COMPOSITIONS AND METHODS FOR THE TREATMENT OF CANCER USING A TET2 ENGINEERED T CELL THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/841,748, filed on May 1, 2019, and U.S. Provisional Application No. 62/841,753, filed on May 1, 2019, the content of which are incorporated in their entirety, and to which priority is claimed.

SEQUENCE LISTINGS

The instant application contains a Sequence Listings which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on April 30, 2020, is named 087520_0146_SL.txt and is 13,867 bytes in size.

BACKGROUND OF THE INVENTION

Tet methylcytosine dioxygenase 2 (TET2) is a gene that encodes the protein methylcytosine dioxygenase that catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine. The encoded protein appears to be involved in myelopoiesis, and defects in this gene have been associated with several myeloproliferative disorders. However, the exact function of the protein is unknown.

The TET2 gene is found on the cytogenetic location 4q24 (the long (q) arm of chromosome 4 at position 24). Other names for the TET2 gene include FLJ20032, KIAA1546, MGC125715, probably methylcytosine dioxygenase TET2, probably methylcytosine dioxygenase TET2 isoform a, probably methylcytosine dioxygenase TET2 isoform b, tet oncogene family member 2, and TET2_HUMAN.

Based on the function of similar proteins, researchers believe the TET2 protein is involved in regulating the process of transcription, which is the first step in protein production. Although this protein is found throughout the body, it may play a particularly important role in the production of blood cells from hematopoietic stem cells. These stem cells are located within the bone marrow and have the potential to develop into red blood cells, white blood cells, and platelets. The TET2 protein appears to act as a tumor suppressor, preventing cells from growing and dividing in an uncontrolled way. For example, somatic TET2 mutations are frequently observed in myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN),

MDS/MPN overlap syndromes including chronic myelomonocytic leukemia (CMML), acute myeloid leukemias (AML) and secondary AML (sAML).

It has also been suggested that TET2 mutations have prognostic value in cytogenetically normal acute myeloid leukemia (CN-AML). "Nonsense" and "frameshift" mutations in this gene are associated with poor outcome on standard therapies in this otherwise favorable-risk patient subset. Loss of function TET2 mutations may also have a possible causal role in atherogenesis.

TET2 may also be a candidate for active DNA demethylation, the catalytic removal of the methyl group added to the fifth carbon on the cytosine base

Some gene mutations are acquired during a person's lifetime and are present only in certain cells. These changes, which are called somatic mutations, are not inherited. Somatic mutations in the TET2 gene have been identified in a small number of people with essential thrombocythemia, which is a condition characterized by high numbers of platelets in the blood. Platelets are the blood cells involved in blood clotting. TET2 gene mutations alter the TET2 protein in different ways; however, all of them appear to result in a nonfunctional protein. The role these mutations play in the development of essential thrombocythemia is unknown.

Somatic mutations in the TET2 gene are associated with polycythemia vera, a disorder characterized by uncontrolled blood cell production. These mutations are thought to result in a nonfunctional protein. Mutations in this gene have been found in approximately 16 percent of people with polycythemia vera. It is unclear what role these mutations play in the development of polycythemia vera.

Somatic mutations in the TET2 gene are associated with primary myelofibrosis. This condition is characterized by scar tissue (fibrosis) in the bone marrow, the tissue that produces blood cells. It is unclear what role the TET2 gene mutations play in the development of primary myelofibrosis.

Somatic TET2 gene mutations are also associated with certain types of cancer of blood-forming cells (leukemia) and a disease of the blood and bone marrow called myelodysplastic syndrome. These mutations are thought to result in a nonfunctional TET2 protein. A loss of TET2 protein in hematopoietic stem cells may lead to uncontrolled growth and division of these cells. Researchers are working to determine exactly what role TET2 gene mutations play in the development of bone marrow disorders.

TET2 mutations have also been shown to be associated with myeloid neoplasia.

TET2-disruption in CAR T cells have also been shown to be associated with cancer remission in subjects and long-term CAR T cell survival post-infusion (Fraietta et al., 2018, Nature, 558(7709), 307–312). Specifically, TET2-disrupted CAR T cells modified by viral

editing methods were identified in a patient with remission and accounted for nearly all edited cells two (2) months post profusion.

Cell therapies designed to express a NeoTCR with a concurrent knockout or knockdown of the TET2 gene have not been previously pursued. Furthermore, there is an unmet need to develop cell therapies that have long persistence times and/or ideally full engraftment as memory stem cells in order to allow for cell therapies to be single or infrequently dosed therapies.

However, given the endogenous suppression of TET2 and its inherent anti-tumor activity, therapies that modulate the expression of TET2 on T cells need to be engineered precisely in order to prevent oncogenic phenotypes of such therapies.

Accordingly, there is an unmet need of a cell therapy that can specifically target tumors through NeoTCR engagement and that persist for extended periods of time within the patient post infusion. Such therapies are that include a knockout or knockdown TET2 that is specific to T cells are described herein.

SUMMARY OF THE INVENTION

The present inventions described herein provide for cells that were engineered to modulate the endogenous expression of the TET2 gene.

In certain embodiments, the presently disclosed subject matter provides a cell, comprising an exogenous T cell receptor (TCR), and a gene disruption of a TET2 locus. In certain embodiments, the gene disruption comprises a substitution, a deletion, an insertion, or any combination thereof. In certain embodiments, the gene disruption comprises a missense mutation, a nonsense mutation, a non-frameshift deletion, a frameshift deletion, a non-frameshift insertion, a frameshift insertion, or any combination thereof. In certain embodiments, the gene disruption of the TET2 locus results in a non-functional TET2 protein. In certain embodiments, the gene disruption of the TET2 locus results in knockout of the TET2 gene expression.

In certain embodiments, the cell comprises a gRNA and a Cas9 nuclease. In certain embodiments, the gRNA comprises a nucleotide sequence set forth in SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, or SEQ ID NO.5.

In certain embodiments, the gene disruption of the TET2 locus enhances cell persistence. In certain embodiments, the gene disruption of the TET2 locus enhances memory cell differentiation.

In certain embodiments, the cell is a primary cell. In certain embodiments, the cell is a patient-derived cell. In certain embodiments, the cell is a lymphocyte. In certain

embodiments, the cell is a T cell. In certain embodiments, the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+. In certain embodiments, the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+. In certain embodiments, the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

In certain embodiments, the exogenous TCR is a patient derived TCR. In certain embodiments, the exogenous TCR comprises a signal sequence, a first and second 2A sequence, and a TCR polypeptide sequence. In certain embodiments, the exogenous TCR recognizes a cancer antigen. In certain embodiments, the cancer antigen is a neoantigen. In certain embodiments, the cancer antigen is a patient specific antigen.

In certain embodiments, the presently disclosed subject matter provides a cell modified by a process, the process comprising introducing into a cell a homologous recombination (HR) template nucleic acid sequence, recombining the HR template nucleic acid into an endogenous locus of the cell, and disrupting a TET2 locus of the cell. In certain embodiments, the HR template comprises first and second homology arms homologous to first and second target nucleic acid sequences, and a TCR gene sequence positioned between the first and second homology arms. In certain embodiments, the HR template comprises a first 2A-coding sequence positioned upstream of the TCR gene sequence and a second 2A-coding sequence positioned downstream of the TCR gene sequence, wherein the first and second 2A-coding sequences code for the same amino acid sequence that are codon-diverged relative to each other. In certain embodiments, the 2A-coding sequence is a P2A-coding sequence. In certain embodiments, a sequence coding for the amino acid sequence Gly Ser Gly is positioned immediately upstream of the 2A-coding sequences. In certain embodiments, the HR template comprises a sequence coding for a Furin cleavage site positioned upstream of the second 2A-coding sequence.

In certain embodiments, the first and second homology arms are each from about 300 bases to about 2,000 bases in length. In certain embodiments, the first and second homology arms are each from about 600 bases to about 2,000 bases in length. In certain embodiments, the HR template further comprises a signal sequence positioned between the first 2A-coding sequence and the TCR gene sequence. In certain embodiments, the HR template comprises a second TCR sequence positioned between the second 2A-coding sequence and the second homology arm.

In certain embodiments, the HR template comprises a first signal sequence positioned between the first 2A-coding sequence and the first TCR gene sequence, and a second signal sequence positioned between the second 2A-coding sequence and the second TCR gene sequence, wherein the first and the second signal sequences encode for the same amino acid

sequence and are codon diverged relative to each other. In certain embodiments, the signal sequence is a human growth hormone signal sequence.

In certain embodiments, the HR template is non-viral. In certain embodiments, the HR template is a circular DNA. In certain embodiments, the HR template is a linear DNA. In certain embodiments, the introducing occurs via electroporation.

In certain embodiments, the recombining comprises cleavage of the endogenous locus by a nuclease, and recombination of the HR template nucleic acid sequence into the endogenous locus by homology directed repair. In certain embodiments, the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof. In certain embodiments, the nuclease comprises a gRNA.

In certain embodiments, the disrupting comprises introducing a substitution, a deletion, an insertion, or any combination thereof. In certain embodiments, the disrupting comprises introducing a missense mutation, a nonsense mutation, a non-frameshift deletion, a frameshift deletion, a non-frameshift insertion, a frameshift insertion, or any combination thereof. In certain embodiments, the disrupting results in a non-functional TET2 protein. In certain embodiments, the disrupting results in knockout of the TET2 gene expression.

