, CD3-zeta significantly enhances the constitutive activation of the PI3K, AKT, mTOR, and glycolysis pathways, and fostered formation of short-lived effector cells over central/stem memory cells. See, e.g., Zhang W. et al., "Modulation of PI3K signaling to improve CAR T cell function," *Oncotarget*, 2018 Nov 9; 9(88): 35807–35808.

Cytokine Gene Deletion or Suppression

[00283] In addition to gene-editing the TCR and cell surface proteins and antigens, genes for secretable proteins such as cytokines and chemokines may be edited. Such editing would be done, e.g., to reduce or prevent the development or maintenance of cytokine release syndrome (CRS). CRS is caused by a large, rapid release of cytokines from immune cells in response to immunotherapy (or other immunological stimulus). Modifying, disrupting, or deleting one or more cytokine or chemokine genes can be accomplished using the methods known in the art, such as genetic ablation (gene silencing) in which gene expression is abolished through the alteration or deletion of genetic sequence information. This can be accomplished using known genetic engineering tools in the art, such as Transcription Activator-like Effector Nucleases (TALENs), Zinc Finger Nucleases (ZFNs), CRISPR, by transduction of small hairpin RNAs (shRNAs), by targeted transduction of a CAR into the gene sequence of the cytokine, and the like.

[00284] Cytokines or chemokines that can be deleted from immune effector cells as disclosed herein, e.g., using Cas9-CRISPR or by targeted transduction of a CAR into the gene sequence of the cytokine, include without limitation the following: XCL1, XCL2, CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CX3CL1, IL-1 α , IL-1 β , IL-1RA, IL-18, IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IL-3, IL-5, GM-CSF, IL-6, IL-11, G-CSF, IL-12, LIF, OSM, IL-10, IL-20, IL-14, IL-16, IL-17, IFN- α , IFN- β , IFN- γ , CD154, LT- β , TNF- α , TNF- β , 4-1BBL, APRIL, CD70, CD153, CD178, GITRL, LIGHT, OX40L, TALL-1, TRAIL, TWEAK, TRANCE, TGF- β 1, TGF- β 2, TGF- β 3, Epo, Tpo, Flt-3L, SCF, M-CSF, MSP, A2M, ACKR1, ACKR2, ACKR3, ACVR1, ACVR2B,

ACVRL1, ADIPOQ, AGER, AGRN, AIMP1, AREG, BMP1, BMP10, BMP15, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8A, BMP8B, BMPR2, C10orf99, C1QTNF4, C5, CCL28, CCR1, CCR2, CCR3, CCR5, CCR6, CCR7, CD109, CD36, CD4, CD40LG, CD74, CER1, CHRD, CKLF, CLCF1, CMTM1, CMTM2, CMTM3, CMTM4, CMTM5, CMTM6, CMTM7, CMTM8, CNTF, CNTFR, COPS5, CRLF1, CSF1, CSF1R, CSF2, CSF3, CSF3R, CTF1, CX3CR1, CXCL16, CXCL17, CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, EBI3, EDN1, ELANE, ENG, FAM3B, FAM3C, FAM3D, FAS, FASLG, FGF2, FLT3LG, FZD4, GBP1, GDF1, GDF10, GDF11, GDF15, GDF2, GDF3, GDF5, GDF6, GDF7, GDF9, GPI, GREM1, GREM2, GRN, HAX1, HFE2, HMGB1, HYAL2, IFNA10, IFNA14, IFNA16, IFNA2, IFNA5, IFNA6, IFNA8, IFNAR1, IFNAR2, IFNB1, IFNE, IFNG, IFNGR1, IFNK, IFNL1, IFNL3, IFNW1, IL10RA, IL11RA, IL12A, IL12B, IL12RB1, IL17A, IL17B, IL17C, IL17D, IL17F, IL18BP, IL-19, IL1F10, IL1R1, IL1R2, IL1RAPL1, IL1RL1, IL1RN, IL20RA, IL20RB, IL21, IL22, IL22RA1, IL22RA2, IL23A, IL23R, IL24, IL25, IL26, IL27, IL2RA, IL2RB, IL2RG, IL31, IL31RA, IL32, IL33, IL34, IL36A, IL36B, IL36G, IL36RN, IL37, IL6R, IL6ST, INHA, INHBA, INHBB, INHBC, INHBE, ITGA4, ITGAV, ITGB1, ITGB3, KIT, KITLG, KLHL20, LEFTY1, LEFTY2, LIFR, LTA, LTB, LTBP1, LTBP3, LTBP4, MIF, MINOS1-, MSTN, NAMPT, NBL1, NDP, NLRP7, NODAL, NOG, NRG1, NRP1, NRP2, OSMR, PARK7, PDPN, PF4, PF4V1, PGLYRP1, PLP2, PPBP, PXDN, SCG2, SCGB3A1, SECTM1, SLURP1, SOSTDC1, SP100, SPP1, TCAP, TGFBR1, TGFBR2, TGFBR3, THBS1, THNSL2, THPO, TIMP1, TNF, TNFRSF11, TNFRSF1A, TNFRSF9, TNFRSF10, TNFSF11, TNFSF12, TNFSF12-, TNFSF13, TNFSF13B, TNFSF14, TNFSF15, TNFSF18, TNFSF4, TNFSF8, TNFSF9, TRIM16, TSLP, TWSG1, TXLNA, VASN, VEGFA, VSTM1, WFIKKN1, WFIKKN2, WNT1, WNT2, WNT5A, WNT7A, and ZFP36.

[00285] The sequences of these genes are known and available in the art.

Indications and Standards of Care in ACT (CAR-T) Therapy

[00286] In some embodiment, the genome-edited immune effector cells disclosed herein, and/or generated using the methods disclosed herein, express one or more chimeric antigen receptors (CARs) and can be used as a medicament, i.e., for the treatment of disease. In many embodiments, the cells are CAR-T cells.

[00287] Cells disclosed herein, and/or generated using the methods disclosed herein, may be used in immunotherapy and adoptive cell transfer, for the treatment, or the manufacture of a

medicament for treatment, of cancers, autoimmune diseases, infectious diseases, and other conditions.

[00288] The cancer may be a hematologic malignancy or solid tumor. Hematologic malignancies include leukemias, lymphomas, multiple myeloma, and subtypes thereof. Lymphomas can be classified various ways, often based on the underlying type of malignant cell, including Hodgkin's lymphoma (often cancers of Reed-Sternberg cells, but also sometimes originating in B cells; all other lymphomas are non-Hodgkin's lymphomas), B-cell lymphomas, T-cell lymphomas, mantle cell lymphomas, Burkitt's lymphoma, follicular lymphoma, and others as defined herein and known in the art.

[00289] B-cell lymphomas include, but are not limited to, diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL) /small lymphocytic lymphoma (SLL) , and others as defined herein and known in the art.

[00290] T-cell lymphomas include T-cell acute lymphoblastic leukemia/lymphoma (T-ALL), , peripheral T-cell lymphoma (PTCL), T-cell chronic lymphocytic leukemia (T-CLL)Sezary syndrome, and others as defined herein and known in the art.

[00291] Leukemias include Acute myeloid (or myelogenous) leukemia (AML), chronic myeloid (or myelogenous) leukemia (CML), acute lymphocytic (or lymphoblastic) leukemia (ALL), chronic lymphocytic leukemia (CLL) hairy cell leukemia (sometimes classified as a lymphoma) , and others as defined herein and known in the art.

[00292] Plasma cell cell malignancies include lymphoplasmacytic lymphoma, plasmacytoma, and multiple myeloma.

[00293] In some embodiments, the medicament can be used for treating cancer in a patient, particularly for the treatment of solid tumors such as melanomas, neuroblastomas, gliomas or carcinomas such as tumors of the brain, head and neck, breast, lung (e.g., non-small cell lung cancer, NSCLC), reproductive tract (e.g., ovary), upper digestive tract, pancreas, liver, renal system (e.g., kidneys), bladder, prostate and colorectum.

[00294] In another embodiment, the medicament can be used for treating cancer in a patient, particularly for the treatment of hematologic malignancies selected from multiple myeloma and acute myeloid leukemia (AML) and for T-cell malignancies selected from T-cell acute lymphoblastic leukemia (T-ALL), non-Hodgkin's lymphoma, and T-cell chronic lymphocytic leukemia (T-CLL).

[00295] In some embodiments, the cells may be used in the treatment of autoimmune diseases such as lupus, autoimmune (rheumatoid) arthritis, multiple sclerosis, transplant rejection, Crohn's disease, ulcerative colitis, dermatitis, and the like. In some embodiments, the cells are chimeric autoantibody receptor T-cells, or CAR-Ts displaying antigens or fragments thereof, instead of antibody fragments; in this version of adoptive cell transfer, the B cells that cause autoimmune diseases will attempt to attack the engineered T cells, which will respond by killing them.

[00296] In some embodiments, the cells may be used in the treatment of infectious diseases such as HIV and tuberculosis.

[00297] In another embodiment, the CAR-T cells of the present disclosure can undergo robust in vivo T cell expansion and can persist for an extended amount of time.

[00298] In some embodiments, the treatment of a patient with CAR-T cells of the present disclosure can be ameliorating, curative or prophylactic. It may be either part of an autologous immunotherapy or part of an allogenic immunotherapy treatment. By autologous, it is meant that cells, cell line or population of cells used for treating patients are originating from said patient or from a Human Leucocyte Antigen (HLA) compatible donor. By allogenic, is meant that the cells or population of cells used for treating patients are not originating from the patient but from a donor.

[00299] The treatment of cancer with CAR-T cells of the present disclosure may be in combination with one or more therapies selected from antibody therapy, chemotherapy, cytokine therapy, dendritic cell therapy, gene therapy, hormone therapy, radiotherapy, laser light therapy, and radiation therapy.

[00300] The administration of CAR-T cells or a population of CAR-T cells of the present disclosure of the present disclosure be carried out by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The CAR-T cells compositions described herein, i.e., mono CAR, dual CAR, tandem CARs, may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous or intralymphatic injection, or intraperitoneally. In one embodiment, the cell compositions of the present disclosure are preferably administered by intravenous injection.

[00301] The administration of CAR-T cells or a population of CAR-T cells can consist of the administration of 10^4 - 10^9 cells per kg body weight, preferably 10^5 to 10^6 cells/kg body weight

including all integer values of cell numbers within those ranges. The CAR-T cells or a population of CAR-T cells can be administrated in one or more doses. In another embodiment, the effective amount of CAR-T cells or a population of CAR-T cells are administrated as a single dose. In another embodiment, the effective amount of cells are administered as more than one dose over a period time. Timing of administration is within the judgment of a health care provider and depends on the clinical condition of the patient. The CAR-T cells or a population of CAR-T cells may be obtained from any source, such as a blood bank or a donor. While the needs of a patient vary, determination of optimal ranges of effective amounts of a given CAR-T cell population(s) for a particular disease or conditions are within the skill of the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit. The dosage administered will be dependent upon the age, health and weight of the patient recipient, type of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

[00302] In another embodiment, the effective amount of CAR-T cells or a population of CAR-T cells or composition comprising those CAR-T cells are administered parenterally. The administration can be an intravenous administration. The administration of CAR-T cells or a population of CAR-T cells or composition comprising those CAR-T cells can be directly done by injection within a tumor.

[00303] In one embodiment of the present disclosure, the CAR-T cells or a population of the CAR-T cells are administered to a patient in conjunction with, e.g., before, simultaneously or following, any number of relevant treatment modalities, including but not limited to, treatment with cytokines, or expression of cytokines from within the CAR-T, that enhance T-cell proliferation and persistence and, include but not limited to, IL-2, IL-7, and IL-15 or analogues thereof.

[00304] In some embodiments, the CAR-T cells or a population of CAR-T cells of the present disclosure may be used in combination with agents that inhibit immunosuppressive pathways, including but not limited to, inhibitors of TGF β , interleukin 10 (IL-10), adenosine, VEGF, indoleamine 2,3 dioxygenase 1 (IDO1), indoleamine 2,3-dioxygenase 2 (IDO2), tryptophan 2–3-dioxygenase (TDO), lactate, hypoxia, arginase, and prostaglandin E2.

[00305] In another embodiment, the CAR-T cells or a population of CAR-T cells of the present disclosure may be used in combination with T-cell checkpoint inhibitors, including but not limited to, anti-CTLA4 (Ipilimumab) anti-PD1 (Pembrolizumab, Nivolumab, Cemiplimab),

anti-PDL1 (Atezolizumab, Avelumab, Durvalumab), anti-PDL2, anti-BTLA, anti-LAG3, anti-TIM3, anti-VISTA, anti-TIGIT, and anti-KIR.

[00306] In another embodiment, the CAR-T cells or a population of CAR-T cells of the present disclosure may be used in combination with T cell agonists, including but not limited to, antibodies that stimulate CD28, ICOS, OX-40, CD27, 4-1BB, CD137, GITR, and HVEM.

[00307] In another embodiment, the CAR-T cells or a population of CAR-T cells of the present disclosure may be used in combination with therapeutic oncolytic viruses, including but not limited to, retroviruses, picornaviruses, rhabdoviruses, paramyxoviruses, reoviruses, parvoviruses, adenoviruses, herpesviruses, and poxviruses.

[00308] In another embodiment, the CAR-T cells or a population of CAR-T cells of the present disclosure may be used in combination with immunostimulatory therapies, such as toll-like receptors agonists, including but not limited to, TLR3, TLR4, TLR7 and TLR9 agonists.

[00309] In another embodiment, the CAR-T cells or a population of CAR-T cells of the present disclosure may be used in combination with stimulator of interferon gene (STING) agonists, such as cyclic GMP-AMP synthase (cGAS).

[00310] Immune effector cell aplasia, particularly T cell aplasia is also a concern after adoptive cell transfer therapy. When the malignancy treated is a T-cell malignancy, and CAR-T cells target a T cell antigen, normal T cells and their precursors expressing the antigen will become depleted, and the immune system will be compromised. Accordingly, methods for managing these side effects are attendant to therapy. Such methods include selecting and retaining non-malignant T cells or precursors, either autologous or allogeneic (optionally engineered not to cause rejection or be rejected), for later expansion and re-infusion into the patient, after CAR-T cells are exhausted or deactivated. Alternatively, CAR-T cells which recognize and kill subsets of TCR-bearing cells, such as normal and malignant TRBC1⁺, but not TRBC2⁺ cells, or alternatively, TRBC2⁺, but not TRBC1⁺ cells, may be used to eradicate a T cell malignancy while preserving sufficient normal T cells to maintain normal immune system function.

Definitions

[00311] Unless specifically defined herein, all technical and scientific terms used have the same meaning as commonly understood by a skilled artisan in the fields of gene therapy, biochemistry, genetics, and molecular biology. All disclosed compositions and methods similar

or equivalent to those described herein can be used in the practice or testing of the present disclosure.

[00312] As used herein, the terms below have the meanings indicated. When ranges of values are disclosed, and the notation “from n_1 ... to n_2 ” or “between n_1 ... and n_2 ” is used, where n_1 and n_2 are the numbers, then unless otherwise specified, this notation is intended to include the numbers themselves and the range between them. This range may be integral or continuous between and including the end values. By way of example, the range “from 2 to 6 carbons” is intended to include two, three, four, five, and six carbons, since carbons come in integer units. Compare, by way of example, the range “from 1 to 3 μM (micromolar),” which is intended to include 1 μM , 3 μM , and everything in between to any number of significant figures (e.g., 1.255 μM , 2.1 μM , 2.9999 μM , etc.).

[00313] The term “about,” as used herein, is intended to qualify the numerical values which it modifies, denoting such a value as variable within a margin of error. When no particular margin of error, such as a standard deviation to a mean value given in a chart or table of data, is recited, the term “about” should be understood to mean that range which would encompass the recited value and the range which would be included by rounding up or down to that figure as well, taking into account significant figures.

[00314] The term “activation” (and other conjugations thereof) in reference to cells is generally understood to be synonymous with “stimulating” and as used herein refers to treatment of cells that results in expansion of cell populations. In T cells, activation is often accomplished by exposure to CD2 and CD28 (and sometimes CD2 as well) agonists, typically antibodies, optionally coated onto magnetic beads or conjugated to a colloidal polymeric matrix.

[00315] The term “antigen” as used herein is a cell surface protein recognized by (i.e., that is the target of) T cell receptor or chimeric antigen receptor. In the classical sense antigens are substances, typically proteins, that are recognized by antibodies, but the definitions overlap insofar as the CAR comprises antibody-derived domains such as light (V_L) and heavy (V_H) chains recognizing one or more antigen(s).

[00316] The term “cancer” refers to a malignancy or abnormal growth of cells in the body. Many different cancers can be characterized or identified by particular cell surface proteins or molecules. Thus, in general terms, cancer in accordance with the present disclosure may refer to any malignancy that may be treated with an immune effector cell, such as a CAR-T cell as

described herein, in which the immune effector cell recognizes and binds to the cell surface protein on the cancer cell. As used herein, cancer may refer to a hematologic malignancy, such as multiple myeloma, a T-cell malignancy, or a B cell malignancy. T cell malignancies may include, but are not limited to, T-cell acute lymphoblastic leukemia (T-ALL) or non-Hodgkin's lymphoma. A cancer may also refer to a solid tumor, such as including, but not limited to, cervical cancer, pancreatic cancer, ovarian cancer, mesothelioma, and lung cancer.

[00317] A “cell surface protein” as used herein is a protein (or protein complex) expressed by a cell at least in part on the surface of the cell. Examples of cell surface proteins include the TCR (and subunits thereof) and CD7.

[00318] A “chimeric antigen receptor” or “CAR” as used herein and generally used in the art, refers to a recombinant fusion protein that has an extracellular ligand-binding domain, a transmembrane domain, and a signaling transducing domain that directs the cell to perform a specialized function upon binding of the extracellular ligand-binding domain to a component present on the target cell. For example, a CAR can have an antibody-based specificity for a desired antigen (e.g., tumor antigen) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits specific anti-target cellular immune activity. First-generation CARs include an extracellular ligand-binding domain and signaling transducing domain, commonly CD3 ζ or Fc ϵ RI γ . Second generation CARs are built upon first generation CAR constructs by including an intracellular costimulatory domain, commonly 4-1BB or CD28. These costimulatory domains help enhance CAR-T cell cytotoxicity and proliferation compared to first generation CARs. The third generation CARs include multiple costimulatory domains, primarily to increase CAR-T cell proliferation and persistence. Chimeric antigen receptors are distinguished from other antigen binding agents by their ability both to bind MHC-independent antigens and transduce activation signals via their intracellular domain.

[00319] A “CAR-bearing immune effector cell” is an immune effector cell which has been transduced with at least one CAR. A “CAR-T cell” is a T cell which has been transduced with at least one CAR; CAR-T cells can be mono, dual, or tandem CAR-T cells. CAR-T cells can be autologous, meaning that they are engineered from a subject's own cells, or allogeneic, meaning that the cells are sourced from a healthy donor, and in many cases, engineered so as not to provoke a host-vs-graft or graft-vs-host reaction. Donor cells may also be sourced from cord blood or generated from induced pluripotent stem cells.

[00320] A dual CAR-T cell (dCAR-T), can be defined as a T cell with two distinct chimeric antigen receptor polypeptides with affinity to different target antigen expressed within the same effector cell, wherein each CAR functions independently. The CAR may be expressed from single or multiple polynucleotide sequences.

[00321] A tandem CAR-T cell (tCAR-T), can be defined as a T cell with a single chimeric antigen polypeptide containing two distinct extracellular ligand-binding domains with affinity to different targets wherein the extracellular ligand-binding domains are linked through a peptide linker and share one or more common costimulatory domains, wherein binding of either extracellular ligand-binding domain will signal through one or more common costimulatory domains and signal transducing domain.

[00322] The term "combination therapy" means the administration of two or more therapeutic agents to treat a therapeutic condition or disorder described in the present disclosure. Such administration encompasses co-administration of these therapeutic agents in a substantially simultaneous manner, such as in a single capsule having a fixed ratio of active ingredients or in multiple, separate capsules for each active ingredient. In addition, such administration also encompasses use of each type of therapeutic agent in a sequential manner. In either case, the treatment regimen will provide beneficial effects of the drug combination in treating the conditions or disorders described herein.

[00323] The term "composition" as used herein refers to an immunotherapeutic cell population combination with one or more therapeutically acceptable carriers.

[00324] The term "disease" as used herein is intended to be generally synonymous, and is used interchangeably with, the terms "disorder," "syndrome," and "condition" (as in medical condition), in that all reflect an abnormal condition of the human or animal body or of one of its parts that impairs normal functioning, is typically manifested by distinguishing signs and symptoms, and causes the human or animal to have a reduced duration or quality of life.

[00325] The term "fratricide" as used herein means a process which occurs when a CAR-T cell becomes the target of, and is killed by, another CAR-T cell comprising the same chimeric antigen receptor as the target of CAR-T cell, because the targeted cell expresses the antigen specifically recognized by the chimeric antigen receptor on both cells. CAR-T cell comprising a chimeric antigen receptor which are deficient in an antigen to which the chimeric antigen receptor specifically binds will be "fratricide-resistant."

[00326] The term “genome-edited” as used herein means having a gene added, deleted, or modified to be non-functional. Thus, in certain embodiments, a “gene-edited CAR-T cell” is an CAR-T cell that has had a gene such as a CAR recognizing at least one antigen added; and/or has had a gene such as the gene(s) to the antigen(s) that are recognized by the CAR deleted; and/or has had a gene such as the TCR, or a subunit thereof (e.g., the α or β chain) deleted or modified to be non-functional, or a subunit of the associated CD3 signal transduction complex, or a subunit thereof (e.g. the γ , δ , ϵ , or ζ chains) deleted or modified to be non-functional.

[00327] As used herein, “suicide gene” refers to a nucleic acid sequence introduced to a CAR-T cell by standard methods known in the art, that when activated result in the death of the CAR-T cell. If required suicide genes may facilitate the tracking and elimination, i.e., killing, of CAR-T cells in vivo. Facilitated killing of CAR-T cells by activating a suicide gene can be accomplished by standard methods known in the art. Suicide gene systems known in the art include, but are not limited to, include (a) herpes simplex virus (HSV)-tk which turns the nontoxic prodrug ganciclovir (GCV) into GCV-triphosphate, leading to cell death by halting DNA replication, (b) iCasp9 can bind to the small molecule AP1903 and result in dimerization, which activates the intrinsic apoptotic pathway, and (c) Targetable surface antigen expressed in the transduced T cells (e.g., CD20 and truncated EGFR), allowing eliminating the modified cells efficiently through complement/antibody-dependent cellular cytotoxicity (CDC/ADCC) after administration of the associated monoclonal antibody.

[00328] A “cancer cell”, for example, is a malignant T cell, malignant B cell, or malignant plasma cell.

[00329] A “malignant B cell” is a B cell derived from a B-cell malignancy. B cell malignancies include, without limitation, (DLBCL), chronic lymphocytic leukemia (CLL) /small lymphocytic lymphoma (SLL), and B cell-precursor acute lymphoblastic leukemia (ALL).

[00330] A “malignant T cell” is a T cell derived from a T-cell malignancy.

[00331] The term “T-cell malignancy” refers to a broad, highly heterogeneous grouping of malignancies derived from T-cell precursors, mature T cells, or natural killer cells. Non-limiting examples of T-cell malignancies include T-cell acute lymphoblastic leukemia/lymphoma (T-ALL), , human T-cell leukemia virus type 1-positive (HTLV-1 +) adult T-cell leukemia/lymphoma (ATL), T-cell prolymphocytic leukemia (T-PLL), Adult T-cell lymphoma / leukemia (HTLV-1 associated), Aggressive NK-cell leukemia, Anaplastic large-cell lymphoma

(ALCL), ALK positive, Anaplastic large-cell lymphoma (ALCL), ALK negative, Angioimmunoblastic T-cell lymphoma (AITL), Breast implant-associated anaplastic large-cell lymphoma, Chronic lymphoproliferative disorder of NK cells, Extra nodal NK / T-cell lymphoma, nasal type, Enteropathy-type T-cell lymphoma, Follicular T-cell lymphoma, Hepatosplenic T-cell lymphoma, Indolent T-cell lymphoproliferative disorder of the GI tract, Monomorphic epitheliotrophic intestinal T-cell lymphoma, Mycosis fungoides, Nodal peripheral T-cell lymphoma with TFH phenotype, Peripheral T-cell lymphoma (PTCL), NOS, Primary cutaneous $\gamma\delta$ T-cell lymphoma, Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma, Primary cutaneous acral CD8+ T-cell lymphoma, Primary cutaneous CD4+ small/medium T-cell lymphoproliferative disorders [Primary cutaneous anaplastic large-cell lymphoma (C-ALCL), lymphoid papulosis], Sezary syndrome, Subcutaneous, panniculitis-like T-cell lymphoma, Systemic EBV+ T-cell lymphoma of childhood, and T-cell large granular lymphocytic leukemia (LGL).

[00332] A “healthy donor,” as used herein, is one who does not have a hematologic malignancy (e.g. a T-cell malignancy).

[00333] The term “therapeutically acceptable” refers to substances which are suitable for use in contact with the tissues of patients without undue toxicity, irritation, and allergic response, are commensurate with a reasonable benefit/risk ratio, and/or are effective for their intended use.

[00334] The term “therapeutically effective” is intended to qualify the amount of active ingredients used in the treatment of a disease or disorder or on the effecting of a clinical endpoint.

[00335] As used herein, a “secretable protein” is a protein secreted by a cell which has an effect on other cells. By way of example, secretable proteins include cytokines, chemokines, and transcription factors.

[00336] The term “donor template” refers to the reference genomic material that the cell uses as a template to repair the a double-stranded break through the homology-directed repair (HDR) DNA repair pathway. The donor template contains the piece of DNA to be inserted into the genome (containing the gene to be expressed, CAR, or marker) with two homology arms flanking the site of the double-stranded break. In some embodiments, a donor template may be an adeno-associated virus, a single-stranded DNA, or a double-stranded DNA.

[00337] The term “exposing to,” as used herein, in the context of bringing compositions of matter (such as antibodies) into intimate contact with other compositions of matter (such as cells), is intended to be synonymous with “incubated with,” and no lengthier period of time in contact is intended by the use of one term instead of the other.

[00338] The term “patient” is generally synonymous with the term “subject” and includes all mammals including humans.

[00339] The invention is further illustrated by the following examples.

EXAMPLES

Example 1 – Method of Making and Testing a Genome-Edited CAR-T Cells by insertion of CAR into CD3e loci

[00340] The following steps may be taken to provide a genome-edited CAR-T cell in which the car is expressed from the gene edited loci (CAR-T) disclosed herein. This example describes the making of a CD7CART Δ CD7 Δ CD3 ϵ cell. As those of skill in the art will recognize, certain of the steps may be conducted sequentially or out of the order listed below, though perhaps leading to different efficiency.

Table 12. Guide RNA sequences for use in removing surface antigens on immune effector cells.