In certain embodiments, the disrupting comprises cleavage of the TET2 locus by a nuclease. In certain embodiments, the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof. In certain embodiments, the nuclease comprises a gRNA. In certain embodiments, the gRNA comprises a sequence set forth in SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, or SEQ ID NO.5. In certain embodiments, the nuclease is expressed by a vector. In certain embodiments, the gRNA is expressed by a vector. In certain embodiments, the vector is a viral vector. In certain embodiments, the vector is a non-viral vector.

In certain embodiments, the disrupting of the TET2 locus enhances cell persistence. In certain embodiments, the disrupting of the TET2 locus enhances memory cell differentiation.

In certain embodiments, the cell is a primary cell. In certain embodiments, the cell is a patient-derived cell. In certain embodiments, the cell is a lymphocyte. In certain embodiments, the cell is a T cell. In certain embodiments, the T cell is a patient-derived cell. In certain embodiments, the cell is a young T cell. In certain embodiments, the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+. In certain embodiments, the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+. In certain embodiments, the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

In certain embodiments, the endogenous locus is within an endogenous TCR gene. In certain embodiments, the TCR gene sequence encodes for a TCR that recognizes a tumor

antigen. In certain embodiments, the tumor antigen is a neoantigen. In certain embodiments, the tumor antigen is a patient specific neoantigen. In certain embodiments, the TCR gene sequence is a patient specific TCR gene sequence.

In certain embodiments, the process further comprises culturing the cell. In certain embodiments, the culturing is conducted in the presence of at least one cytokine. In certain embodiments, the culturing is conducted in the presence of IL2, IL7, IL15, or any combination thereof. In certain embodiments, the culturing is conducted in the presence of IL7 and IL15.

In certain embodiments, the presently disclosed subject matter provides a method of modifying a cell, the method comprising introducing into a cell a homologous recombination (HR) template nucleic acid sequence, recombining the HR template nucleic acid into an endogenous locus of the cell, and disrupting a TET2 locus of the cell. In certain embodiments, the HR template comprises first and second homology arms homologous to first and second target nucleic acid sequences, and a TCR gene sequence positioned between the first and second homology arms. In certain embodiments, the HR template comprises a first 2A-coding sequence positioned upstream of the TCR gene sequence and a second 2A-coding sequence positioned downstream of the TCR gene sequence, wherein the first and second 2A-coding sequences code for the same amino acid sequence that are codon-diverged relative to each other. In certain embodiments, the 2A-coding sequence is a P2A-coding sequence. In certain embodiments, a sequence coding for the amino acid sequence Gly Ser Gly is positioned immediately upstream of the 2A-coding sequences. In certain embodiments, the HR template comprises a sequence coding for a Furin cleavage site positioned upstream of the second 2A-coding sequence.

In certain embodiments, the first and second homology arms are each from about 300 bases to about 2,000 bases in length. In certain embodiments, the first and second homology arms are each from about 600 bases to about 2,000 bases in length. In certain embodiments, the HR template further comprises a signal sequence positioned between the first 2A-coding sequence and the TCR gene sequence. In certain embodiments, the HR template comprises a second TCR sequence positioned between the second 2A-coding sequence and the second homology arm.

In certain embodiments, the HR template comprises a first signal sequence positioned between the first 2A-coding sequence and the first TCR gene sequence, and a second signal sequence positioned between the second 2A-coding sequence and the second TCR gene sequence, wherein the first and the second signal sequences encode for the same amino acid sequence and are codon diverged relative to each other. In certain embodiments, the signal sequence is a human growth hormone signal sequence.

In certain embodiments, the HR template is non-viral. In certain embodiments, the HR template is a circular DNA. In certain embodiments, the HR template is a linear DNA. In certain embodiments, the introducing occurs via electroporation.

In certain embodiments, the recombining comprises cleavage of the endogenous locus by a nuclease, and recombination of the HR template nucleic acid sequence into the endogenous locus by homology directed repair. In certain embodiments, the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof. In certain embodiments, the nuclease comprises a gRNA.

In certain embodiments, the disrupting comprises introducing a substitution, a deletion, an insertion, or any combination thereof. In certain embodiments, the disrupting comprises introducing a missense mutation, a nonsense mutation, a non-frameshift deletion, a frameshift deletion, a non-frameshift insertion, a frameshift insertion, or any combination thereof. In certain embodiments, the disrupting results in a non-functional TET2 protein. In certain embodiments, the disrupting results in knockout of the TET2 gene expression.

In certain embodiments, the disrupting comprises cleavage of the TET2 locus by a nuclease. In certain embodiments, the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof. In certain embodiments, the nuclease comprises a gRNA. In certain embodiments, the gRNA comprises a sequence set forth in SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, or SEQ ID NO.5. In certain embodiments, the nuclease is expressed by a vector. In certain embodiments, the gRNA is expressed by a vector. In certain embodiments, the vector is a viral vector. In certain embodiments, the vector is a non-viral vector.

In certain embodiments, the disrupting of the TET2 locus enhances cell persistence. In certain embodiments, the disrupting of the TET2 locus enhances memory cell differentiation.

In certain embodiments, wherein the cell is a primary cell. In certain embodiments, the cell is a patient-derived cell. In certain embodiments, the cell is a lymphocyte. In certain embodiments, the cell is a T cell. In certain embodiments, the T cell is a patient-derived cell. In certain embodiments, the cell is a young T cell. In certain embodiments, the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+. In certain embodiments, the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+. In certain embodiments, the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

In certain embodiments, the endogenous locus is within an endogenous TCR gene. In certain embodiments, the TCR gene sequence encodes for a TCR that recognizes a tumor antigen. In certain embodiments, the tumor antigen is a neoantigen. In certain embodiments,

the tumor antigen is a patient specific neoantigen. In certain embodiments, the TCR gene sequence is a patient specific TCR gene sequence.

In certain embodiments, the method further comprises culturing the cell. In certain embodiments, the culturing is conducted in the presence of at least one cytokine. In certain embodiments, the culturing is conducted in the presence of IL2, IL7, IL15, or any combination thereof. In certain embodiments, the culturing is conducted in the presence of IL7 and IL15.

In certain embodiments, the presently disclosed subject matter provides a method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell disclosed herein. In certain embodiments, prior to administering the therapeutically effective amount of cells provided herein, a non-myeloablative lymphodepletion regimen is administered to the subject. In certain embodiments, the cancer is a solid tumor. In certain embodiments, the cancer is liquid tumor. In certain embodiments, the solid tumor is selected from the group consisting of melanoma, thoracic cancer, lung cancer, ovarian cancer, breast cancer, pancreatic cancer, head and neck cancer, prostate cancer, gynecological cancer, central nervous system cancer, cutaneous cancer, HPV+ cancer, esophageal cancer, thyroid cancer, gastric cancer, hepatocellular cancer, cholangiocarcinomas, renal cell cancers, testicular cancer, sarcomas, and colorectal cancer. In certain embodiments, the liquid tumor is selected from the group consisting of follicular lymphoma, leukemia, and multiple myeloma.

In certain embodiments, the presently disclosed subject matter provides a composition comprising an effective amount of a cell disclosed herein. In certain embodiments, the composition is a pharmaceutical composition that further comprises a pharmaceutically acceptable excipient. In certain embodiments, the composition is administered to a patient in need thereof for the treatment of cancer. In certain embodiments, the composition comprises a cryopreservation agent. In certain embodiments, the composition comprises serum albumin. In certain embodiments, the composition comprises Plasma-Lyte A, HSA, and CryoStor CS10.

In certain embodiments, the presently disclosed subject matter provides a kit comprising a cell, reagents for performing a method, or a composition disclosed herein. In certain embodiments, the kit further comprises written instructions for treating a cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Figure 1 provides a high-level diagram of the knock-out and knock-in at the endogenous TCR locus and the knock-out of the TET2 gene accomplished by the gene editing technology described herein.

Figures 2A-2C. **Figures 2A-2C** show an example of a NeoE TCR cassette and gene editing methods that can be used to make NeoTCR Products. **Figure 2A** shows a schematic representing the general targeting strategy used for integrating neoantigen-specific TCR constructs (neoTCRs) into the TCRα locus. **Figures 2B** and **2C** show a neoantigen-specific TCR construct design used for integrating a NeoTCR into the TCRα locus wherein the cassette is shown with signal sequences ("SS"), protease cleavage sites ("P"), and 2A peptides ("2A"). **Figure 2B** shows a target TCRα locus (endogenous TRAC, top panel) and its CRISPR Cas9 target site (horizontal stripes, cleavage site designated by the arrow), and the circular plasmid HR template (bottom panel) with the polynucleotide encoding the neoTCR, which is located between left and right homology arms ("LHA" and "RHA" respectively) prior to integration. **Figure 2C** shows the integrated neoTCR in the TCRα locus (top panel), the transcribed and spliced neoTCR mRNA (middle panel), and translation and processing of the expressed neoTCR (bottom panel).

Figure 3. **Figure 3** shows the results of an In-Out PCR confirming precise target integration of the NeoE TCR cassette. Agarose gels show the results of a PCR using primers specific to the NeoE TCR cassette and relative site generate products of the expected size only for cells treated with both nuclease and DNA template (knock-out-knock-in (KOKI) and knock-out-knock-in-knock-out (KOKIKO)), demonstrating site-specific and precise integration.

Figures 4A and 4B. **Figure 4A** shows results from a FACS experiment showing that the endogenous TCR has reduced signal and that there is a strong NeoE TCR signal in cells that were electroporated with the NeoE TCR cassette. **Figure 4B** shows the results from a series of multiple transfection experiments with the NeoE TCR cassette showing a high degree of reproducibility between experiments.