Target gene	gRNA sequence
CD7	5’_2’OMe(A(ps)U(ps)C(ps))ACGGAGGUCAAUGUCUAGUUUUAGAGCUAGA AAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG GCACCGAGUCGGUGC2’OMe(U(ps)U(ps)U(ps)U_3’ (SEQ ID NO:47)
CD7g10	5’_2’OMe(G(ps)U(ps)A(ps))GACAUUGACCUCCGUGAGUUUUAGAGCUAGA AAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG GCACCGAGUCGGUGC2’OMe(U(ps)U(ps)U(ps)U_3’ (SEQ ID NO:48)

CD7g4	5'_2'OMe(<u>A(ps)U(ps)C(ps)</u>)ACGGAGGUCAAUGUCUAGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG GCACCGAGUCGGUGC2'OMe(U(ps)U(ps)U(ps)U_3' (SEQ ID NO:49)
TRACg	5'_2'OMe(<u>G(ps)A(ps)G(ps)</u>)AAUCAAAAUCGGUGAAUGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG GCACCGAGUCGGUGC2'OMe(U(ps)U(ps)U(ps)U_3' (SEQ ID NO:50)
CS1	5'_2'OMe(<u>G(ps)A(ps)C(ps)</u>)CAAUCUGACAUGCUGCAGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG GCACCGAGUCGGUGC2'OMe(U(ps)U(ps)U(ps)U_3' (SEQ ID NO:51)
CD2	5'_2'OMe(<u>A(ps)C(ps)A(ps)</u>)GCUGACAGGCUCGACACGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG GCACCGAGUCGGUGC2'OMe(U(ps)U(ps)U(ps)U_3' (SEQ ID NO:52)
CD2g	5'_2'OMe(<u>G(ps)A(ps)G(ps)</u>)AAUCAAAAUCGGUGAAUGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG GCACCGAGUCGGUGC2'OMe(U(ps)U(ps)U(ps)U_3' (SEQ ID NO:53)
CD3eg	5'_2'OMe(<u>A(ps)G(ps)G(ps)</u>)GCAUGUCAAUUUACUGGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG GCACCGAGUCGGUGC2'OMe(U(ps)U(ps)U(ps)U_3' (SEQ ID NO:54)
CD5	5'_2'OMe(<u>C(ps)G(ps)U(ps)</u>)UCCAACUCGAAGUGCCAGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG GCACCGAGUCGGUGC2'OMe(U(ps)U(ps)U(ps)U3' (SEQ ID NO:55)
CD5g	5'_2'OMe(<u>C(ps)G(ps)U(ps)</u>)uCCAACUCGAAGUGCCAGUUUUAGAGCUAGA AAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG GCACCGAGUCGGUGC2'OMe(U(ps)U(ps)U(ps)U_3' (SEQ ID NO:56)

RNA; (ps) indicate phosphorothioate. Underlined bases denote target sequence.

Step 1 – T Cell Activation (Day 0).

[00341] Purify T cells from leukapheresis chamber using Miltenyi human PanT isolation kit.

Resuspend in media. Count cells. Determine number of human T cell activation CD3/CD28

beads required to obtain 3:1 bead:cell ratio. Wash beads 2x with T cell media. Dilute cells at

1.256 cells/mL in hXcyte media. Add human T cell activation CD3/CD28 beads. Aliquot 4 mL/well of 1.256 cell/mL solution into 6 well plate. Incubate cells at 37C.

Step 2 – CRISPR (Day 2).

[00342] The target gene is genetically deleted and the CAR inserted into the gene edited loci. The DNA double strand break can be repaired using homology directed repair using a donor template to repair the break and insert the desired sequence into the edited loci. Target deletion may be accomplished by electroporating with Cas9 mRNA and gRNA against the target(s). The donor template may be, a DNA plasmid, or double stranded linear DNA containing homology to the DNA surrounding the double strand breaks electroporated with the Cas9/gRNA. Additionally, a viral vector such as AAV may be used as the source of the donor template. Other techniques, however, could be used to induce DNA double strand breaks. These include other genome editing techniques such as TALENs and mega-nucleases.

Table 13. CRISPR Protocol

Sample ID	gRNA#1	gRNA#2	Cas9	Nucleofection Buffer P3
UCART7	20ug gCD7	20ug gCD3ε	15ug Cas9 mRNA	100ul

[00343] *Protocol – Nucleofection using nucleofector 4D - 4×10^6 cells per reaction.* Program EO-115 - 100ul transfection volume. The entire supplement needs to be added to the Nucleofector™ Solution P3. Prepare cell culture plates by filling appropriate number of wells with desired volume of recommended culture media (2ml in 6 well plate) and pre-incubate/equilibrate plates in a humidified 37 °C/5 % CO₂ incubator. Magnetically Remove beads (do this twice to ensure complete removal). Count cells and determine cell density. Centrifuge the required number of cells at 90xg for 10 minutes at room temperature. Remove supernatant completely. Resuspend in PBS (1ml) and transfer to a microcentrifuge tube and centrifuge the required number of cells at 90xg for 10 minutes at room temperature. Remove supernatant completely. Resuspend the cell pellet carefully in complete room temperature 4DNucleofector™ Solution P3 4×10^6 per 100ul). Add 20ug of each gRNA (gCD7 and gCD3ε) to each tube of 15ug cas9. Add 100ul of cells to each tube of Cas9/gRNA, gently mix and

transfer everything into the Nucleocuvette™. Gently tap to remove bubbles. Electroporate using program (Human T cell stim EO-115). After run completion, carefully remove the Nucleocuvette™ Vessel from the retainer using special. Resuspend cells with pre-warmed medium. Take up media from destination well, add to cuvette and gently pipetting up and down two to three times. Transfer to well. Repeat with media from same well. Incubate at 37°C.

Step 3 – Transduction of T cells with AAV vector containing HDR repair construct.

[00344] Recombinant AAV6 donor vector is added to the cell culture 2-4hrs after electroporation with a MOI between 1e4 and 1e6.

Step 4 - Assessment of CRISPR activity and Td efficiency (Day 10).

[00345] Take 5×10^5 cells from each sample and analyze by flow cytometry. Wash samples with RB. Add 3ul of anti-CD34 PE antibody (This detects the CAR as our construct contains human truncated CD34). Add 5ul of CD3 APC and 2ul of CD2 BV421. Wash. Perform Flow cytometry. Cells should be CD3ε negative, CD7 negative. Harvest T cells (**Day 11**).

[00346] Purification of CAR-T cells. CD34+ (CAR+) and TCR negative cells can be purified in a single step using a positive selection of CD34+ cells on the Miltenyi Automacs. This enriches the CAR+ cells and removes and TCR+ cells (as CAR insertion disrupts TCR signaling)

Step 5 – Assessment of CAR-T activity in vivo

[00347] Inject tumor in NSG mice (5e5 MOLT3 or HH: containing Luciferase) if performing in vivo imaging experiment. (**Day 7**)

[00348] Image tumor burden in mouse using bioluminescent imaging. Inject 2×10^6 CD34+ CAR-T per mouse I.V. via tail vein or perform a 4hr chromium release assay against targets cell (MOLT3 or HH) (**Day 11**). Those of skill in the art will appreciate that some flexibility is possible in the time frames specified in Example 1.

Example 2 – Method of Making a Genome-Edited Tandem tCAR-T Cells

[00349] In a variation of the protocol in Example 1, a tandem CAR-T cell recognizing two antigens can be made. In Step 2, the two antigens can be deleted from the cell surface, or suppressed as described above, by electroporating with gRNA for each of the two targets and Cas9 mRNA. In Step 3, This CAR-T cell is then transduced with a CAR that recognizes two targets. The variations of a tandem CAR-T cell shown in the schematic in **FIG. 2**. Additional examples of tCAR-T cells are shown in Table 6.

Example 3 – Genome-Edited Dual CAR-T Cells or Genome-Edited Tandem CAR-T Cells

[00350] Several types of genome-edited dual or tandem CAR-T cells may be made using the methods above. **FIG. 1** and **FIG. 2** show the examples of tandem and dual CAR-T cells. The figures state the antigens to be targeted and does not indicate order of scFv expression in tandem CAR construct. Further examples are provided below in Tables 6-11.

[00351] Additional examples of tandem and dual CAR-T cells are provided herein with deletion, without deletion, or suppression of one or more surface proteins that are target antigens of the CARs and expressed on CAR-T cells. In general, examples with deletion or suppression of more than one antigen will more likely have the benefit of greater fratricide-resistance for these CAR-T cells. It should be further noted that the order in which the scFvs are oriented in the tandem CARs are set forth in Tables 6-11 and is not limiting. For example, the CD2*CD3 ϵ encompasses a tCAR with the orientation CD2*CD3 ϵ or the orientation CD3 ϵ *CD2.

[00352] Additional examples of mono, tandem, and dual CAR-T cells targeting antigens expresses on multiple myeloma cells are provided herein, without deletion, with deletion, or suppression of one or more surface proteins that are target antigens of the CARs and expressed on CAR-T cells. In general, examples with deletion or suppression of more than one antigen will more likely have the benefit of greater fratricide-resistance for these CAR-T cells.

Example 4 – Treatment of Patient(s) with Genome-Edited Dual or Tandem CAR-T Cells

[00353] Patients may be treated using cells made by the methods above, as shown in **FIG. 1** and **FIG. 2**. For example, an expanded population of dual or tandem CAR-T cells may be infused into a patient

[00354] Dual or Tandem CAR-T cells target cancer cells without inducing alloreactivity. For example, CD2*CD3 ϵ -dCART Δ CD2 Δ CD3 ϵ cells would target cancer cells (and other non-cancer cells) bearing the CD2 and CD ϵ surface antigens.

Example 5 – Testing efficacy of CD2*CD3 Δ -dCART Δ CD2 Δ CD3 ϵ in a xenogeneic model of T-ALL

[00355] Testing efficacy of CD2*CD3 Δ -dCART Δ CD2 Δ CD3 ϵ in a xenogeneic model of T-ALL: 5x10⁵ Click Beetle Red luciferase (CBR) labeled Jurkat (T-ALL - 99% CD2+, 99% % CD3% by FACS) cells were injected I.V. into NSG recipients prior to infusion of CD2*CD3 ϵ -

dCART Δ CD2 Δ CD3 ϵ (WC5 or WC13), CD3CART Δ CD2 Δ CD3 ϵ (UCART3), CD2CART Δ CD2 Δ CD3 ϵ (UCART2) or non-targeting CD19-CAR Δ CD2 Δ CD3 ϵ (UCART19) control cells i.v. on day +4. In contrast to mice receiving CD19-CAR Δ CD2 Δ CD3 ϵ or mice injected with tumor only, mice receiving CD2*CD3 ϵ -dCART Δ CD2 Δ CD3 ϵ demonstrate significantly prolonged survival and reduced tumor burden as determined by bioluminescent imaging shown in **FIG. 13**. In future models, CD2*CD3 ϵ -dCART Δ CD2 Δ CD3 ϵ would provide a survival advantage over CD3CART Δ CD2 Δ CD3 ϵ , CD2CART Δ CD2 Δ CD3 ϵ , and reduce tumor burden in a version of this model in which the target cell is missing either CD2 (CD2CART Δ CD2 Δ CD3 ϵ) or CD3 (CD3CART Δ CD2 Δ CD3 ϵ).

Example 6 – Genome-Edited Mono CAR-T cells

[00356] Examples of genome-edited mono CAR-T cells targeting antigens expresses on hematologic malignancies are provided below, without deletion, with deletion, or suppression of one or more surface proteins that are target antigens of the CARs and expressed on CAR-T cells. In general, examples with deletion or suppression of more than one antigen will more likely have the benefit of greater fratricide-resistance for these CAR-T cells.

Example 7 – Genome-Edited UCART2/3 Cells Made by Editing Before Activation

[00357] On Day 0, cells were thawed in a thaw buffer. Thereafter, cells were resuspended in media and allowed to rest for two hours. Cells were harvested and counted. The required number of cells were centrifuged at 100xg for 10 minutes at room temperature. Supernatant was removed completely, cells resuspended in PBS (1ml) and transfer to a microcentrifuge tube, and centrifuged at 100xg for 10 minutes at room temperature. Supernatant was removed completely, and cells then resuspended in a buffer P3, counted, and the count adjusted to 5×10^7 per mL. A cell pool volume of 100 μ L was added to a tube containing Cas9/gRNA, gently mixed, and everything transferred into the Nucleocuvette™, which was gently tapped to remove bubbles. Electroporation was thereafter commenced using program (Human T cell stim EO-115). After this procedure, the activated cells were transferred to pre-warmed media and distributed in 2 mL aliquots in a 12-well plate. Aliquoted samples were rested for 24 hours.

[00358] On day 1, cells were activated with T Cell TransAct™ as shown in Table 14.

[00359] **Table 14. T Cell TransAct™ Activation.**

	<u>Name</u>	<u>Media</u>	<u>Stimulation</u>	<u>Cas9 p</u>	<u>gRNA</u>	<u>Virus</u>
1	WT	TexMacs	T Cell TransAct™ (50μl)	-	-	
2	WC5	TexMacs	T Cell TransAct™ (50μl)	2 ul	20ug iDT CD2 + CD3ε	WC5
3	WC6	TexMacs	T Cell TransAct™ (50μl)	2 ul	20ug iDT CD2 + CD3ε	WC6
4	WC7	TexMacs	T Cell TransAct™ (50μl)	2 ul	20ug iDT CD2 + CD3ε	WC7
5	WC8	TexMacs	T Cell TransAct™ (50μl)	2 ul	20ug iDT CD2 + CD3ε	WC8
6	WC13	TexMacs	T Cell TransAct™ (50μl)	2 ul	20ug iDT CD2 + CD3ε	WC13
7	WC14	TexMacs	T Cell TransAct™ (50μl)	2 ul	20ug iDT CD2 + CD3ε	WC14
8	WC15	TexMacs	T Cell TransAct™ (50μl)	2 ul	20ug iDT CD2 + CD3ε	WC15
9	WC16	TexMACS	T Cell TransAct™ (50μl)	2 ul	20ug iDT CD2 + CD3ε	WC16

[00360] On day 2, 1μl of polybrene was added for each ml media (8mg/ml stock). The required amount of virus was added to give required M.O.I (Multiplicity of Infection). Cells and virus were mixed and placed back in incubator at 37°C.

[00361] On day 3, activated cells were washed to remove stimulation.