Figures 5A-5E. **Figures 5A-5E** depict five (5) exemplary RNP knockout strategies for knocking out the TET2 gene. **Figures 5A** and **5E** depict sgRNAs that target the negative strand. **Figures 5B**, **5C**, and **5D** depict sgRNAs that target the positive strand.

Figures 6A and **6B**. **Figures 6A** and **6B** show agarose gels of amplified DNA extracted from lysed TET2 gRNA-Cas9 RNP edited CD4 and CD8 T cells. The primers used for the amplification are provided in **Table 3**. **Figure 6A** shows PCR amplicons that were uncut. **Figure 6B** shows PCR amplicons that were cut by incubation with T7 endonuclease I (NEB). The lanes of both **Figure 6A** and **Figure 6B** are: 1. Marker, 2. WT (TET2_Fwd1, TET2_Rev1), 3. WT (TET2_Fwd2, TET2_Rev2), 4. gRNA1 (TET2_Fwd1, TET2_Rev1), 5. gRNA2 (TET2_Fwd2, TET2_Rev2), 6. gRNA3 (TET2_Fwd2, TET2_Rev2), 7. gRNA4 (TET2_Fwd1, TET2_Rev1), 8. gRNA5 (TET2_Fwd1, TET2_Rev1).

Figures 7A-7E. **Figures 7A-7E** show that coverage of the PCR amplicons was robust for all amplicons surrounding the guide site. **Figure 7A** shows results of gRNA 1 (SEQ ID NO:1). **Figure 7B** shows results of gRNA 4 (SEQ ID NO:4). **Figure 7C** shows results ofgRNA 5 (SEQ ID NO:5). **Figure 7D** shows results of gRNA 3 (SEQ ID NO:3). **Figure 7E** shows results of gRNA 2 (SEQ ID NO:2).

Figures 8A and **8B**. **Figures 8A** and **8B** show that sgRNA 3 (SEQ ID NO:3) provides the best disruption of TET2 and does not interfere with the neoTCR gene editing and the insertion of a neoTCR. **Figure 8A** shows the indel characterization and **Figure 8B** shows the gene editing (insertion of the neoTCR) rates. The gene editing insertion of the neoTCR as provided in **Figure 8B** is measured by dextramer binding to the gene edited cells. Because the dextramer specifically binds to the neoTCR, binding is a direct correlation to neoTCR expression.

Figure 9. **Figure 9** shows that there is no appreciable reduction in 5-hmC in the absence of TET2 (i.e., in TET2 Products) with or without 1.5h stimulation with cognate comPACT plate coating. Anti-5-hmC antibody staining of samples resting or stimulated for 1.5h with cognate comPACT (EXP19001222) was detected using flow cytometry.

Figure 10. **Figure 10** shows the TET2 Products had the equivalent expression of the TCF7 and TBET transcription factors as NeoTCR Products (i.e., cells that did not have a TET2 deletion). The cells used in this flow cytometry experiment were resting edited cells.

Figures 11A and **11B**. **Figures 11A** and **11B** show that TET2 Disruption by gRNA3 results in reduced terminal effector differentiation as evidenced by percent IFNγ+ cells (**Figure 11A**) and percent CD107a+ cells (**Figure 11B**).

Figures 12A and **12B**. As shown in **Figures 12A** and **12B**, there are no differences between the tumor cell killing ability of TET2 Products and NeoTCR Products kept in culture for 14-15 days. IncuCyte assays with PACT035-TCR089 neoTCR edited T cells with and without TET2 gRNA3 were co-cultured with SW620 COX6C-R20Q tumor cells (**Figure 12A**) or SW620 tumor cells lacking the COX6C-R20Q mutation (**Figure 12B**). The R20Q mutation directly correlates with TCR089 such that TCR089 is designed to kill cells that express the R20Q mutation.

Figures 13A and **13B**. **Figures 13A** and **13B** show that TET2 Products maintain killing activity longer than NeoTCR Products (cells without a knockout of the TET2 gene). NeoTCR Products (PACT035-TCR089 as shown in the figures) and TET2 Products (PACT035-TCR089 TET2 gRNA 3 as shown in the figures) were kept in culture for 30 days before being tested. IncuCyte assays with NeoTCR Products (PACT035-TCR089) and TET2 Products

(PACT035-TCR089 TET2 gRNA 3) were co-cultured with SW620 COX6C-R20Q tumor cells (**Figure 13A**) or SW620 tumor cells lacking the COX6C-R20Q mutation (**Figure 13B**).

Figures 14A and **14B**. **Figures 14A** and **14B** shows that TET2 disruption enhances CD8 memory cell differentiation. Geometric mean fluorescence intensity of surface markers associated with memory and exhaustion among CD8+ dextramer+ neoTCR edited T cells with or without TET2 gRNA3 stimulated with cognate comPACT for 7 days is shown for NeoTCR Products (PACT035-TCR089 as shown in the figures) and TET2 Products (PACT035-TCR089 TET2 gRNA 3 as shown in the figures). The CCR7 and CD27 plots shown in the **Figures 14A** and **14B** show that TET2 distruption enhances CD8 memory cell differentiation. Specifically, CCR7 and CD27 are highly expressed on central memory CD8 T cells. The fact that the TET2 Cells increase the expression (higher MFI) compared to NeoTCR Cells is represents this effect.

Figures 15A-15D. **Figures 15A-15D** show that expression of TOX (**Figures 15B** and **15D**) and TCF7 (**Figures 15A** and **15C**) were unchanged between NeoTCR Products (PACT035-TCR089 as shown in the figures) and TET2 Products (PACT035-TCR089 TET2 gRNA 3 as shown in the figures). Frequency of TCF7+ (**Figures 15A** and **15C**) and TOX+ (**Figures 15B** and **15D**) CD8+ dextramer+ T cells in response to comPACT stimulation for 7 days (**Figures 15A** and **15B**) or stimulation for 7 days followed by re-stimulation on fresh comPACT coated plates for 2 additional days (**Figures 15C** and **15D**).

DETAILED DESCRIPTION

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art. The following references provide one of skill with a general definition of many of the terms used in the presently disclosed subject matter: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments. The terms “comprises” and “comprising” are intended to have the broad meaning ascribed to them in U.S. Patent Law and can mean “includes”, “including” and the like.

As used herein, the term “about” or “approximately” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, “about” can mean within 3 or more than 3 standard deviations, per the practice in the art. Alternatively, “about” can mean a range of up to 20%, e.g., up to 10%, up to 5%, or up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, e.g., within 5-fold or within 2-fold, of a value.

The terms “Cancer” and “Tumor” are used interchangeably herein. As used herein, the terms “Cancer” or “Tumor” refer to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms are further used to refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Cancer can affect a variety of cell types, tissues, or organs, including but not limited to an organ selected from the group consisting of bladder, bone, brain, breast, cartilage, glia, esophagus, fallopian tube, gallbladder, heart, intestines, kidney, liver, lung, lymph node, nervous tissue, ovaries, pancreas, prostate, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, urogenital tract, ureter, urethra, uterus, and vagina, or a tissue or cell type thereof. Cancer includes cancers, such as sarcomas, carcinomas, or plasmacytomas (malignant tumor of the plasma cells). Examples of cancer include, but are not limited to, those described herein. The terms “Cancer” or “Tumor” and “Proliferative Disorder” are not mutually exclusive as used herein.

“Dextramer” as used herein means a multimerized neoepitope-HLA complex that specifically binds to its cognate NeoTCR.

As used herein, the terms "neoantigen", “neoepitope” or “neoE” refer to a newly formed antigenic determinant that arises, e.g., from a somatic mutation(s) and is recognized as "non-self.” A mutation giving rise to a "neoantigen", “neoepitope” or “neoE” can include a frameshift or non-frameshift indel, missense or nonsense substitution, splice site alteration (e.g., alternatively spliced transcripts), genomic rearrangement or gene fusion, any genomic or expression alterations, or any post-translational modifications.

“NeoTCR” and “NeoE TCR” as used herein mean a neoepitope-specific T cell receptor that is introduced into a T cell, e.g., by gene editing methods.

“NeoTCR cells” as used herein means one or more cells precision engineered to express one or more NeoTCRs. In certain embodiments, the cells are T cells. In certain embodiments, the T cells are CD8+ and/or CD4+ T cells. In certain embodiments, the CD8+ and/or CD4+ T cells are autologous cells from the patient for whom a NeoTCR Product will be

administered. The terms "NeoTCR cells" and "NeoTCR-P1 T cells" and "NeoTCR-P1 cells" are used interchangeably herein. As used herein, NeoTCR cells are not engineered to knockout expression of the TET2 gene.

"NeoTCR Product" as used herein means a pharmaceutical formulation comprising one or more NeoTCR cells. NeoTCR Product consists of autologous precision genome-engineered CD8+ and CD4+ T cells. Using a targeted DNA-mediated *non-viral* precision genome engineering approach, expression of the endogenous TCR is eliminated and replaced by a patient-specific NeoTCR isolated from peripheral CD8+ T cells targeting the tumor-exclusive neoepitope. In certain embodiments, the resulting engineered CD8+ or CD4+ T cells express NeoTCRs on their surface of native sequence, native expression levels, and native TCR function. The sequences of the NeoTCR external binding domain and cytoplasmic signaling domains are unmodified from the TCR isolated from native CD8+ T cells. Regulation of the NeoTCR gene expression is driven by the native endogenous TCR promoter positioned upstream of where the NeoTCR gene cassette is integrated into the genome. Through this approach, native levels of NeoTCR expression are observed in unstimulated and antigen-activated T cell states.