[00362] On Day 12, FACS analysis showed the high purity of CD3⁺CD2⁺/CAR-T cells; see **FIG. 5** (clone 5 and clone 6), **FIG. 6** (clone 7 and clone 8), **FIG. 7** (clone 13 and clone 14), and **FIG. 8** (clone 15 and clone 16). Standard four-hour chromium release (⁵¹Cr) assays were performed using (⁵¹Cr) labeled genome-edited Jurkat cells (ΔCD2, ΔCD3, and ΔCD2ΔCD3). These experiments showed a functional tumor killing response to CD2 and CD3 targets independent of one another (see **FIG. 9A**, **FIG. 9B**, **FIG. 9C**, and **FIG. 9D**).

Example 8 - Tumor cell killing of BCMA-CAR-T cells

[00363] BCMA CAR-Ts were first tested in vitro for efficacy using a standard four-hour chromium release (^{51}Cr) assays using ^{51}Cr labeled MM.1S target cells. To enable in vivo tracking, the human myeloma cell line (BCMA+/CD19-), was modified to express click beetle red luciferase fused to GFP (MM.1S-CG). The CAR-T cells were incubated with ^{51}Cr -labeled MM.1S-CG cells for four hours at a range of effector (CAR-T) to target (MM.1S-CG) ratios and released ^{51}Cr was measured as a marker of MM.1S-CG cell death (**FIG. 10B**). Efficient killing was observed at multiple Effector to Target (E:T) ratios. Non-transduced activated T cells and CD19-CAR-Ts were used as negative controls and did not induce killing of MM.1S-CG cells. Next, in vivo efficacy was tested by engrafting NSG mice with 500,000 MM.1S-CG human myeloma cells (i.v.). Twenty-eight days later, when tumor burden was high, mice were left untreated or were treated with 2×10^6 CD19-CAR-Ts or BCMA CAR-Ts. All seven mice treated with BCMA CAR-Ts lived to almost 150 days or more compared to controls which died around day 50 (**FIG. 10C**). The cause of death of the one mouse that died in the BCMA CAR-T cohort is unknown. Flow cytometry analysis revealed no GFP+ tumor cells in that mouse. Serial bioluminescent imaging (BLI) revealed a robust reduction of signal to background levels that never increased throughout the duration of the experiment (**FIG. 10D**).

Example 9 - Tumor cell killing of CS1-CAR-T cells

[00364] *In vivo* efficacy of CS1-CAR-T cells by injecting 5×10^5 MM.1S-CG into NSG mice and 28 days later when tumor burden was high (BLI signal 1010 photon flux), injected 2×10^6 CS1-CAR-T cells or negative control CD19-CAR-T cells. Mice were also engrafted with MM.1S-CG cells lacking CS1 (using CAS9/CRISPR technology; MM.1S-CG Δ CS1) as a method to test the specificity of CS1-CAR-T cells. All mice treated with CS1-CAR-T cells (n=10) lived >90 days (**FIG. 11B**) while median survival of CD19-control mice (n=8) was 43 days. We treated mice engrafted with MM.1S-CG Δ CS1 with CS1-CAR-T or CD19 CAR-T, as above. Survival of those mice was similar to control mice (49 days), demonstrating in vivo specificity. Serial Bioluminescent imaging (BLI) showed CS1-CAR-Ts treated mice had a three log decrease in photon flux and clearance of marrow tumor (**FIG. 11C**). A subset of CS1-CAR-T mice

developed extramedullary tumors that retained expression of CS1, suggesting antigen escape did not occur.

Example 10 – Efficacy and cell killing of a tandem (tCAR) which targets BCMA and CS1

[00365] Bi-targeted CAR-T that express two scFvs in a tandem (tCAR) that target BCMA and CS1 were designed in an attempt to improve efficacy and killing of myeloma CAR-T cells. For a control, the tandem CAR was tested side by side with single-targeted BCMA-CAR-T cells and single-targeted CS1-CAR-T cells. CD19-CAR-T cells were used as a negative control. First, each scFv was confirmed to be expressed in the tCAR. To accomplish this, Jurkat cells were infected with lentivirus expressing each CAR construct as described in **FIG. 12A**. The CAR-T cells were incubated with human recombinant BCMA and CS1 proteins each labeled with separate fluorescent fluorophores. Negative control CAR-T cells were gated (blue color) and the experimental CAR-T cells were overlaid (red color). As expected, Jurkat cells expressing CD19 CAR did not bind to either BCMA or CS1 protein (lower left quadrant, **FIG. 12B**). Jurkat cells expressing BCMA CAR protein bound BCMA protein (upper left quadrant, **FIG. 12B**). Jurkat cells expressing CS1 CAR protein bound CS1 protein (lower right quadrant, **FIG. 12B**). Jurkat cells expressing the tandem BCMA-CS1 CAR protein bound to both recombinant proteins (upper right quadrant, **FIG. 12B**), suggesting expression of both scFvs.

[00366] Single and tandem CAR-T cells were tested for *in vitro* efficacy with standard four-hour chromium release (^{51}Cr) assays. For these experiments, CAR-T cells were incubated with a range of effector to target cells (E:T ratio). BCMA-CS1 tCAR T cells killed MM.1S-CG cells with similar efficacy of both single targeted CAR-T cells. Additional experiments will optimize bi-targeted BCMA-CS1 CAR-T cells for *in vivo* efficacy.

Example 11 – Off Target Analysis for gRNA Selection

[00367] Guide RNA were designed and validated for activity by Washington University Genome Engineering & iPSC. Guide RNA were designed and validated for activity by Washington University Genome Engineering & iPSC. Sequences complementary to a given gRNA may exist throughout the genome, including but not limited to the target locus. A short sequence is likelier to hybridize off-target. Similarly, some long sequences within the gRNA may have exact matches (long_0) or near matches (long_1, long_2, representing, respectively, a

single or two nucleotide difference) throughout the genome. These may also hybridize off-target, in effect leading to editing of the wrong gene and diminishing editing efficiency.

[00368] Off target analysis of selected gRNA was performed for 2 exons of hCD2 (CF58 and CF59) to determine the number of sites in human genome which are an exact match or contains up to 1 or 2 mismatches, which may include the target site. The results are listed in Table 15 for Exon CF58 and Table 16 for Exon CF59.

Table 15. Guide RNA (gRNA) Off Target Analysis for hCD2 (Exon CF58)

Name	gRNA	long_ 0	long_ 1	long_ 2	short_ 0	SNP
CF58.CD2.g1	CAAAGAGATTACGAATGCCTN GG (SEQ ID NO:57)	1	1	1	3	NA
CF58.CD2.g2 3	CAAGGCATTTCGTAATCTCTT GG (SEQ ID NO:58)	1	1	1	5	NA
CF58.CD2.g1 8	CTTGTAGATATCCTGATCATNG G (SEQ ID NO:59)	1	1	1	13	NA
CF58.CD2.g8	CTTGGGTCAGGACATCAACTNG G (SEQ ID NO:60)	1	1	1	14	NA
CF58.CD2.g1 4	CGATGATCAGGATATCTACANG G (SEQ ID NO:61)	1	1	1	17	NA
CF58.CD2.g2	TTACGAATGCCTTGGAAACCNG G (SEQ ID NO:62)	1	1	1	27	NA
CF58.CD2.g3	TACGAATGCCTTGGAAACCTNG G (SEQ ID NO:63)	1	1	1	34	NA
CF58.CD2.g4	ACGAATGCCTTGGAAACCTGNG G (SEQ ID NO:64)	1	1	1	40	NA
CF58.CD2.g1 0	TGATATTGACGATATAAAATNG G (SEQ ID NO:65)	1	1	2	3	NA
CF58.CD2.g9	ATGATATTGACGATATAAAANG G (SEQ ID NO:66)	1	1	2	4	NA
CF58.CD2.g1 3	GCATCTGAAGACCGATGATCNG G (SEQ ID NO:67)	1	1	2	4	NA
CF58.CD2.g7	AACCTGGGGTGCCTTGGGTCNG G (SEQ ID NO:68)	1	1	2	22	NA
CF58.CD2.g6	TTGGAAACCTGGGGTGCCTTNG G (SEQ ID NO:69)	1	1	2	33	NA
CF58.CD2.g1 5	GTATCAATATATGATACAAANG G (SEQ ID NO:70)	1	1	2	35	NA
CF58.CD2.g2 2	CAAGGCACCCCAGGTTTCCANG G (SEQ ID NO:71)	1	1	2	45	NA
CF58.CD2.g5	CTTGGAAACCTGGGGTGCCTNG G (SEQ ID NO:72)	1	1	2	62	NA
CF58.CD2.g1	TCATCACTCATTTGAAAACCTNG	1	1	3	56	NA

9	G (SEQ ID NO:73)					
CF58.CD2.g20	CAAGTTGATGTCCTGACCCANG G (SEQ ID NO:74)	1	1	4	27	NA
CF58.CD2.g21	GTCCTGACCCAAGGCACCCNG G (SEQ ID NO:75)	1	1	4	33	NA
CF58.CD2.g17	ATATTTGATTTGAAGATTCANG G (SEQ ID NO:76)	1	1	6	35	NA
CF58.CD2.g16	TACAAAAGGAAAAAATGTGTN GG (SEQ ID NO:77)	1	1	7	64	NA
CF58.CD2.g12	ACATATAAGCTATTTAAAAANG G (SEQ ID NO:78)	1	1	8	58	NA
CF58.CD2.g11	AAAAGAGAAAGAGACTTTCAN GG (SEQ ID NO:79)	1	1	15	42	NA

Table 16. Guide RNA (gRNA) Off Target Analysis for hCD2 (CF59)

Name	gRNA	long_ 0	long_ 1	long_ 2	short_ 0	SNP
CF59.CD2.g20	CTTGATACAGGTTTAATTCTG NGG (SEQ ID NO:80)	1	1	1	2	NA
CF59.CD2.g13	ACAGCTGACAGGCTCGACAC NGG (SEQ ID NO:81)	1	1	1	4	NA
CF59.CD2.g17	GATGTTTCCCATCTTGATAC NGG (SEQ ID NO:82)	1	1	1	8	NA
CF59.CD2.g12	GTCGAGCCTGTCAGCTGTCCN GG (SEQ ID NO:83)	1	1	1	24	NA
CF59.CD2.g10	CAAAATTCAAGTGCACAGCAN GG (SEQ ID NO:84)	1	1	1	33	NA
CF59.CD2.g16	GAATTTTGCACCTCAGGCTGGN GG (SEQ ID NO:85)	1	1	1	245	NA
CF59.CD2.g4	GAATTAAACCTGTATCAAGAN GG (SEQ ID NO:86)	1	1	2	7	NA
CF59.CD2.g5	AATTAAACCTGTATCAAGATN GG (SEQ ID NO:87)	1	1	2	7	NA
CF59.CD2.g21	AGTTCCATTTCATTACCTCACNG G (SEQ ID NO:88)	1	1	2	14	NA
CF59.CD2.g8	AGAGGGTCATCACACACAAGN GG (SEQ ID NO:89)	1	1	2	20	NA
CF59.CD2.g25	ATACAAGTCCAGGAGATCTTN GG (SEQ ID NO:90)	1	1	2	21	NA
CF59.CD2.g19	TCTTGATACAGGTTTAATTCNG G (SEQ ID NO:91)	1	1	2	25	NA
CF59.CD2.g3	CTGACCTGTGAGGTAATGAAN GG (SEQ ID NO:92)	1	1	2	29	NA
CF59.CD2.g7	ACATCTAAACTTTCTCAGAN GG (SEQ ID NO:93)	1	1	2	41	NA

CF59.CD2.g9	GCAAAATTCAAGTGCACAGCNGG (SEQ ID NO:94)	1	1	2	46	NA
CF59.CD2.g24	GGTTGTGTTGATACAAGTCCNGG (SEQ ID NO:95)	1	1	3	8	NA
CF59.CD2.g18	ATCTTGATACAGGTTTAATTNGG (SEQ ID NO:96)	1	1	3	24	NA
CF59.CD2.g23	ATTCATTACCTCACAGGTCANGG (SEQ ID NO:97)	1	1	3	35	NA
CF59.CD2.g6	AACATCTAAAACCTTCTCAGNGG (SEQ ID NO:98)	1	1	3	43	NA
CF59.CD2.g11	AGCAGGGAACAAAGTCAGCANGG (SEQ ID NO:99)	1	1	3	45	NA
CF59.CD2.g2	CAACACAACCCTGACCTGTGNGG (SEQ ID NO:100)	1	1	3	47	NA
CF59.CD2.g15	CTTGAATTTTGCACCTCAGGCNGG (SEQ ID NO:101)	1	1	4	21	NA
CF59.CD2.g22	CATTCATTACCTCACAGGTCNGG (SEQ ID NO:102)	1	1	10	29	NA
CF59.CD2.g14	TGCACTTGAATTTTGCACCTCNGG (SEQ ID NO:103)	1	2	3	26	NA
CF59.CD2.g1	TCTCAAAACCAAAGATCTCCNGG (SEQ ID NO:104)	1	2	5	19	NA

[00369] The gRNA sequences in Table 15 and Table 16 were normalized (% Normalization to NHEJ) for gRNA activity via next generation sequencing (NGS). GFP was used as a control. Following sequencing analysis, the following gRNAs were recommended based on off-target profile: CF58.CD2.g1 (41.2%), CF58.CD2.g23 (13.2%), CF59.CD2.g20 (26.6%), CF59.CD2.g13 (66.2%), CF59.CD2.g17 (17.5%). Guide RNA (gRNA) with normalized NHEJ frequencies equal to or greater than 15% are good candidates for cell line and animal model creation projects.

[00370] Off target analysis of selected gRNA was performed for hCD3E to determine the number of sites in human genome which are an exact match or contains up to 1 or 2 mismatches, which may include the target site. The results are listed in Table 17 for hCD3E.

Table 17. Guide RNA (gRNA) Off Target Analysis for hCD3E

Name	gRNA	long _0	long _1	long _2	long _3	short _0	SN P
MS1044.CD3E. sp2	TTGACATGCCCTCAGTATC CNGG (SEQ ID NO:105)	1	1	1	21	73	NA
MS1044.CD3E. sp17	CTGGATTACCTCTTGCCCT CNGG (SEQ ID NO:106)	1	1	1	24	114	NA

MS1044.CD3E.sp28	GAGATGGAGACTTTATAT GCNGG (SEQ ID NO:107)	1	1	1	30	44	NA
MS1044.CD3E.sp29	AGATGGAGACTTTATATGC TNGG (SEQ ID NO:108)	1	1	1	33	55	NA
MS1044.CD3E.sp26	AGGGCATGTCAATATTACT GNGG (SEQ ID NO:109)	1	1	1	23	60	NA
MS1044.CD3E.sp30	GATGGAGACTTTATATGCT GNGG (SEQ ID NO:110)	1	1	2	26	64	NA
MS1044.CD3E.sp12	TATTATGTCTGCTACCCC AGNGG (SEQ ID NO:111)	1	1	2	20	61	NA
MS1044.CD3E.sp23	TGCCATAGTATTTTCAGATC CNGG (SEQ ID NO:112)	1	1	2	21	55	NA
MS1044.CD3E.sp18	AGATAAAAGTTCGCATCTT CNGG (SEQ ID NO:113)	1	1	2	33	6	NA
MS1044.CD3E.sp22	CTGAAAATTCCTTCAGTGA CNGG (SEQ ID NO:114)	1	1	2	44	60	NA
MS1044.CD3E.sp16	CTGAGGGCAAGAGGTAAT CCNGG (SEQ ID NO:115)	1	1	3	30	41	NA
MS1044.CD3E.sp25	TTTCAGATCCAGGATACTG ANG G (SEQ ID NO:116)	1	1	3	38	63	NA
MS1044.CD3E.sp15	TATCTCTACCTGAGGGCAA GNGG (SEQ ID NO:117)	1	1	3	22	134	NA
MS1044.CD3E.sp9	TGAGGATCACCTGTCACTG ANG G (SEQ ID NO:118)	1	1	3	44	54	NA

[00371] The gRNA sequences in Table 17 were normalized (% Normalization to NHEJ) for gRNA activity via next generation sequencing (NGS). GFP was used as a control. Following sequencing analysis, the following gRNAs were recommended based on off-target profile: MS1044.CD3E.sp28 (>15%) and MS1044.CD3E.sp12 (>15%). Guide RNA (gRNA) with normalized NHEJ frequencies equal to or greater than 15% are good candidates for cell line and animal model creation projects.

[00372] Off target analysis of selected gRNA was performed for 3 exons of hCD5 (Exon 3, Exon 4, and Exon 5) to determine the number of sites in human genome which are an exact match or contains up to 1 or 2 mismatches, which may include the target site. The results are listed in Table 18 for Exon 3, Table 19 for Exon 4, and Table 20 for Exon 5.

Table 18. Guide RNA (gRNA) Off Target Analysis for hCD5 (Exon 3)

Name	gRNA	long_ 0	long_ 1	long_ 2	short_ 0	SNP
SP597.CD5.g22	AATCATCTGCTACGGACAAC NGG (SEQ ID NO:119)	1	1	1	1	NA

SP597.CD5.g 39	GCAGACTTTTGACGCTTGAC NGG (SEQ ID NO:120)	1	1	1	1	NA
SP597.CD5.g 1	CCGTTCCAACCTCGAAGTGCCN GG (SEQ ID NO:121)	1	1	1	2	NA
SP597.CD5.g 2	CGTTCCAACCTCGAAGTGCCA NGG (SEQ ID NO:122)	1	1	1	2	NA
SP597.CD5.g 50	CTGGCACTTCGAGTTGGAACN GG (SEQ ID NO:123)	1	1	1	2	NA
SP597.CD5.g 17	GTCTGCCAGCGGCTGAACTGN GG (SEQ ID NO:124)	1	1	1	3	NA
SP597.CD5.g 23	ATCATCTGCTACGGACAACN GG (SEQ ID NO:125)	1	1	1	3	NA
SP597.CD5.g 41	AGACTTTTGACGCTTGACTGN GG (SEQ ID NO:126)	1	1	1	3	NA
SP597.CD5.g 40	CAGACTTTTGACGCTTGACTN GG (SEQ ID NO:127)	1	1	1	5	NA
SP597.CD5.g 49	CCTGGCACTTCGAGTTGGAAN GG (SEQ ID NO:128)	1	1	1	5	NA
SP597.CD5.g 38	GCACCCACAGTTCAGCCGCN GG (SEQ ID NO:129)	1	1	1	8	NA
SP597.CD5.g 46	CCTTGAGGTAGACCTCCAG CNGG (SEQ ID NO:130)	1	1	1	9	NA
SP597.CD5.g 7	AGGTCTACCTCAAGGACGGA NGG (SEQ ID NO:131)	1	1	1	11	NA
SP597.CD5.g 51	TGGAACGGGTGAGCCTTGCCN GG (SEQ ID NO:132)	1	1	1	13	NA
SP597.CD5.g 20	TGTGGGGTGCCCTTAAGCCTN GG (SEQ ID NO:133)	1	1	1	19	NA
SP597.CD5.g 16	AAGCGTCAAAAGTCTGCCAG NGG (SEQ ID NO:134)	1	1	1	20	NA
SP597.CD5.g 29	TAGCAGATGATTGAGCTCTGN GG (SEQ ID NO:135)	1	1	1	25	NA
SP597.CD5.g 30	GATTGAGCTCTGAGGTGTGTN GG (SEQ ID NO:136)	1	1	1	33	NA
SP597.CD5.g 13	GGGGCCGGAGCTCCAAGCAG NGG (SEQ ID NO:137)	1	1	1	42	NA
SP597.CD5.g 33	GGTGTGTAGGTGACAAGGAA NGG (SEQ ID NO:138)	1	1	1	48	NA
SP597.CD5.g 15	CCGGAGCTCCAAGCAGTGGG NGG (SEQ ID NO:139)	1	1	1	58	NA
SP597.CD5.g 47	GGTAGACCTCCAGCTGGCCCN GG (SEQ ID NO:140)	1	1	1	78	NA
SP597.CD5.g 3	CTCGAAGTGCCAGGGCCAGC NGG (SEQ ID NO:141)	1	1	1	121	NA
SP597.CD5.g 48	CTGGCCCTGGCACTTCGAGTN GG (SEQ ID NO:142)	1	1	2	1	NA

SP597.CD5.g 18	TCTGCCAGCGGCTGAACTGTN GG (SEQ ID NO:143)	1	1	2	5	NA
SP597.CD5.g 45	CCATGTGCCATCCGTCCTTGN GG (SEQ ID NO:144)	1	1	2	5	NA
SP597.CD5.g 5	CCAGCTGGAGGTCTACCTCAN GG (SEQ ID NO:145)	1	1	2	14	NA
SP597.CD5.g 31	TCTGAGGTGTGTAGGTGACAN GG (SEQ ID NO:146)	1	1	2	18	NA
SP597.CD5.g 37	AGGAAGGGGCCAAGGCTTAA NGG (SEQ ID NO:147)	1	1	2	18	NA
SP597.CD5.g 21	CAGAGCTCAATCATCTGCTAN GG (SEQ ID NO:148)	1	1	2	19	NA
SP597.CD5.g 14	GGGCCGGAGCTCCAAGCAGT NGG (SEQ ID NO:149)	1	1	2	23	NA
SP597.CD5.g 43	CCTCCCCTGCTTGGAGCTCN GG (SEQ ID NO:150)	1	1	2	30	NA
SP597.CD5.g 44	TGGAGCTCCGGCCCCAGCTCN GG (SEQ ID NO:151)	1	1	2	38	NA
SP597.CD5.g 34	GTGTGTAGGTGACAAGGAAG NGG (SEQ ID NO:152)	1	1	2	48	NA
SP597.CD5.g 11	ATGGTTTGCAGCCAGAGCTGN GG (SEQ ID NO:153)	1	1	2	108	NA
SP597.CD5.g 6	CTGGAGGTCTACCTCAAGGAN GG (SEQ ID NO:154)	1	1	3	16	NA
SP597.CD5.g 19	CTGCCAGCGGCTGAACTGTGN GG (SEQ ID NO:155)	1	1	3	25	NA
SP597.CD5.g 25	AATGACATGTGTCCTCTCTN GG (SEQ ID NO:156)	1	1	3	25	NA
SP597.CD5.g 9	ACATGGTTTGCAGCCAGAGCN GG (SEQ ID NO:157)	1	1	3	30	NA
SP597.CD5.g 10	CATGGTTTGCAGCCAGAGCTN GG (SEQ ID NO:158)	1	1	3	52	NA
SP597.CD5.g 26	GACACATGTCATTTCTGCTGN GG (SEQ ID NO:159)	1	1	3	53	NA
SP597.CD5.g 42	ACTGGGGTCCTCCCTGCTN GG (SEQ ID NO:160)	1	1	3	91	NA
SP597.CD5.g 8	CCTCAAGGACGGATGGCACA NGG (SEQ ID NO:161)	1	1	4	5	NA
SP597.CD5.g 32	AGGTGTGTAGGTGACAAGGA NGG (SEQ ID NO:162)	1	1	4	49	NA
SP597.CD5.g 36	AAGGAAGGGGCCAAGGCTTA NGG (SEQ ID NO:163)	1	1	5	16	NA
SP597.CD5.g 4	GAAGTGCCAGGGCCAGCTGG NGG (SEQ ID NO:164)	1	1	5	93	NA
SP597.CD5.g 12	TTTGCAGCCAGAGCTGGGGCN GG (SEQ ID NO:165)	1	1	8	257	NA

SP597.CD5.g 24	AAATGACATGTGTCACTCTCN GG (SEQ ID NO:166)	1	1	10	33	NA
SP597.CD5.g 35	AGGTGACAAGGAAGGGGCCA NGG (SEQ ID NO:167)	1	1	10	202	NA
SP597.CD5.g 27	ATTTCTGCTGTGGCTGCAGTN GG (SEQ ID NO:168)	1	2	4	70	NA
SP597.CD5.g 28	GCTGTGGCTGCAGTTGGAGAN GG (SEQ ID NO:169)	1	2	19	49	NA

Table 19. Guide RNA (gRNA) Off Target Analysis for hCD5 (Exon 4)

Name	gRNA	long_ 0	long_ 1	long_ 2	short_ 0	SNP
SP598.CD5.g 10	GGCGGGGGCCTTGTCGTTG GNGG (SEQ ID NO:170)	1	1	1	1	NA
SP598.CD5.g 7	CTCTGGAGTTGTGGTGGGC GNGG (SEQ ID NO:171)	1	1	1	16	NA
SP598.CD5.g 8	TCTGGAGTTGTGGTGGGCGGN GG (SEQ ID NO:172)	1	1	1	40	NA
SP598.CD5.g 12	CGTTGGAGGTGTTGTCTTCTN GG (SEQ ID NO:173)	1	1	1	46	NA
SP598.CD5.g 1	AGACAACACCTCCAACGACAN GG (SEQ ID NO:174)	1	1	2	2	NA
SP598.CD5.g 9	GTGGGCGGGGGCCTTGTCGTN GG (SEQ ID NO:175)	1	1	2	5	NA
SP598.CD5.g 11	TCGTTGGAGGTGTTGTCTTCN GG (SEQ ID NO:176)	1	1	2	13	NA
SP598.CD5.g 2	ACCACAACCTCCAGAGCCACN GG (SEQ ID NO:177)	1	1	2	60	NA
SP598.CD5.g 6	GCTCTGGAGTTGTGGTGGGCN GG (SEQ ID NO:178)	1	1	4	74	NA
SP598.CD5.g 4	GTGGGCTCTGGAGTTGTGGTN GG (SEQ ID NO:179)	1	1	6	35	NA
SP598.CD5.g 3	TGTGGGCTCTGGAGTTGTGGN GG (SEQ ID NO:180)	1	1	8	54	NA
SP598.CD5.g 13	GTTGGAGGTGTTGTCTTCTGN GG (SEQ ID NO:181)	1	2	2	48	NA
SP598.CD5.g 5	GGCTCTGGAGTTGTGGTGGGN GG (SEQ ID NO:182)	1	3	9	51	NA

Table 20. Guide RNA (gRNA) Off Target Analysis for hCD5 (Exon 5)

Name	gRNA	long_ 0	long_ 1	long_ 2	short_ 0	SNP
SP599.CD5. g58	CATAGCTGATGGTACCCC CCNGG (SEQ ID NO:183)	1	1	1	1	NA