The NeoTCR Product manufactured for each patient represents a defined dose of autologous CD8+ and/or CD4+ T cells that are precision genome engineered to express a single neoE-specific TCR cloned from neoE-specific CD8+ T cells individually isolated from the peripheral blood of that same patient.

As used herein, NeoTCR Products are not engineered to knockout expression of the TET2 gene.

"NeoTCR Viral Product" as used herein has the same definition of NeoTCR Product except that the genome engineering is performed using viral mediated methods.

"Pharmaceutical Formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. For clarity, DMSO at quantities used in a NeoTCR Product is not considered unacceptably toxic.

A "subject," "patient," or an "individual" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

"TCR" as used herein means T cell receptor.

"TET2" is a gene that encodes the protein methylcytosine dioxygenase that catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine. TET2 is also referred to as Tet

methylcytosine dioxygenase 2, FLJ20032, KIAA1546, MGC125715, probably methylcytosine dioxygenase TET2, probably mehtylcytosine dioxygenase TET2 isoform a, probably methylcytosine dioxygenase TET2 isoform b, tet oncogene family member 2, and TET2_HUMAN.

"TET2 cells" as used herein means one or more cells precision engineered to express one or more NeoTCRs and a knockout of the TET2 gene. In certain embodiments, the cells are T cells. In certain embodiments, the T cells are CD8+ and/or CD4+ T cells. In certain embodiments, the CD8+ and/or CD4+ T cells are autologous cells from the patient for whom a TET2 Product will be administered.

"TET2 NeoTCR Product" as used herein means a product comprising TET2 cells.

"Treat," "Treatment," and "treating" are used interchangeably and as used herein mean obtaining beneficial or desired results including clinical results. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, the TET2 Products of this disclosure are used to delay development of a proliferative disorder (e.g., cancer) or to slow the progression of such disease.

"Treat," "Treatment," and "treating" are used interchangeably and as used herein mean obtaining beneficial or desired results including clinical results. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, the TET2 Products of the disclosure is used to delay the development of a proliferative disorder (e.g., cancer) or to slow the progression of such disease.

The term "tumor antigen" as used herein refers to an antigen (e.g., a polypeptide) that is uniquely or differentially expressed on a tumor cell compared to a normal or non-neoplastic cell. In certain embodiments, a tumor antigen includes any polypeptide expressed by a tumor that is capable of activating or inducing an immune response via an antigen-recognizing receptor or capable of suppressing an immune response via receptor-ligand binding.

"2A" and "2A peptide" are used interchangeably herein and mean a class of 18-22 amino acid long, viral, self-cleaving peptides that are able to mediate cleavage of peptides during translation in eukaryotic cells.

Four well-known members of the 2A peptide class are T2A, P2A, E2A, and F2A. The T2A peptide was first identified in the Thosea asigna virus 2A. The P2A peptide was first identified in the porcine teschovirus-1 2A. The E2A peptide was first identified in the equine rhinitis A virus. The F2A peptide was first identified in the foot-and-mouth disease virus.

The self-cleaving mechanism of the 2A peptides is a result of ribosome skipping the formation of a glycyl-prolyl peptide bond at the C-terminus of the 2A. Specifically, the 2A peptides have a C-terminal conserved sequence that is necessary for the creation of steric hindrance and ribosome skipping. The ribosome skipping can result in one of three options: 1) successful skipping and recommencement of translation resulting in two cleaved proteins (the upstream of the 2A protein which is attached to the complete 2A peptide except for the C-terminal proline and the downstream of the 2A protein which is attached to one proline at the N-terminal; 2) successful skipping but ribosome fall-off that results in discontinued translation and only the protein upstream of the 2A; or 3) unsuccessful skipping and continued translation (i.e., a fusion protein).

The term "endogenous" as used herein refers to a nucleic acid molecule or polypeptide that is normally expressed in a cell or tissue.

The term "exogenous" as used herein refers to a nucleic acid molecule or polypeptide that is not endogenously present in a cell. The term "exogenous" would therefore encompass any recombinant nucleic acid molecule or polypeptide expressed in a cell, such as foreign, heterologous, and over-expressed nucleic acid molecules and polypeptides. By "exogenous" nucleic acid is meant a nucleic acid not present in a native wild-type cell; for example an exogenous nucleic acid may vary from an endogenous counterpart by sequence, by position/location, or both. For clarity, an exogenous nucleic acid may have the same or different sequence relative to its native endogenous counterpart; it may be introduced by genetic engineering into the cell itself or a progenitor thereof, and may optionally be linked to alternative control sequences, such as a non-native promoter or secretory sequence.

"Young" or "Younger" or "Young T cell" as it relates to T cells means memory stem cells (T_{MSC}) and central memory cells (T_{CM}). These cells have T cell proliferation upon specific activation and are competent for multiple cell divisions. They also have the ability to engraft after re-infusion, to rapidly differentiate into effector T cells upon exposure to their cognate antigen and target and kill tumor cells, as well as to persist for ongoing cancer surveillance and control.

NeoTCR PRODUCTS

In some embodiments, using the gene editing technology and neoTCR isolation technology described in PCT/US2020/017887 and PCT/US2019/025415, which are incorporated herein in their entireties, NeoTCRs are cloned in autologous CD8+ and CD4+ T cells from the same patient with cancer by precision genome engineered (using a DNA-mediated (non-viral) method as described in **Figures 2A-2C**) to express the neoTCR. In other words, the NeoTCRs are tumor specific and identified in cancer patients. These NeoTCRs are then cloned and the cloned NeoTCRs are inserted into the cancer patient's T cells. NeoTCR expressing T cells are then expanded in a manner that preserves a "young" T cell phenotypes, resulting in a NeoTCR-P1 product (i.e., a NeoTCR Product) in which the majority of the T cells exhibit T memory stem cell and T central memory phenotypes.

These 'young' or 'younger' or less-differentiated T cell phenotypes are described to confer improved engraftment potential and prolonged persistence post-infusion. Thus, the administration of NeoTCR Product, consisting significantly of 'young' T cell phenotypes, has the potential to benefit patients with cancer, through improved engraftment potential, prolonged persistence post-infusion, and rapid differentiation into effector T cells to eradicate tumor cells throughout the body.

Ex vivo mechanism-of-action studies were also performed with NeoTCR Product manufactured with T cells from patients with cancer. Comparable gene editing efficiencies and functional activities, as measured by antigen-specificity of T cell killing activity, proliferation, and cytokine production, were observed demonstrating that the manufacturing process described herein is successful in generating product with T cells from patients with cancer as starting material.

In certain embodiments, the NeoTCR Product manufacturing process involves electroporation of dual ribonucleoprotein species of CRISPR-Cas9 nucleases bound to guide RNA sequences, with each species targeting the genomic TCRα and the genomic TCRβ loci. The specificity of targeting Cas9 nucleases to each genomic locus has been previously described in the literature as being highly specific. Comprehensive testing of the NeoTCR Product was performed *in vitro* and *in silico* analyses to survey possible off-target genomic cleavage sites, using COSMID and GUIDE-seq, respectively. Multiple NeoTCR Product or comparable cell products from healthy donors were assessed for cleavage of the candidate off-target sites by deep sequencing, supporting the published evidence that the selected nucleases are highly specific.

Further aspects of the precision genome engineering process have been assessed for safety. No evidence of genomic instability following precision genome engineering was found in assessing multiple NeoTCR Products by targeted locus amplification (TLA) or standard FISH

cytogenetics. No off-target integration anywhere into the genome of the NeoTCR sequence was detected. No evidence of residual Cas9 was found in the cell product.

The comprehensive assessment of the NeoTCR Product and precision genome engineering process indicates that the NeoTCR Product will be well tolerated following infusion back to the patient.

The genome engineering approach described herein enables highly efficient generation of bespoke NeoTCR T cells (i.e., NeoTCR Products) for personalized adoptive cell therapy for patients with solid and liquid tumors. Furthermore, the engineering method is not restricted to the use in T cells and has also been applied successfully to other primary cell types, including natural killer and hematopoietic stem cells.

TET2 PRODUCTS

In some embodiments, the NeoTCR Products described above are further modified to knock out TET2 expression from the cells of the product (i.e., a TET2 Product). Specifically, using the gene editing technology and neoTCR isolation technology described in PCT/US2020/017887 and PCT/US2019/025415, which are incorporated herein in their entireties, NeoTCRs are cloned in autologous CD8+ and CD4+ T cells from the same patient with cancer by precision genome engineered (using a DNA-mediated (non-viral) method as described in **Figures 2A-2C**) to express the neoTCR.

In certain embodiments, the TET2 gene is disrupted using a gRNA to knockout expression of TET2 in the same reaction as the reaction used to knock out the TCRβ and insertion of the neoTCRα and neoTCRβ (see **Figure 1**). In certain embodiments, the knock out the TCRβ, insertion of the neoTCRα and neoTCRβ, and knockout of TET2 are performed concurrently in the same reaction.

In certain embodiments, the TET2 gene is disrupted using a gRNA to knockout expression of TET2 in a different reaction as the reaction used to knock out the TCRβ and insertion of the neoTCRα and neoTCRβ (see **Figure 1**). In certain embodiments, the knockout of the TCRβ and insertion of the neoTCRα and neoTCRβ are performed in separate and consecutive reactions from the knockout of TET2. In certain embodiments, the knockout of the TCRβ and insertion of the neoTCRα and neoTCRβ are performed in a first reaction and the knockout of TET2 is performed in a second reaction. In certain embodiments, the knockout of TET2 is performed in a first reaction and the knockout of the TCRβ and insertion of the neoTCRα and neoTCRβ are performed in a second reaction.

In some embodiments, the TET2 Products comprise cells that were engineered to express one or more NeoTCRs using viral methods (i.e., NeoTCR Viral Product) and a knockout of the TET2 gene. In certain embodiments, the cells of the NeoTCR Viral Product are further engineered to knockout the TET2 gene using non-viral methods. In certain embodiments, the cells of the NeoTCR Viral Product are further engineered to knockout the TET2 gene using viral methods.

The TET2 Product manufacturing process involves electroporation of 1) dual ribonucleoprotein species of CRISPR-Cas9 nucleases bound to guide RNA sequences, with each species targeting the genomic TCRα and the genomic TCRβ loci, and 2) a ribonucleoprotein species of CRISPR-Cas9 nucleases bound to guide RNA sequences that target the TET2 gene. The specificity of targeting Cas9 nucleases to each genomic locus has been previously described in the literature as being highly specific. Comprehensive testing of the TET2 Product can be performed *in vitro* and *in silico* analyses to survey possible off-target genomic cleavage sites, using COSMID and GUIDE-seq, respectively. In certain embodiments, testing can be performed using COSMID-based in silico prediction of off-targets and GUIDE-seq-based in vitro prediction of off-targets, followed by testing of those putative off-targets by targeted deep sequencing.

In certain embodiments, TET2 cells are expanded in a manner that preserves a "young" T cell phenotypes, resulting in a TET2 Product in which the majority of the T cells exhibit T memory stem cell and T central memory phenotypes

These 'young' or 'younger' or less-differentiated T cell phenotypes are described to confer improved engraftment potential and prolonged persistence post-infusion. Thus, the administration of TET2 Product, consisting significantly of 'young' T cell phenotypes, has the potential to benefit patients with cancer, through improved engraftment potential, prolonged persistence post-infusion, and rapid differentiation into effector T cells to eradicate tumor cells throughout the body.

In certain embodiments, the TET2 cells of the TET2 Products predominantly comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 25% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 30% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 35% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 40% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 45% of the TET2 cells of the TET2

Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 50% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 55% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 60% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 65% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 70% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, at least 75% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). In certain embodiments, greater than 75% of the TET2 cells of the TET2 Products comprise memory stem cells (Tmsc) and/or central memory cells (Tcm). Tmsc are characterized as cells that are CD45RA+CD62L+, CD28+CD95+, and CCR7+CD27+. Tcm are characterized as cells that are CD45RO+CD62L+, CD28+CD95+, and CCR7+CD27+CD127+. Both Tmsc and Tcm are characterized as having weak effector T cell function, robust proliferation, robust engraftment, and long telomeres.

METHODS OF PRODUCING TET2 PRODUCTS WITH A YOUNG PHENOTYPE

In certain embodiments, the present disclosure relates, in part, on the production of engineered “young” T cells. In certain embodiments, the present disclosure comprises methods for producing antigen-specific cells, e.g., T cells, *ex vivo*, comprising activating, engineering, and expanding antigen-specific cells originally obtained from a subject or isolated from such sample.

In certain embodiments, the methods for activating cells comprise the steps of activating the TCR/CD3 complex. For example, without limitation, the T cells can be incubated and/or cultured with CD3 agonists, CD28 agonists, or a combination thereof.

In certain embodiments, engineered activated antigen-specific cells, e.g., engineered activated T cells, can be expanded by culturing the engineered activated antigen-specific cells, e.g., T cells, with cytokines, chemokine, soluble peptides, or combination thereof. In certain embodiments, the engineered activated antigen-specific cells, e.g., engineered activated T cells, can be cultured with one or more cytokines. In certain embodiments, the cytokines can be IL2, IL7, IL15, or combinations thereof. For example, engineered activated antigen-specific cells, e.g., engineered activated T cells, can be cultured with IL7 and IL15. In certain embodiments, the cytokine used in connection with the engineered activated antigen-specific cell, e.g.,

engineered activated T cell, culture can be present at a concentration from about 1 pg/ml to about 1 g/ml, from about 1 ng/ml to about 1 g/ml, from about 1 μg/ml to about 1 g/ml, or from about 1 mg/ml to about 1g/ml, and any values in between.

PHARMACEUTICAL FORMULATIONS.

Pharmaceutical formulations of the TET2 Product are prepared by combining the TET2 cells in a solution that can preserve the 'young' phenotype of the cells in a cryopreserved state. Table 1 provides an example of one such pharmaceutical formulation. Alternatively, pharmaceutical formulations of the TET2 Product can be prepared by combining the TET2 cells in a solution that can preserve the 'young' phenotype of the cells without the need to freeze or cryopreserve the product (*i.e.,* the TET2 Product is maintained in an aqueous solution or as a non-frozen/cryopreserved cell pellet).

Additional pharmaceutically acceptable carriers, buffers, stabilizers, and/or preservatives can also be added to the cryopreservation solution or the aqueous storage solution (if the TET2 Product is not cryopreserved). Any cryopreservation agent and/or media can be used to cryopreserve the TET2 Product, including but not limited to CryoStor, CryoStor CS5, CELLBANKER, and custom cryopreservation media that optionally include DMSO.

GENE-EDITING METHODS

In certain embodiments, the present disclosure involves, in part, methods of engineering human cells, e.g., engineered T cells or engineered human stem cells. In certain embodiments, such engineering involves genome editing. For example, but not by way of limitation, such genome editing can be accomplished with nucleases targeting one or more endogenous loci, e.g., TCR alpha (TCRα) locus and TCR beta (TCRβ) locus, along with the TET2 locus. In certain embodiments, the nucleases can generate single-stranded DNA nicks or double-stranded DNA breaks in an endogenous target sequence. In certain embodiments, the nuclease can target coding or non-coding portions of the genome, e.g., exons, introns. In certain embodiments, the nucleases contemplated herein comprise homing endonuclease, meganuclease, megaTAL nuclease, transcription activator-like effector nuclease (TALEN), zinc-finger nuclease (ZFN), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas nuclease. In certain embodiments, the nucleases can themselves be engineered, e.g., via the introduction of amino acid substitutions and/or deletions, to increase the efficiency of the cutting activity.

In certain embodiments, a CRISPR/Cas nuclease system is used to engineer human cells. In certain embodiments, the CRISPR/Cas nuclease system comprises a Cas nuclease and

one or more RNAs that recruit the Cas nuclease to the endogenous target sequence, *e.g.*, single guide RNA. In certain embodiments, the Cas nuclease and the RNA are introduced in the cell separately, e.g. using different vectors or compositions, or together, e.g., in a polycistronic construct or a single protein-RNA complex. In certain embodiments, the Cas nuclease is Cas9 or Cas12a. In certain embodiments, the Cas9 polypeptide is obtained from a bacterial species including, without limitation, *Streptococcus pyogenes* or *Neisseria menengitidis*. Additional examples of CRISPR/Cas systems are known in the art. See Adli, Mazhar. "The CRISPR tool kit for genome editing and beyond." Nature communications vol. 9,1 1911 (2018), herein incorporated by reference for all that it teaches.

In certain embodiments, genome editing occurs at one or more genome loci that regulate immunological responses. In certain embodiments, the loci include, without limitation, TCR alpha (TCRα) locus, TCR beta (TCRβ) locus, TCR gamma (TCRγ), TCR delta (TCRδ), and TET2. In certain embodiments, one of the loci is the TET2 locus.

In certain embodiments, genome editing is performed by using non-viral delivery systems. For example, a nucleic acid molecule can be introduced into a cell by administering the nucleic acid in the presence of lipofection (Feigner et al., Proc. Natl. Acad. Sci. U.S.A. 84:7413, 1987; Ono et al., Neuroscience Letters 17:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Methods in Enzymology 101:512, 1983), asialoorosomucoid-polylysine conjugation (Wu et al., Journal of Biological Chemistry 263:14621, 1988; Wu et al., Journal of Biological Chemistry 264:16985, 1989), or by micro-injection under surgical conditions (Wolff et al., Science 247:1465, 1990). Other non-viral means for gene transfer include transfection in vitro using calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell. Transplantation of normal genes into the affected tissues of a subject can also be accomplished by transferring a normal nucleic acid into a cultivatable cell type ex vivo (e.g., an autologous or heterologous primary cell or progeny thereof), after which the cell (or its descendants) are injected into a targeted tissue or are injected systemically.

In certain embodiments, genome editing is performed by using viral delivery systems. In certain embodiments, the viral methods include targeted integration (including but not limited to AAV) and random integration (including but not limited to lentiviral approaches). In certain embodiments, the viral delivery would be accomplished without integration of the nuclease. In such embodiments, the viral delivery system can be Lentiflash or another similar delivery system.