SP599.CD5. g5	CGGCCAGCACTGTGCCGG CGNGG (SEQ ID NO:184)	1	1	1	2	NA
SP599.CD5. g30	CAAGAACTCGGCCACTTT TCNGG (SEQ ID NO:185)	1	1	1	6	NA
SP599.CD5. g44	GGTGTTCCTGGTCCCT NGG (SEQ ID NO:186)	1	1	1	11	rs2241002:0. 158
SP599.CD5. g6	CCAGCACTGTGCCGGCGTG GNGG (SEQ ID NO:187)	1	1	1	13	NA
SP599.CD5. g42	GGCAAGGGCTGGTGTTC CGNGG (SEQ ID NO:188)	1	1	1	13	NA
SP599.CD5. g7	GGCGTGGTGGAGTTCTACA GNGG (SEQ ID NO:189)	1	1	1	14	NA
SP599.CD5. g60	CCACCACGCCGGCACAGTG CNGG (SEQ ID NO:190)	1	1	1	15	NA
SP599.CD5. g8	GGAGTTCTACAGCGGCAGC CNGG (SEQ ID NO:191)	1	1	1	17	NA
SP599.CD5. g11	GTTCTACAGCGGCAGCCTG GNGG (SEQ ID NO:192)	1	1	1	18	NA
SP599.CD5. g25	ACCAGCCCTTGCCAATCCA ANGG (SEQ ID NO:193)	1	1	1	20	NA
SP599.CD5. g10	AGTTCTACAGCGGCAGCCT GNGG (SEQ ID NO:194)	1	1	1	24	NA
SP599.CD5. g55	CCAGGTCCTGGGTCTTGTC CNGG (SEQ ID NO:195)	1	1	1	25	NA
SP599.CD5. g43	TGGTGTTCCTGGTCCCC NGG (SEQ ID NO:196)	1	1	1	25	rs2241002:0. 158
SP599.CD5. g9	GAGTTCTACAGCGGCAGCC TNGG (SEQ ID NO:197)	1	1	1	26	NA
SP599.CD5. g26	GAAGTCAAGCTGTACCTCC CNGG (SEQ ID NO:198)	1	1	1	29	NA
SP599.CD5. g31	AAGAACTCGGCCACTTTTC TNGG (SEQ ID NO:199)	1	1	1	29	NA
SP599.CD5. g41	TCCATTGGATTGGCAAGGG CNGG (SEQ ID NO:200)	1	1	1	32	NA
SP599.CD5. g12	TTCTACAGCGGCAGCCTGG GNGG (SEQ ID NO:201)	1	1	1	33	NA
SP599.CD5. g32	AGAACTCGGCCACTTTTCT GNGG (SEQ ID NO:202)	1	1	1	37	NA
SP599.CD5. g49	GCTTCAAGAAGGAGCCACA CNGG (SEQ ID NO:203)	1	1	1	48	NA
SP599.CD5. g39	GATCTTCCATTGGATTGGC ANGG (SEQ ID NO:204)	1	1	2	7	NA
SP599.CD5. g59	GCTGTAGAACTCCACCACG CNGG (SEQ ID NO:205)	1	1	2	11	NA
SP599.CD5. g57	GTCCTGGGCCTCATAGCTG ANGG (SEQ ID NO:206)	1	1	2	13	NA

SP599.CD5. g14	TACCATCAGCTATGAGGCC CNGG (SEQ ID NO:207)	1	1	2	14	NA
SP599.CD5. g13	GGGGGGTACCATCAGCTAT GNGG (SEQ ID NO:208)	1	1	2	16	NA
SP599.CD5. g35	CCTGAAGCAATGCTCCAGG GNGG (SEQ ID NO:209)	1	1	2	18	NA
SP599.CD5. g33	TTTCCTGAAGCAATGCTCC NGG (SEQ ID NO:210)	1	1	2	24	NA
SP599.CD5. g48	CTCTGGCAGATGCTTCAAG ANGG (SEQ ID NO:211)	1	1	2	25	NA
SP599.CD5. g53	AGAGGAAGTTCTCCAGGTC CNGG (SEQ ID NO:212)	1	1	2	53	NA
SP599.CD5. g4	TCTGGCGGCCAGCACTGTG CNGG (SEQ ID NO:213)	1	1	2	166	NA
SP599.CD5. g37	TTGAGTTCTGGATCTTCCAT NGG (SEQ ID NO:214)	1	1	3	9	NA
SP599.CD5. g38	TTCTGGATCTTCCATTGGAT NGG (SEQ ID NO:215)	1	1	3	13	NA
SP599.CD5. g40	ATCTTCCATTGGATTGGCA ANGG (SEQ ID NO:216)	1	1	3	18	NA
SP599.CD5. g50	TCAAGAAGGAGCCCACTG GNGG (SEQ ID NO:217)	1	1	3	31	NA
SP599.CD5. g36	GGGAGGTACAGTTGAGTT CNGG (SEQ ID NO:218)	1	1	3	37	NA
SP599.CD5. g45	CCCGTGGCTCCCCTGGGTC TNGG (SEQ ID NO:219)	1	1	3	43	rs2241002:0. 158
SP599.CD5. g16	CCAGGACAAGACCCAGGAC CNGG (SEQ ID NO:220)	1	1	3	57	NA
SP599.CD5. g17	CTCTGCAACAACCTCCAGT GNGG (SEQ ID NO:221)	1	1	3	67	NA
SP599.CD5. g52	TGTTGCAGAGGAAGTTCTC CNGG (SEQ ID NO:222)	1	1	3	236	NA
SP599.CD5. g56	CAGGTCCTGGGTCTTGTCT NGG (SEQ ID NO:223)	1	1	4	24	NA
SP599.CD5. g15	TGAGGCCCAAGGACAAGACC CNGG (SEQ ID NO:224)	1	1	4	30	NA
SP599.CD5. g61	CTGTGCCACCAGCTGCAGC CNGG (SEQ ID NO:225)	1	1	4	133	NA
SP599.CD5. g62	TGTGCCACCAGCTGCAGCC TNGG (SEQ ID NO:226)	1	1	4	139	NA
SP599.CD5. g19	CATCTGCCAGAGACTGAGG CNGG (SEQ ID NO:227)	1	1	4	1253	NA
SP599.CD5. g2	CTGCAGCTGGTGGCACAGT CNGG (SEQ ID NO:228)	1	1	5	17	NA
SP599.CD5. g51	CACACTGGAGGTTGTTGCA GNGG (SEQ ID NO:229)	1	1	5	28	NA

SP599.CD5.g3	CAGCTGGTGGCACAGTCTG GNGG (SEQ ID NO:230)	1	1	5	31	NA
SP599.CD5.g29	AGCAAAGGAGGGCAAGAA CTNGG (SEQ ID NO:231)	1	1	6	53	NA
SP599.CD5.g54	GAGGAAGTTCTCCAGGTCC TNGG (SEQ ID NO:232)	1	1	6	53	NA
SP599.CD5.g63	GCCACCAGCTGCAGCCTGG GNGG (SEQ ID NO:233)	1	1	6	287	NA
SP599.CD5.g20	GCAGGCAGAGCCCAAGACC CNGG (SEQ ID NO:234)	1	1	7	40	rs2241002:0.158
SP599.CD5.g21	CAGGCAGAGCCCAAGACCC ANGG (SEQ ID NO:235)	1	1	8	45	rs2241002:0.158
SP599.CD5.g1	TCCTCCCAGGCTGCAGCTG GNGG (SEQ ID NO:236)	1	1	8	140	NA
SP599.CD5.g47	GCTCTGCCTGCCTCAGTCTC NGG (SEQ ID NO:237)	1	1	26	412	NA
SP599.CD5.g27	CCTCCCTGGAGCATTGCTTC NGG (SEQ ID NO:238)	1	2	3	22	NA
SP599.CD5.g34	TTTCCTGAAGCAATGCTCC ANGG (SEQ ID NO:239)	1	2	4	32	NA
SP599.CD5.g46	CCGTGGCTCCCCTGGGTCTT NGG (SEQ ID NO:240)	1	2	5	37	rs2241002:0.158
SP599.CD5.g28	AAAATCAAGCCCCAGAAAA GNGG (SEQ ID NO:241)	1	2	5	60	NA
SP599.CD5.g18	GAAGCATCTGCCAGAGACT GNGG (SEQ ID NO:242)	1	2	7	98	NA
SP599.CD5.g24	CCAAGACCCAGGGGAGCCA CNGG (SEQ ID NO:243)	1	2	8	56	rs2241002:0.158
SP599.CD5.g22	AGGCAGAGCCCAAGACCCA GNGG (SEQ ID NO:244)	1	2	10	41	rs2241002:0.158
SP599.CD5.g23	CCCAAGACCCAGGGGAGCC ANGG (SEQ ID NO:245)	1	2	10	99	rs2241002:0.158

[00373] The gRNA sequences in Table 18, Table 19, and Table 20 were normalized (% Normalization to NHEJ) for gRNA activity via next generation sequencing (NGS). GFP was used as a control. Following sequencing analysis, the following gRNAs were recommended based on off-target profile: Exon 3: SP597.hCD5.g2 (76.5%), SP597.hCD5.g22 (36.3%), SP597.hCD5.g39 (16.0%), SP597.hCD5.g46. Exon4: SP598.hCD5.g7, SP598.hCD5.g10 (58.5%). Exon5: SP599.hCD5.g5 (51.0%), SP599.hCD5.g30, SP599.hCD5.g42, SP599.hCD5.g58 (41.0%)

[00374] Off target analysis of selected gRNA was performed for hCSF2 to determine the number of sites in human genome which are an exact match or contains up to 1 or 2 mismatches, which may include the target site. The results are listed in Table 21 for hCSF2.

Table 21. Guide RNA (gRNA) Off Target Analysis for hCSF2

Name	gRNA	long _0	long _1	long _2	long _3	short _0	SNP
MS1086.CSF2 .sp8	TACTCAGGTTTCAGGAGACGCNNGG (SEQ ID NO:)	1	1	1	10	11	NA
MS1086.CSF2 .sp10	TCAGGAGACGCCGGGCCTCCNNGG (SEQ ID NO:)	1	1	1	20	38	NA
MS1086.CSF2 .sp9	ACTCAGGTTTCAGGAGACGCCNNGG (SEQ ID NO:)	1	1	1	20	16	NA
MS1086.CSF2 .sp7	CAGTGTCTCTACTCAGGTTTCNNGG (SEQ ID NO:)	1	1	2	22	29	NA
MS1086.CSF2 .sp14	ATGCTCCAGGGCTGCGTGCNNGG (SEQ ID NO:)	1	1	2	42	34	rs2069622
MS1086.CSF2 .sp11	GAGACGCCGGGCCTCCTGGANGG (SEQ ID NO:)	1	1	2	26	146	NA
MS1086.CSF2 .sp6	CAGCAGCAGTGTCTCTACTCNNGG (SEQ ID NO:)	1	1	3	39	24	NA
MS1086.CSF2 .sp12	GATGGCATTACATGCTCCCNNGG (SEQ ID NO:)	1	1	3	28	59	NA
MS1086.CSF2 .sp2	GGAGCATGTGAATGCCATCCCNNGG (SEQ ID NO:)	1	1	3	26	48	NA
MS1086.CSF2 .sp5	TAGAGACACTGCTGCTGAGANGG (SEQ ID NO:)	1	1	3	56	168	NA
MS1086.CSF2 .sp3	GCATGTGAATGCCATCCAGGNGG (SEQ ID NO:)	1	1	3	41	56	NA
MS1086.CSF2 .sp13	ATGGCATTACATGCTCCCANNGG (SEQ ID NO:)	1	1	4	30	80	NA
MS1086.CSF2 .sp4	TGAATGCCATCCAGGAGGCCNNGG (SEQ ID NO:)	1	1	5	65	180	NA
MS1086.CSF2 .sp15	TGCTCCCAGGGCTGCGTGTCTNNGG (SEQ ID NO:)	1	1	6	57	29	rs2069622
MS1086.CSF2 .sp1	CAGCCCCAGCACGCAGCCCTNNGG (SEQ ID NO:)	1	1	15	146	41	rs2069622
MS1086.CSF2 .sp16	GCTCCCAGGGCTGCGTGCTGNGG (SEQ ID NO:)	1	2	9	85	37	rs2069622

[00375] The gRNA sequences in Table 21 were normalized (% Normalization to NHEJ) for gRNA activity via next generation sequencing (NGS). GFP was used as a control.

Following sequencing analysis, the following gRNAs were recommended based on off-target profile: MS1086.CSF2.sp8 (>15%) and MS1086.CSF2.sp10 (>15%).

[00376] Off target analysis of selected gRNA was performed for 2 exons of hCTLA4 (Exon 1 and Exon 2) to determine the number of sites in human genome which are an exact match or contains up to 1 or 2 mismatches, which may include the target site. The results are listed in Table 22 for Exon 1 and Table 23 for Exon 2 for hCTLA4.