HOMOLOGY RECOMBINATION TEMPLATES

In certain embodiments, the present disclosure provides genome editing of a cell by introducing and recombining a homologous recombination (HR) template nucleic acid sequence into an endogenous locus of a cell. In certain embodiments, the HR template nucleic acid sequence is linear. In certain embodiments, the HR template nucleic acid sequence is circular. In certain embodiments, the circular HR template can be a plasmid, minicircle, or nanoplasmid. In certain embodiments, the HR template nucleic acid sequence comprises a first and a second homology arms. In certain embodiments, the homology arms can be of about 300 bases to about 2,000 bases. For example, each homology arm can be 1,000 bases. In certain embodiments, the homology arms can be homologous to a first and second endogenous sequences of the cell. In certain embodiments, the endogenous locus is a TCR locus. For example, the first and second endogenous sequences are within a TCR alpha locus or a TCR beta locus. In certain embodiments, the HR template comprises a TCR gene sequences. In non-limiting embodiments, the TCR gene sequence is a patient specific TCR gene sequence. In non-limiting embodiments, the TCR gene sequence is tumor-specific. In non-limiting embodiments, the TCR gene sequence can be identified and obtained using the methods described in PCT/US2020/017887, the content of which is herein incorporated by reference. In certain embodiments, the HR template comprises a TCR alpha gene sequence and a TCR beta gene sequence.

In certain embodiments, the HR template is a polycistronic polynucleotide. In certain embodiments, the HR template comprises sequences encoding for flexible polypeptide sequences (e.g., Gly-Ser-Gly sequence). In certain embodiments, the HR template comprises sequences encoding an internal ribosome entry site (IRES). In certain embodiments, the HR template comprises a 2A peptide (e.g., P2A, T2A, E2A, and F2A). Additional information on the HR template nucleic acids and methods of modifying a cell thereof can be found in International Patent Application no. PCT/US2018/058230, the content of which is herein incorporated by reference.

METHODS OF TREATMENT

The presently disclosed subject matter provides methods for inducing and/or increasing an immune response in a subject in need thereof. The TET2 Products can be used for treating and/or preventing a cancer in a subject. The TET2 Products can be used for prolonging the survival of a subject suffering from a cancer. The TET2 Products can also be used for treating and/or preventing a cancer in a subject. The TET2 Products can also be used for reducing tumor burden in a subject. Such methods comprise administering the TET2 Products in an amount effective or a composition (*e.g.*, a pharmaceutical composition) comprising thereof

to achieve the desired effect, be it palliation of an existing condition or prevention of recurrence. For treatment, the amount administered is an amount effective in producing the desired effect. An effective amount can be provided in one or a series of administrations. An effective amount can be provided in a bolus or by continuous perfusion.

In certain embodiments, an effective amount of the TET2 Products are delivered through intravenous (IV) administration. In certain embodiments, the TET2 Products are delivered through IV administration in a single administration. In certain embodiments, the TET2 Products are delivered through IV administration in multiple administrations. In certain embodiments, the TET2 Products are delivered through IV administration in two or more administrations. In certain embodiments, the TET2 Products are delivered through IV administration in two administrations. In certain embodiments, the TET2 Products are delivered through IV administration in three administrations.

The presently disclosed subject matter provides methods for treating and/or preventing cancer in a subject. In certain embodiments, the method comprises administering an effective amount of the TET2 Products to a subject having cancer.

Non-limiting examples of cancer include blood cancers (e.g. leukemias, lymphomas, and myelomas), ovarian cancer, breast cancer, bladder cancer, brain cancer, colon cancer, intestinal cancer, liver cancer, lung cancer, pancreatic cancer, prostate cancer, skin cancer, stomach cancer, glioblastoma, throat cancer, melanoma, neuroblastoma, adenocarcinoma, glioma, soft tissue sarcoma, and various carcinomas (including prostate and small cell lung cancer). Suitable carcinomas further include any known in the field of oncology, including, but not limited to, astrocytoma, fibrosarcoma, myxosarcoma, liposarcoma, oligodendroglioma, ependymoma, medulloblastoma, primitive neural ectodermal tumor (PNET), chondrosarcoma, osteogenic sarcoma, pancreatic ductal adenocarcinoma, small and large cell lung adenocarcinomas, chordoma, angiosarcoma, endotheliosarcoma, squamous cell carcinoma, bronchoalveolarcarcinoma, epithelial adenocarcinoma, and liver metastases thereof, lymphangiosarcoma, lymphangioendotheliosarcoma, hepatoma, cholangiocarcinoma, synovioma, mesothelioma, Ewing's tumor, rhabdomyosarcoma, colon carcinoma, basal cell carcinoma, sweat gland carcinoma, papillary carcinoma, sebaceous gland carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, testicular tumor, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, neuroblastoma, retinoblastoma, leukemia, multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease, breast tumors such as ductal and lobular adenocarcinoma, squamous and

adenocarcinomas of the uterine cervix, uterine and ovarian epithelial carcinomas, prostatic adenocarcinomas, transitional squamous cell carcinoma of the bladder, B and T cell lymphomas (nodular and diffuse) plasmacytoma, acute and chronic leukemias, malignant melanoma, soft tissue sarcomas and leiomyosarcomas. In certain embodiments, the cancer is selected from the group consisting of blood cancers (e.g. leukemias, lymphomas, and myelomas), ovarian cancer, prostate cancer, breast cancer, bladder cancer, brain cancer, colon cancer, intestinal cancer, liver cancer, lung cancer, pancreatic cancer, prostate cancer, skin cancer, stomach cancer, glioblastoma, and throat cancer. In certain embodiments, the presently disclosed young T cells and compositions comprising thereof can be used for treating and/or preventing blood cancers (e.g., leukemias, lymphomas, and myelomas) or ovarian cancer, which are not amenable to conventional therapeutic interventions.

In certain embodiments, the cancer is a solid cancer or a solid tumor. In certain embodiments, the solid tumor or solid cancer is selected from the group consisting of glioblastoma, prostate adenocarcinoma, kidney papillary cell carcinoma, sarcoma, ovarian cancer, pancreatic adenocarcinoma, rectum adenocarcinoma, colon adenocarcinoma, esophageal carcinoma, uterine corpus endometrioid carcinoma, breast cancer, skin cutaneous melanoma, lung adenocarcinoma, stomach adenocarcinoma, cervical and endocervical cancer, kidney clear cell carcinoma, testicular germ cell tumors, and aggressive B-cell lymphomas.

The subjects can have an advanced form of disease, in which case the treatment objective can include mitigation or reversal of disease progression, and/or amelioration of side effects. The subjects can have a history of the condition, for which they have already been treated, in which case the therapeutic objective will typically include a decrease or delay in the risk of recurrence.

Suitable human subjects for therapy typically comprise two treatment groups that can be distinguished by clinical criteria. Subjects with “advanced disease” or “high tumor burden” are those who bear a clinically measurable tumor. A clinically measurable tumor is one that can be detected on the basis of tumor mass (e.g., by palpation, CAT scan, sonogram, mammogram or X-ray; positive biochemical or histopathologic markers on their own are insufficient to identify this population). A pharmaceutical composition is administered to these subjects to elicit an anti-tumor response, with the objective of palliating their condition. Ideally, reduction in tumor mass occurs as a result, but any clinical improvement constitutes a benefit. Clinical improvement includes decreased risk or rate of progression or reduction in pathological consequences of the tumor.

ARTICLES OF MANUFACTURE

The TET2 Products can be used in combination with articles of manufacture. Such articles of manufacture can be useful for the prevention or treatment of proliferative disorders (e.g., cancer). Examples of articles of manufacture include but are not limited to containers (e.g., infusion bags, bottles, storage containers, flasks, vials, syringes, tubes, and IV solution bags) and a label or package insert on or associated with the container. The containers may be made of any material that is acceptable for the storage and preservation of the TET2 cells within the TET2 Products. In certain embodiments, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle. For example, the container may be a CryoMACS freezing bag. The label or package insert indicates that the TET2 Products are used for treating the condition of choice and the patient of origin. The patient is identified on the container of the TET2 Product because the TET2 Products is made from autologous cells and engineered as a patient-specific and individualized treatment.

The article of manufacture may comprise: 1) a first container with a TET2 Product contained therein.

The article of manufacture may comprise: 1) a first container with a TET2 Product contained therein; and 2) a second container with the same TET2 Product as the first container contained therein. Optionally, additional containers with the same TET2 Product as the first and second containers may be prepared and made. Optionally, additional containers containing a composition comprising a different cytotoxic or otherwise therapeutic agent may also be combined with the containers described above.

The article of manufacture may comprise: 1) a first container with a TET2 Product contained therein; and 2) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent.

The article of manufacture may comprise: 1) a first container with two TET2 Products contained therein; and 2) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent.

The article of manufacture may comprise: 1) a first container with a TET2 Product contained therein; 2) a second container with a second TET2 Product contained therein; and 3) optionally a third container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. In certain embodiments, the first and second TET2 Products are different TET2 Products. In certain embodiments, the first and second TET2 Products are the same TET2 Products.

The article of manufacture may comprise: 1) a first container with three TET2 Products contained therein; and 2) optionally a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent.

The article of manufacture may comprise: 1) a first container with a TET2 Product contained therein; 2) a second container with a second TET2 Product contained therein; 3) a third container with a third TET2 Product contained therein; and 4) optionally a fourth container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. In certain embodiments, the first, second, and third TET2 Products are different TET2 Products. In certain embodiments, the first, second, and third TET2 Products are the same TET2 Products. In certain embodiments, two of the first, second, and third TET2 Products are the same TET2 Products.

The article of manufacture may comprise: 1) a first container with four TET2 Products contained therein; and 2) optionally a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent.

The article of manufacture may comprise: 1) a first container with a TET2 Product contained therein; 2) a second container with a second TET2 Product contained therein; 3) a third container with a third TET2 Product contained therein; 4) a fourth container with a fourth TET2 Product contained therein; and 5) optionally a fifth container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. In certain embodiments, the first, second, third, and fourth TET2 Products are different TET2 Products. In certain embodiments, the first, second, third, and fourth TET2 Products are the same TET2 Products. In certain embodiments, two of the first, second, third, and fourth TET2 Products are the same TET2 Products. In certain embodiments, three of the first, second, third, and fourth TET2 Products are the same TET2 Products.

The article of manufacture may comprise: 1) a first container with five or more TET2 Products contained therein; and 2) optionally a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent.

The article of manufacture may comprise: 1) a first container with a TET2 Product contained therein; 2) a second container with a second TET2 Product contained therein; 3) a third container with a third TET2 Product contained therein; 4) a fourth container with a fourth TET2 Product contained therein; 5) a fifth container with a fifth TET2 Product contained therein; 6) optionally a sixth or more additional containers with a sixth or more TET2 Product contained therein; and 7) optionally an additional container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. In certain embodiments, the all of the containers of TET2 Products are different TET2 Products. In certain embodiments, all of the containers of TET2 Products are the same TET2 Products. In certain embodiments, there can be any combination of same or different TET2 Products in the five or more containers based on the availability of detectable TET2s in a patient's tumor

sample(s), the need and/or desire to have multiple TET2 Products for the patient, and the availability of any one TET2 Product that may require or benefit from one or more container.

The article of manufacture may comprise: 1) a first container with a TET2 Product contained therein; 2) a second container with a second TET2 Product contained therein; and 3) a third container with a third TET2 Product contained therein.

The article of manufacture may comprise: 1) a first container with a TET2 Product contained therein; 2) a second container with a second TET2 Product contained therein; 3) a third container with a third TET2 Product contained therein; and 4) optionally a fourth container with a fourth TET2 Product contained therein.

The article of manufacture may comprise: 1) a first container with a TET2 Product contained therein; 2) a second container with a second TET2 Product contained therein; 3) a third container with a third TET2 Product contained therein; 4) a fourth container with a fourth TET2 Product contained therein; and 5) optionally a fifth container with a fourth TET2 Product contained therein.

The article of manufacture may comprise a container with one TET2 Product contained therein. The article of manufacture may comprise a container with two TET2 Products contained therein. The article of manufacture may comprise a container with three TET2 Products contained therein. The article of manufacture may comprise a container with four TET2 Products contained therein. The article of manufacture may comprise a container with five TET2 Products contained therein.

The article of manufacture may comprise 1) a first container with one TET2 Product contained therein, and 2) a second container with two TET2 Products contained therein. The article of manufacture may comprise 1) a first container with two TET2 Products contained therein, and 2) a second container with one TET2 Product contained therein. In the examples above, a third and/or fourth container comprising one or more additional TET2 Products may be included in the article of manufacture. Additionally, a fifth container comprising one or more additional TET2 Products may be included in the article of manufacture.

Furthermore, any container of TET2 Product described herein can be split into two, three, or four separate containers for multiple time points of administration and/or based on the appropriate dose for the patient.

In certain embodiments, the TET2 Products are provided in a kit. The kit can, by means of non-limiting examples, contain package insert(s), labels, instructions for using the TET2 Product(s), syringes, disposal instructions, administration instructions, tubing, needles, and anything else a clinician would need in order to properly administer the TET2 Product(s).

THERAPEUTIC COMPOSITIONS AND METHODS OF MANUFACTURING

As described herein, plasmid DNA-mediated precision genome engineering process for Good Manufacturing Practice (GMP) manufacturing of TET2 Product was developed. Targeted integration of the patient-specific neoTCR was accomplished by electroporating CRISPR endonuclease ribonucleoproteins (RNPs) together with the personalized neoTCR gene cassette, encoded by the plasmid DNA. In addition to the neoTCR, the TET2 knockout was accomplished by electroporating CRISPR endonuclease ribonucleoproteins (RNPs) that target the TET2 locus.

The TET2 Product can be formulated into a drug product using the clinical manufacturing process. Under this process, the TET2 Product is cryopreserved in CryoMACS Freezing Bags. One or more bags may be shipped to the site for each patient depending on patient needs. The product is composed of apheresis-derived, patient-autologous, CD8 and CD4 T cells that have been precision genome engineered to express one or more autologous neoTCRs targeting a neoepitope complexed to one of the endogenous HLA receptors presented exclusively on the surface of that patient's tumor cells.

The final product will contain 5% dimethyl sulfoxide (DMSO), human serum albumin, and Plasma-Lyte. The final cell product will contain the list of components provided in Table 1.

Table 1: Composition of the TET2 Product

Component	Specification/Grade
Total nucleated NeoTCR cells	cGMP manufactured
Plasma-Lyte A	USP
Human Serum Albumin in 0.02 - 0.08 M sodium caprylate and sodium tryptophanate	USP
CryoStor CS10	cGMP manufactured with USP grade materials

COMPOSITIONS AND VECTORS

The presently disclosed subject matter provides compositions comprising cells (e.g., immunoresponsive cells) disclosed herein.

In certain embodiments, the presently disclosed subject matter provides nucleic acid compositions comprising a polynucleotide encoding the NeoTCR disclosed herein. In certain embodiments, the nucleic acid compositions disclosed herein comprise a polynucleotide encoding a nuclease for the gene disruption of a TET2 locus. In certain embodiments, the

nucleic acid compositions disclosed herein comprise a Cas nuclease and a gRNA for the gene disruption of a TET2 locus. In certain embodiments, the gRNA has a sequence comprising the nucleotide sequence set forth in SEQ ID NOs: 1-5. Also provided are cells comprising such nucleic acid compositions.

In certain embodiments, the nucleic acid composition further comprises a promoter that is operably linked to the nuclease for the gene disruption of a TET2 locus.

In certain embodiments, the promoter is endogenous or exogenous. In certain embodiments, the exogenous promoter is selected from the group consisting of an elongation factor (EF)-1 promoter, a CMV promoter, a SV40 promoter, a PGK promoter, a long terminal repeat (LTR) promoter and a metallothionein promoter. In certain embodiments, the promoter is an inducible promoter. In certain embodiments, the inducible promoter is selected from the group consisting of a NFAT transcriptional response element (TRE) promoter, a CD69 promoter, a CD25 promoter, an IL-2 promoter, an IL-12 promoter, a p40 promoter, and a Bcl-xL promoter.

The compositions and nucleic acid compositions can be administered to subjects or and/delivered into cells by art-known methods or as described herein. Genetic modification of a cell (e.g., a T cell) can be accomplished by transducing a substantially homogeneous cell composition with a recombinant DNA construct. In certain embodiments, a retroviral vector (either a gamma-retroviral vector or a lentiviral vector) is employed for the introduction of the DNA construct into the cell. For example, a polynucleotide encoding a nuclease for the gene disruption of a TET2 locus can be cloned into a viral vector and expression can be driven from its endogenous promoter, or from a promoter specific for a target cell type of interest. Non-viral vectors may be used as well.

Possible methods of transduction also include direct co-culture of the cells with producer cells, e.g., by the method of Bregni, *et al.* (1992) *Blood* 80:1418-1422, or culturing with viral supernatant alone or concentrated vector stocks with or without appropriate growth factors and polycations, e.g., by the method of Xu, *et al.* (1994) *Exp. Hemat.* 22:223-230; and Hughes, *et al.* (1992) *J. Clin. Invest.* 89:1817.

Other transducing viral vectors can be used to modify a cell. In certain embodiments, the chosen vector exhibits high efficiency of infection and stable integration and expression (see, e.g., Cayouette et al., Human Gene Therapy 8:423-430, 1997; Kido et al., Current Eye Research 15:833-844, 1996; Bloomer et al., Journal of Virology 71:6641-6649, 1997; Naldini et al., Science 272:263-267, 1996; and Miyoshi et al., Proc. Natl. Acad. Sci. U.S.A. 94:10319, 1997). Other viral vectors that can be used include, for example, adenoviral, lentiviral, and adena-associated viral vectors, vaccinia virus, a bovine papilloma virus, or a

herpes virus, such as Epstein-Barr Virus (also see, for example, the vectors of Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis et al., BioTechniques 6:608-614, 1988; Tolstoshev et al., Current Opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; Miller et al., Biotechnology 7:980-990, 1989; LeGal La Salle et al., Science 259:988-990, 1993; and Johnson, Chest 107:77S- 83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323:370, 1990; Anderson et al., U.S. Pat. No. 5,399,346).

Non-viral approaches can also be employed for genetic modification of a cell. For example, a nucleic acid molecule can be introduced into a cell by administering the nucleic acid in the presence of lipofection (Feigner et al., Proc. Natl. Acad. Sci. U.S.A. 84:7413, 1987; Ono et al., Neuroscience Letters 17:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Methods in Enzymology 101:512, 1983), asialoorosomucoid-polylysine conjugation (Wu et al., Journal of Biological Chemistry 263:14621, 1988; Wu et al., Journal of Biological Chemistry 264:16985, 1989), or by micro-injection under surgical conditions (Wolff et al., Science 247:1465, 1990). Other non-viral means for gene transfer include transfection *in vitro* using calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes can also be potentially beneficial for delivery of DNA into a cell. Transplantation of normal genes into the affected tissues of a subject can also be accomplished by transferring a normal nucleic acid into a cultivatable cell type *ex vivo* (e.g., an autologous or heterologous primary cell or progeny thereof), after which the cell (or its descendants) are injected into a targeted tissue or are injected systemically.