Table 22. Guide RNA (gRNA) Off Target Analysis for hCTLA4 (Exon 1)

Name	gRNA	long_ 0	long_ 1	long_ 2	short_ 0	SNP
SP621.CTLA4.g2	CCTTGGATTTCAGCGGCACANGG (SEQ ID NO:)	1	1	1	5	NA
SP621.CTLA4.g12	CCTTGTGCCGCTGAAATC CANGG (SEQ ID NO:)	1	1	1	5	NA
SP621.CTLA4.g5	TGAACCTGGCTACCAGGACCNGG (SEQ ID NO:)	1	1	1	11	rs231775:0.452
SP621.CTLA4.g11	AGGGCCAGGTCCTGGTAGCCNGG (SEQ ID NO:)	1	1	3	16	rs231775:0.452
SP621.CTLA4.g4	CTCAGCTGAACCTGGCTAC CNGG (SEQ ID NO:)	1	1	3	17	rs231775:0.452
SP621.CTLA4.g8	AGAAAAAACAGGAGAGTGCANGG (SEQ ID NO:)	1	1	3	39	NA
SP621.CTLA4.g3	GCACAAGGCTCAGCTGAACCNGG (SEQ ID NO:)	1	1	4	29	NA
SP621.CTLA4.g1	TGGCTTGCCTTGGATTTCAGNGG (SEQ ID NO:)	1	1	6	33	NA
SP621.CTLA4.g9	AAACAGGAGAGTGCAGGGCCNGG (SEQ ID NO:)	1	1	6	69	NA
SP621.CTLA4.g10	GAGAGTGCAGGGCCAGGTCCNGG (SEQ ID NO:)	1	1	7	50	NA
SP621.CTLA4.g6	GGATGAAGAGAAGAAAAACNGG (SEQ ID NO:)	1	1	8	173	NA
SP621.CTLA4.g7	AAGAAAAAACAGGAGAGTGCNGG (SEQ ID NO:)	1	2	8	33	NA

Table 23. Guide RNA (gRNA) Off Target Analysis for hCTLA4 (Exon 2)

Name	gRNA	long_ 0	long_ 1	long_ 2	short_ 0	SNP
SP622.CTLA4.g9	CCGGGTGACAGTGCTTCGG CNGG (SEQ ID NO:)	1	1	1	2	NA
SP622.CTLA4.g33	ACACAAAGCTGGCGATGCC TNGG (SEQ ID NO:)	1	1	1	4	NA

SP622.CTLA4. g21	CCCTCAGTCCTTGGATAGTG NGG (SEQ ID NO:)	1	1	1	8	NA
SP622.CTLA4. g14	GTGCGGCAACCTACATGATG NGG (SEQ ID NO:)	1	1	1	9	NA
SP622.CTLA4. g12	CTGTGCGGCAACCTACATGA NGG (SEQ ID NO:)	1	1	1	13	NA
SP622.CTLA4. g2	GGCCCAGCCTGCTGTGGTA CNGG (SEQ ID NO:)	1	1	1	17	NA
SP622.CTLA4. g23	GTTCACTTGATTTCCTGCGN GG (SEQ ID NO:)	1	1	1	17	NA
SP622.CTLA4. g27	CAACTCATTCCCCATCATGTN GG (SEQ ID NO:)	1	1	1	18	NA
SP622.CTLA4. g28	CCGCACAGACTTCAGTCACC NGG (SEQ ID NO:)	1	1	1	20	NA
SP622.CTLA4. g13	TGTGCGGCAACCTACATGAT NGG (SEQ ID NO:)	1	1	1	30	NA
SP622.CTLA4. g20	CCTCACTATCCAAGGACTGA NGG (SEQ ID NO:)	1	1	1	30	NA
SP622.CTLA4. g31	CGGACCTCAGTGGCTTTGCC NGG (SEQ ID NO:)	1	1	1	34	NA
SP622.CTLA4. g22	GAGGTTCACTTGATTTCAC NGG (SEQ ID NO:)	1	1	1	40	NA
SP622.CTLA4. g11	CCAGGTGACTGAAGTCTGTG NGG (SEQ ID NO:)	1	1	1	45	NA
SP622.CTLA4. g24	ACTGGAGGTGCCCCGTGCAGA NGG (SEQ ID NO:)	1	1	2	15	NA
SP622.CTLA4. g18	CAAGTGAACCTCACTATCCA NGG (SEQ ID NO:)	1	1	2	16	NA
SP622.CTLA4. g3	GTGGTACTGGCCAGCAGCCG NGG (SEQ ID NO:)	1	1	2	29	NA
SP622.CTLA4. g8	AGGTCCGGGTGACAGTGCTT NGG (SEQ ID NO:)	1	1	2	29	NA
SP622.CTLA4. g17	ATCTGCACGGGCACCTCCAG NGG (SEQ ID NO:)	1	1	2	29	NA
SP622.CTLA4. g25	CCGTGCAGATGGAATCATCT NGG (SEQ ID NO:)	1	1	2	36	NA
SP622.CTLA4. g16	CTAGATGATTCCATCTGCAC NGG (SEQ ID NO:)	1	1	2	39	NA
SP622.CTLA4. g19	ACCTCACTATCCAAGGACTG NGG (SEQ ID NO:)	1	1	2	40	NA
SP622.CTLA4. g29	CCTGCCGAAGCACTGTCACC NGG (SEQ ID NO:)	1	1	2	47	NA
SP622.CTLA4. g36	TGGCCAGTACCACAGCAGGC NGG (SEQ ID NO:)	1	1	2	74	NA
SP622.CTLA4. g5	ATCTCCAGGCAAAGCCACTG NGG (SEQ ID NO:)	1	1	2	80	NA

SP622.CTLA4.g1	GCACGTGGCCCAGCCTGCTG NGG (SEQ ID NO:)	1	1	2	121	NA
SP622.CTLA4.g4	GTGTGTGAGTATGCATCTCC NGG (SEQ ID NO:)	1	1	3	8	NA
SP622.CTLA4.g30	CACTGTCACCCGACCTCAG NGG (SEQ ID NO:)	1	1	3	9	NA
SP622.CTLA4.g34	GCTGGCGATGCCTCGGCTGC NGG (SEQ ID NO:)	1	1	3	17	NA
SP622.CTLA4.g35	CTGCTGGCCAGTACCACAGC NGG (SEQ ID NO:)	1	1	3	22	NA
SP622.CTLA4.g7	AGGCAAAGCCACTGAGGTCC NGG (SEQ ID NO:)	1	1	3	40	NA
SP622.CTLA4.g26	GCAGATGGAATCATCTAGGA NGG (SEQ ID NO:)	1	1	4	20	NA
SP622.CTLA4.g15	CCTAGATGATTCCATCTGCA NGG (SEQ ID NO:)	1	1	4	40	NA
SP622.CTLA4.g37	GGCCAGTACCACAGCAGGCT NGG (SEQ ID NO:)	1	1	4	65	NA
SP622.CTLA4.g32	TGCATACTCACACAAAGC NGG (SEQ ID NO:)	1	1	7	71	NA
SP622.CTLA4.g10	GCTTCGGCAGGCTGACAGCC NGG (SEQ ID NO:)	1	1	8	58	NA
SP622.CTLA4.g6	CAGGCAAAGCCACTGAGGTC NGG (SEQ ID NO:)	1	1	11	30	NA

[00377] The gRNA sequences in Table 22 and Table 23 were normalized (% Normalization to NHEJ) for gRNA activity via next generation sequencing (NGS). GFP was used as a control. Following sequencing analysis, the following gRNAs were recommended based on off-target profile: Exon 1: SP621.hCTLA4.g2 (>15%) and SP621.hCTLA4.g12 (>15%). Exon 2: SP622.hCTLA4.g2 (>15%), SP622.hCTLA4.g9 (>15%), and SP622.hCTLA4.g33 (>15%).

[00378] Off target analysis of selected gRNA was performed for 2 exons of hPDCD1 (CF60 and CF61) to determine the number of sites in human genome which are an exact match or contains up to 1 or 2 mismatches, which may include the target site. The results are listed in Table 24 for Exon CF60 and Table 25 for Exon CF61.

Table 24. Guide RNA (gRNA) Off Target Analysis for hPDCD1 (Exon CF60)

Name	gRNA	long_ 0	long_ 1	long_ 2	short_ 0	SNP
CF60.PDCD1.g12	TGTAGCACCGCCCAGACGA CNGG (SEQ ID NO:)	1	1	1	1	NA

CF60.PDCD1.g3	GGCGCCCTGGCCAGTCGTC TNGG (SEQ ID NO:)	1	1	1	3	NA
CF60.PDCD1.g5	CGTCTGGGCGGTGCTACAAC NGG (SEQ ID NO:)	1	1	1	3	NA
CF60.PDCD1.g2	AGGCGCCCTGGCCAGTCGTC NGG (SEQ ID NO:)	1	1	1	5	NA
CF60.PDCD1.g13	CACCGCCCAGACGACTGGCC NGG (SEQ ID NO:)	1	1	1	5	NA
CF60.PDCD1.g14	ACCGCCCAGACGACTGGCCA NGG (SEQ ID NO:)	1	1	1	5	NA
CF60.PDCD1.g7	GGGCGGTGCTACAAC TGGG NGG (SEQ ID NO:)	1	1	1	7	NA
CF60.PDCD1.g6	GTCTGGGCGGTGCTACAAC TNGG (SEQ ID NO:)	1	1	1	9	NA
CF60.PDCD1.g16	CGACTGGCCAGGGCGCCTGT NGG (SEQ ID NO:)	1	1	1	15	NA
CF60.PDCD1.g8	CGGTGCTACAAC TGGGCTGG NGG (SEQ ID NO:)	1	1	1	33	NA
CF60.PDCD1.g11	TGGCGGCCAGGATGGTTCTT NGG (SEQ ID NO:)	1	1	1	33	NA
CF60.PDCD1.g15	ACGACTGGCCAGGGCGCCTGT NGG (SEQ ID NO:)	1	1	1	45	NA
CF60.PDCD1.g9	CTACAAC TGGGCTGGCGGCC NGG (SEQ ID NO:)	1	1	1	57	NA
CF60.PDCD1.g4	GCCCTGGCCAGTCGTCTGGG NGG (SEQ ID NO:)	1	1	2	2	NA
CF60.PDCD1.g1	TGCAGATCCCACAGGCGCCCC NGG (SEQ ID NO:)	1	1	2	23	NA
CF60.PDCD1.g10	AACTGGGCTGGCGGCCAGGA NGG (SEQ ID NO:)	1	1	3	17	NA

Table 25. Guide RNA (gRNA) Off Target Analysis for hPDCD1 (CF61)

Name	gRNA	long_0	long_1	long_2	short_0	SNP
CF61.PDCD1.g6	CGGAGAGCTTCGTGCTAAA CNGG (SEQ ID NO:)	1	1	1	1	NA
CF61.PDCD1.g14	GCGTGACTTCCACATGAGCG NGG (SEQ ID NO:)	1	1	1	2	NA
CF61.PDCD1.g17	ATGTGGAAGTCACGCCCGTT NGG (SEQ ID NO:)	1	1	1	2	NA
CF61.PDCD1.g2	GCCCTGCTCGTGGTGACCG ANG (SEQ ID NO:)	1	1	1	3	NA
CF61.PDCD1.g35	CACGAAGCTCTCCGATGTG TNGG (SEQ ID NO:)	1	1	1	3	NA
CF61.PDCD1.g	CCTGCTCGTGGTGACCGAAG	1	1	1	4	NA

4	NGG (SEQ ID NO:)					
CF61.PDCD1.g 20	TGACACGGAAGCGGCAGTCC NGG (SEQ ID NO:)	1	1	1	5	NA
CF61.PDCD1.g 40	CCCCTTCGGTCACCACGAGC NGG (SEQ ID NO:)	1	1	1	5	NA
CF61.PDCD1.g 8	CAGCAACCAGACGGACAAGC NGG (SEQ ID NO:)	1	1	1	6	NA
CF61.PDCD1.g 19	GCAGTTGTGTGACACGGAAG NGG (SEQ ID NO:)	1	1	1	6	NA
CF61.PDCD1.g 41	CCCTTCGGTCACCACGAGCA NGG (SEQ ID NO:)	1	1	1	6	NA
CF61.PDCD1.g 26	CCGGGCTGGCTGCGGTCCTC NGG (SEQ ID NO:)	1	1	1	8	NA
CF61.PDCD1.g 30	AGGCGGCCAGCTTGTCGTC NGG (SEQ ID NO:)	1	1	1	8	NA
CF61.PDCD1.g 31	CAGCTTGTCCTGCTGGTTGCN GG (SEQ ID NO:)	1	1	1	8	NA
CF61.PDCD1.g 43	CGGTCACCACGAGCAGGGCT NGG (SEQ ID NO:)	1	1	1	10	NA
CF61.PDCD1.g 13	GTGTCACACAACTGCCCAAC NGG (SEQ ID NO:)	1	1	1	13	NA
CF61.PDCD1.g 5	CTGCAGCTTCTCCAACACATN GG (SEQ ID NO:)	1	1	1	23	NA
CF61.PDCD1.g 9	CAAGCTGGCCGCCTTCCCCG NGG (SEQ ID NO:)	1	1	1	23	NA
CF61.PDCD1.g 12	CGTGTACACAACTGCCCAA NGG (SEQ ID NO:)	1	1	1	28	NA
CF61.PDCD1.g 18	CGTTGGGCAGTTGTGTGACA NGG (SEQ ID NO:)	1	1	1	32	NA
CF61.PDCD1.g 33	GCTTGTCCGTCTGGTTGCTGN GG (SEQ ID NO:)	1	1	1	41	NA
CF61.PDCD1.g 22	CGGAAGCGGCAGTCCTGGCC NGG (SEQ ID NO:)	1	1	1	61	NA
CF61.PDCD1.g 36	CGATGTGTTGGAGAAGCTGC NGG (SEQ ID NO:)	1	1	1	135	NA
CF61.PDCD1.g 16	CATGTGGAAGTCACGCCCCGT NGG (SEQ ID NO:)	1	1	2	2	NA
CF61.PDCD1.g 3	CCCTGCTCGTGGTGACCGAA NGG (SEQ ID NO:)	1	1	2	3	NA
CF61.PDCD1.g 27	CGGGCTGGCTGCGGTCCTCG NGG (SEQ ID NO:)	1	1	2	3	NA
CF61.PDCD1.g 32	AGCTTGTCCGTCTGGTTGCTN GG (SEQ ID NO:)	1	1	2	4	NA
CF61.PDCD1.g 39	GAAGGTGGCGTTGTCCCCTT NGG (SEQ ID NO:)	1	1	2	4	NA

CF61.PDCD1.g 15	ACTTCCACATGAGCGTGGTC NGG (SEQ ID NO:)	1	1	2	6	NA
CF61.PDCD1.g 25	GCCGGGCTGGCTGCGGTCCT NGG (SEQ ID NO:)	1	1	2	17	NA
CF61.PDCD1.g 42	TCGGTCACCACGAGCAGGGC NGG (SEQ ID NO:)	1	1	2	23	NA
CF61.PDCD1.g 34	TCTGGTTGCTGGGGCTCATGN GG (SEQ ID NO:)	1	1	2	31	NA
CF61.PDCD1.g 21	ACGGAAGCGGCAGTCCTGGC NGG (SEQ ID NO:)	1	1	2	41	NA
CF61.PDCD1.g 10	CCCGAGGACCGCAGCCAGCC NGG (SEQ ID NO:)	1	1	2	46	NA
CF61.PDCD1.g 28	CTGGCTGCGGTCCTCGGGGA NGG (SEQ ID NO:)	1	1	3	16	NA
CF61.PDCD1.g 7	CATGAGCCCCAGCAACCAGA NGG (SEQ ID NO:)	1	1	3	33	NA
CF61.PDCD1.g 24	AGTCCTGGCCGGGCTGGCTG NGG (SEQ ID NO:)	1	1	3	42	NA
CF61.PDCD1.g 55	GGGGGTTCCAGGGCCTGTCT NGG (SEQ ID NO:)	1	1	3	126	NA
CF61.PDCD1.g 44	GGTCACCACGAGCAGGGCTG NGG (SEQ ID NO:)	1	1	4	26	NA
CF61.PDCD1.g 29	GCTGCGGTCCTCGGGGAAGG NGG (SEQ ID NO:)	1	1	4	35	NA
CF61.PDCD1.g 11	GGACCGCAGCCAGCCCGGCC NGG (SEQ ID NO:)	1	1	4	47	NA
CF61.PDCD1.g 53	GAGAAGGTGGGGGGGTTCCA NGG (SEQ ID NO:)	1	1	5	8	NA
CF61.PDCD1.g 52	GGAGAAGGTGGGGGGGTTCC NGG (SEQ ID NO:)	1	1	5	15	NA
CF61.PDCD1.g 23	AGCGGCAGTCCTGGCCGGGC NGG (SEQ ID NO:)	1	1	5	39	NA
CF61.PDCD1.g 56	GGGGTTCCAGGGCCTGTCTG NGG (SEQ ID NO:)	1	1	5	97	NA
CF61.PDCD1.g 1	CTTCTCCCCAGCCCTGCTCGN GG (SEQ ID NO:)	1	1	6	22	NA
CF61.PDCD1.g 37	GTTGGAGAAGCTGCAGGTGA NGG (SEQ ID NO:)	1	1	6	88	NA
CF61.PDCD1.g 54	GGGGGGTTCCAGGGCCTGTC NGG (SEQ ID NO:)	1	1	6	1286	NA
CF61.PDCD1.g 38	GGAGAAGCTGCAGGTGAAGG NGG (SEQ ID NO:)	1	1	9	66	NA
CF61.PDCD1.g 45	CACGAGCAGGGCTGGGGAGA NGG (SEQ ID NO:)	1	1	10	448	NA
CF61.PDCD1.g 48	GCAGGGCTGGGGAGAAGGTG NGG (SEQ ID NO:)	1	1	21	125	NA

CF61.PDCD1.g 49	CAGGGCTGGGGAGAAGGTGG NGG (SEQ ID NO:)	1	1	29	214	NA
CF61.PDCD1.g 46	GAGCAGGGCTGGGGAGAAG GNGG (SEQ ID NO:)	1	1	30	202	NA
CF61.PDCD1.g 47	AGCAGGGCTGGGGAGAAGGT NGG (SEQ ID NO:)	1	2	11	136	NA
CF61.PDCD1.g 50	AGGGCTGGGGAGAAGGTGGG NGG (SEQ ID NO:)	1	2	31	179	NA
CF61.PDCD1.g 51	GGGCTGGGGAGAAGGTGGGG NGG (SEQ ID NO:)	1	2	49	130	NA

[00379] The gRNA sequences in Table 24 and Table 25 were normalized (% Normalization to NHEJ) for gRNA activity via next generation sequencing (NGS). GFP was used as a control. Following sequencing analysis, the following gRNAs were recommended based on off-target profile: CF60.PDCD1.g12 (65.6%), CF60.PDCD1.g3 (69.2%), CF61.PDCD1.g6, CF61.PDCD1.g2 (72.7%), and CF61.PDCD1.g35 (24.0%).

[00380] Off target analysis of selected gRNA was performed for 2 exons of hTIM3 (Exon 2 and Exon 3) to determine the number of sites in human genome which are an exact match or contains up to 1 or 2 mismatches, which may include the target site. The results are listed in Table 26 for Exon 2 and Table 27 for Exon 3.

Table 26. Guide RNA (gRNA) Off Target Analysis for hTIM3 (Exon 2)

Name	gRNA	long_ 0	long_ 1	long_ 2	short_ 0	SNP
SP619.TIM3.g 2	AGAAGTGGAATACAGAGCGG NGG (SEQ ID NO:)	1	1	1	2	NA
SP619.TIM3. g12	AATGTGGCAACGTGGTGCT CNGG (SEQ ID NO:)	1	1	1	3	NA
SP619.TIM3. g20	CTAAATGGGGATTTCGCAA NGG (SEQ ID NO:)	1	1	1	4	NA
SP619.TIM3.g 18	CATCCAGATACTGGCTAAATN GG (SEQ ID NO:)	1	1	1	8	NA
SP619.TIM3.g 41	CAGACGGGCACGAGGTTCCC NGG (SEQ ID NO:)	1	1	1	8	NA
SP619.TIM3. g49	GCGGCTGGGGTGTAGAAGC ANGG (SEQ ID NO:)	1	1	1	8	NA
SP619.TIM3.g 7	GAACCTCGTGCCCGTCTGCTN GG (SEQ ID NO:)	1	1	1	10	NA
SP619.TIM3.g 43	GACGGGCACGAGGTTCCCTG NGG (SEQ ID NO:)	1	1	1	10	NA
SP619.TIM3.g 35	ATCCCCATTAGCCAGTATCN GG (SEQ ID NO:)	1	1	1	11	NA

SP619.TIM3.g 3	GTGGAATACAGAGCGGAGGT NGG (SEQ ID NO:)	1	1	1	12	NA
SP619.TIM3.g 42	AGACGGGCACGAGGTTCCT NGG (SEQ ID NO:)	1	1	1	12	NA
SP619.TIM3.g 6	GGAACCTCGTGCCCGTCTGCN GG (SEQ ID NO:)	1	1	1	13	NA
SP619.TIM3.g 32	GAGTCACATTCTCTATGGTCN GG (SEQ ID NO:)	1	1	1	14	NA
SP619.TIM3.g 22	ATGTGACTCTAGCAGACAGTN GG (SEQ ID NO:)	1	1	1	16	NA
SP619.TIM3.g 27	TTTTCATCATTCAATTATGCCN GG (SEQ ID NO:)	1	1	1	16	NA
SP619.TIM3.g 21	AATGTGACTCTAGCAGACAG NGG (SEQ ID NO:)	1	1	1	17	NA
SP619.TIM3.g 19	ATCCAGATACTGGCTAAATGN GG (SEQ ID NO:)	1	1	1	18	NA
SP619.TIM3.g 24	TGCTGCCGGATCCAAATCCCN GG (SEQ ID NO:)	1	1	1	22	NA
SP619.TIM3.g 5	TCTACACCCAGCCGCCCAN GG (SEQ ID NO:)	1	1	1	30	NA
SP619.TIM3.g 30	TTATGCCTGGGATTTGGATCN GG (SEQ ID NO:)	1	1	1	35	NA
SP619.TIM3.g 51	CGCTCTGTATCCACTTCTGN GG (SEQ ID NO:)	1	1	1	83	NA
SP619.TIM3.g 47	GAGGTTCCCTGGGGCGGCTGN GG (SEQ ID NO:)	1	1	1	85	NA
SP619.TIM3.g 40	TGCCCCAGCAGACGGGCACG NGG (SEQ ID NO:)	1	1	2	5	NA
SP619.TIM3.g 23	ACAGTGGGATCTACTGCTGCN GG (SEQ ID NO:)	1	1	2	8	NA
SP619.TIM3.g 11	TGTGTTTGAATGTGGCAACGN GG (SEQ ID NO:)	1	1	2	9	NA
SP619.TIM3.g 25	TGAAAAATTAACTGAAGTN GG (SEQ ID NO:)	1	1	2	16	NA
SP619.TIM3.g 17	ACATCCAGATACTGGCTAAAN GG (SEQ ID NO:)	1	1	2	19	NA
SP619.TIM3.g 15	ATGAAAGGGATGTGAATTAT NGG (SEQ ID NO:)	1	1	2	22	NA
SP619.TIM3.g 13	TGGTGCTCAGGACTGATGAAN GG (SEQ ID NO:)	1	1	2	25	NA
SP619.TIM3.g 50	GGTGTAGAAGCAGGGCAGAT NGG (SEQ ID NO:)	1	1	2	36	NA
SP619.TIM3.g 36	ACGTTGCCACATTCAAACACN GG (SEQ ID NO:)	1	1	2	37	NA
SP619.TIM3.g 45	ACGAGGTTCCCTGGGGCGGC NGG (SEQ ID NO:)	1	1	2	40	NA

SP619.TIM3.g 10	GCCTGTCCTGTGTTTGAATGN GG (SEQ ID NO:)	1	1	2	47	NA
SP619.TIM3.g 9	GTGCCCCGTCTGCTGGGGCAAN GG (SEQ ID NO:)	1	1	2	58	NA
SP619.TIM3.g 8	AACCTCGTGCCCGTCTGCTGN GG (SEQ ID NO:)	1	1	3	15	NA
SP619.TIM3.g 48	GGCGGCTGGGGTGTAGAAGC NGG (SEQ ID NO:)	1	1	3	15	NA
SP619.TIM3.g 33	AGTCACATTCTCTATGGTCAN GG (SEQ ID NO:)	1	1	3	19	NA
SP619.TIM3.g 26	CTGGTTTGATGACCAACTTCN GG (SEQ ID NO:)	1	1	3	21	NA
SP619.TIM3.g 29	CATTCATTATGCCTGGGATTN GG (SEQ ID NO:)	1	1	3	24	NA
SP619.TIM3.g 31	TGCTAGAGTCACATTCTCTAN GG (SEQ ID NO:)	1	1	3	49	NA
SP619.TIM3.g 44	GGGCACGAGGTTCCCTGGGG NGG (SEQ ID NO:)	1	1	3	53	NA
SP619.TIM3.g 38	GGCTCCTTTGCCCCAGCAGAN GG (SEQ ID NO:)	1	1	3	58	NA
SP619.TIM3.g 16	ATTATTGGACATCCAGATACN GG (SEQ ID NO:)	1	1	3	106	NA
SP619.TIM3.g 28	TTTCATCATTATTATGCCTN GG (SEQ ID NO:)	1	1	4	23	NA
SP619.TIM3.g 4	TTCTACACCCCAGCCGCCCN GG (SEQ ID NO:)	1	1	4	29	NA
SP619.TIM3.g 34	TCAGGGACACATCTCCTTTGN GG (SEQ ID NO:)	1	1	4	41	NA
SP619.TIM3.g 39	GCTCCTTTGCCCCAGCAGACN GG (SEQ ID NO:)	1	1	4	42	NA
SP619.TIM3.g 1	CTCAGAAGTGGAATACAGAG NGG (SEQ ID NO:)	1	1	5	35	NA
SP619.TIM3.g 46	CGAGGTTCCCTGGGGCGGCTN GG (SEQ ID NO:)	1	2	2	18	NA
SP619.TIM3.g 37	GCCACATTCAAACACAGGAC NGG (SEQ ID NO:)	1	2	2	25	NA
SP619.TIM3.g 14	GGTGCTCAGGACTGATGAAA NGG (SEQ ID NO:)	1	2	3	28	NA

Table 27. Guide RNA (gRNA) Off Target Analysis for hTIM3 (Exon 3)

Name	gRNA	long_ 0	long_ 1	long_ 2	short_ 0	SNP
SP620.TIM3. g1	AGGTCACCCCTGCACCGAC TNGG (SEQ ID NO:)	1	1	1	4	rs1036199: 0.13

SP620.TIM3.g11	CTCTCTGCCGAGTCGGTGC ANGG (SEQ ID NO:)	1	1	1	4	rs1036199: 0.13
SP620.TIM3.g10	TCTCTCTGCCGAGTCGGTGC CNGG (SEQ ID NO:)	1	1	1	6	rs1036199: 0.13
SP620.TIM3.g5	CCAAGGATGCTTACCACC AGNGG (SEQ ID NO:)	1	1	1	8	NA
SP620.TIM3.g12	TCTCTGCCGAGTCGGTGCA GNGG (SEQ ID NO:)	1	1	1	9	rs1036199: 0.13
SP620.TIM3.g7	CCCCTGGTGGTAAGCATC CTNGG (SEQ ID NO:)	1	1	1	10	NA
SP620.TIM3.g4	TCCAAGGATGCTTACCACC ANGG (SEQ ID NO:)	1	1	1	16	NA
SP620.TIM3.g8	GGTGGTAAGCATCCTTGGA ANGG (SEQ ID NO:)	1	1	1	20	NA
SP620.TIM3.g9	GTGAAGTCTCTCTGCCGAG TNGG (SEQ ID NO:)	1	1	2	6	rs1036199: 0.13
SP620.TIM3.g6	ATGCTTACCACAGGGGAC ANGG (SEQ ID NO:)	1	1	2	34	NA
SP620.TIM3.g3	TTCCAAGGATGCTTACCAC CNGG (SEQ ID NO:)	1	1	2	36	NA
SP620.TIM3.g13	AGTCGGTGCAGGGGTGACC TNGG (SEQ ID NO:)	1	1	2	45	NA
SP620.TIM3.g2	ACTTCACTGCAGCCTTTCC ANGG (SEQ ID NO:)	1	1	4	38	NA

[00381] The gRNA sequences in Table 26 and Table 27 were normalized (% Normalization to NHEJ) for gRNA activity via next generation sequencing (NGS). GFP was used as a control. Following sequencing analysis, the following gRNAs were recommended based on off-target profile: Exon 2: SP619.hTIM3.g12 (45.0%), SP619.hTIM3.g20 (60.9%), and SP619.hTIM3.g49 (45.4%). Exon 3: SP620.hTIM3.g5 (58.0%) and SP620.hTIM3.g7 (2.9%).

[00382] The methods disclosed above can be varied appropriately by those skilled in the art to make and confirm activity of other mono, dual, and tandem CAR-T cells disclosed herein.

[00383] Although the present invention has been described with reference to specific details of certain embodiments thereof in the above examples, it will be understood that modification and variation are encompassed within the spirit and scope of the invention.