Polynucleotide therapy methods can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element or intron (e.g. the elongation factor 1a enhancer/promoter/intron structure). For example, if desired, enhancers known to preferentially direct gene expression in specific cell types can be used to direct the expression of a nucleic acid. The enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers. Alternatively, if a genomic clone is used as a therapeutic construct, regulation can be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

The resulting cells can be grown under conditions similar to those for unmodified cells, whereby the modified cells can be expanded and used for a variety of purposes.

KITS

The presently disclosed subject matter provides kits for inducing and/or enhancing an immune response and/or treating and/or preventing a cancer or a pathogen infection in a subject. In certain embodiments, the kit comprises an effective amount of presently disclosed cells or a pharmaceutical composition comprising thereof. In certain embodiments, the kit comprises a sterile container; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments.

If desired, the cells and/or nucleic acid molecules are provided together with instructions for administering the cells or nucleic acid molecules to a subject having or at risk of developing a cancer or pathogen or immune disorder. The instructions generally include information about the use of the composition for the treatment and/or prevention of a neoplasm or a pathogen infection. In certain embodiments, the instructions include at least one of the following: description of the therapeutic agent; dosage schedule and administration for treatment or prevention of a cancer, pathogen infection, or immune disorder or symptoms thereof; precautions; warnings; indications; counter-indications; over-dosage information; adverse reactions; animal pharmacology; clinical studies; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container. The resulting cells can be grown under conditions similar to those for unmodified cells, whereby the modified cells can be expanded and used for a variety of purposes.

EXEMPLARY EMBODIMENTS

A. In certain non-limiting embodiments, the presently disclosed subject matter provides for a cell, comprising an exogenous T cell receptor (TCR), and a gene disruption of a TET2 locus.

A1. The foregoing cell of A, wherein the gene disruption comprises a substitution, a deletion, an insertion, or any combination thereof.

A2. The foregoing cell of A or A1, wherein the gene disruption comprises a missense mutation, a nonsense mutation, a non-frameshift deletion, a frameshift deletion, a non-frameshift insertion, a frameshift insertion, or any combination thereof.

A3. The foregoing cell of A-A2, wherein the gene disruption of the TET2 locus results in a non-functional TET2 protein.

A4. The foregoing cell of A-A3, wherein the gene disruption of the TET2 locus results in knockout of the TET2 gene expression.

A5. The foregoing cell of A-A4, comprising a gRNA and a Cas9 nuclease.

A6. The foregoing cell of A5, wherein the gRNA comprises a nucleotide sequence set forth in SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, or SEQ ID NO.5.

A7. The foregoing cell of A-A6, wherein the gene disruption of the TET2 locus enhances cell persistence.

A8. The foregoing cell of A-A7, wherein the gene disruption of the TET2 locus enhances memory cell differentiation.

A9. The foregoing cell of A-A8, wherein the cell is a primary cell.

A10. The foregoing cell of A-A9, wherein the cell is a patient-derived cell.

A11. The foregoing cell of A-A10, wherein the cell is a lymphocyte.

A12. The foregoing cell of A-A11, wherein the cell is a T cell.

A13. The foregoing cell of A12, wherein the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+.

A14. The foregoing cell of A12, wherein the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+.

A15. The foregoing cell of A12, wherein the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

A16. The foregoing cell of A-A15, wherein the exogenous TCR is a patient-derived TCR.

A17. The foregoing cell of A-A16, wherein the exogenous TCR comprises a signal sequence, a first and second 2A sequence, and a TCR polypeptide sequence.

A18. The foregoing cell of A-A17, wherein the exogenous TCR recognizes a cancer antigen.

A19. The foregoing cell of A18, wherein the cancer antigen is a neoantigen.

A20. The foregoing cell of A18, wherein the cancer antigen is a patient specific antigen.

B. In certain non-limiting embodiments, the presently disclosed subject matter provides for a cell modified by a process, the process comprising introducing into a cell a homologous recombination (HR) template nucleic acid sequence; recombining the HR template nucleic acid into an endogenous locus of the cell; and disrupting a TET2 locus of the cell.

B1. The foregoing cell of B, wherein the HR template comprises first and second homology arms homologous to first and second target nucleic acid sequences; and a TCR gene sequence positioned between the first and second homology arms.

B2. The foregoing cell of B or B1, wherein the HR template comprises a first 2A-coding sequence positioned upstream of the TCR gene sequence and a second 2A-coding sequence positioned downstream of the TCR gene sequence, wherein the first and second 2A-coding sequences code for the same amino acid sequence that are codon-diverged relative to each other.

B3. The foregoing cell of B2, wherein the 2A-coding sequence is a P2A-coding sequence.

B4. The foregoing cell of B2 or B3, wherein a sequence coding for the amino acid sequence Gly Ser Gly is positioned immediately upstream of the 2A-coding sequences.

B5. The foregoing cell of B-B4, wherein the HR template comprises a sequence coding for a Furin cleavage site positioned upstream of the second 2A-coding sequence.

B6. The foregoing cell of B1-B5, wherein the first and second homology arms are each from about 300 bases to about 2,000 bases in length.

B7. The foregoing cell of B1-B5, wherein the first and second homology arms are each from about 600 bases to about 2,000 bases in length.

B8. The foregoing cell of B2-B7, wherein the HR template further comprises a signal sequence positioned between the first 2A-coding sequence and the TCR gene sequence.

B9. The foregoing cell of B2-B8, wherein the HR template comprises a second TCR sequence positioned between the second 2A-coding sequence and the second homology arm.

B10. The foregoing cell of B9, wherein the HR template comprises a first signal sequence positioned between the first 2A-coding sequence and the first TCR gene sequence; and a second signal sequence positioned between the second 2A-coding sequence and the second TCR gene sequence; wherein the first and the second signal sequences encode for the same amino acid sequence and are codon diverged relative to each other.

B11. The foregoing cell of B8 or B10, wherein the signal sequence is a human growth hormone signal sequence.

B12. The foregoing cell of B-B11, wherein the HR template is non-viral.

B13. The foregoing cell of B-B12, wherein the HR template is a circular DNA.

B14. The foregoing cell of B-B12, wherein the HR template is a linear DNA.

B15. The foregoing cell of B-B14, wherein the introducing occurs via electroporation.

B16. The foregoing cell of B-B15, wherein the recombining comprises cleavage of the endogenous locus by a nuclease; and recombination of the HR template nucleic acid sequence into the endogenous locus by homology directed repair.

B17. The foregoing cell of B16, wherein the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof.

B18. The foregoing cell of B17, further comprising a gRNA.

B19. The foregoing cell of B-B18, wherein the disrupting comprises introducing a substitution, a deletion, an insertion, or any combination thereof.

B20. The foregoing cell of B-B19, wherein the disrupting comprises introducing a missense mutation, a nonsense mutation, a non-frameshift deletion, a frameshift deletion, a non-frameshift insertion, a frameshift insertion, or any combination thereof.

B21. The foregoing cell of B-B20, wherein the disrupting results in a non-functional TET2 protein.

B22. The foregoing cell of B-B21, wherein the disrupting results in knockout of the TET2 gene expression.

B23. The foregoing cell of B-B22, wherein the disrupting comprises cleavage of the TET2 locus by a nuclease.

B24. The foregoing cell of B23, wherein the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof.

B25. The foregoing cell of B24, further comprising a gRNA.

B26. The foregoing cell of B25, wherein the gRNA comprises a sequence set forth in SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, or SEQ ID NO.5.

B27. The foregoing cell of B23-B26, wherein the nuclease is expressed by a vector.

B28. The foregoing cell of B25-B27, wherein the gRNA is expressed by a vector.

B29. The foregoing cell of B27 or B28, wherein the vector is a viral vector.

B30. The foregoing cell of B27 or B28, wherein the vector is a non-viral vector.

B31. The foregoing cell of B-B30, wherein the disrupting of the TET2 locus enhances cell persistence.

B32. The foregoing cell of B-B31, wherein the disrupting of the TET2 locus enhances memory cell differentiation.

B33. The foregoing cell of B-B32, wherein the cell is a primary cell.

B34. The foregoing cell of B-B33, wherein the cell is a patient-derived cell.

B35. The foregoing cell of B-B34, wherein the cell is a lymphocyte.

B36. The foregoing cell of B-B35, wherein the cell is a T cell.

B37. The foregoing cell of B36, wherein the T cell is a patient-derived cell.

B38. The foregoing cell of B-B37, wherein the cell is a young T cell.

B39. The foregoing cell of B38, wherein the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+.

B40. The foregoing cell of B38, wherein the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+.

B41. The foregoing cell of B38, wherein the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

B42. The foregoing cell of B-B41, wherein the endogenous locus is within an endogenous TCR gene.

B43. The foregoing cell of B1-B42, wherein the TCR gene sequence encodes for a TCR that recognizes a tumor antigen.

B44. The foregoing cell of B43, wherein the tumor antigen is a neoantigen.

B45. The foregoing cell of B43, wherein the tumor antigen is a patient specific neoantigen.

B46. The foregoing cell of B1-B45, wherein the TCR gene sequence is a patient specific TCR gene sequence.

B47. The foregoing cell of B-B46, wherein the process further comprises culturing the cell.

B48. The foregoing cell of B47, wherein the culturing is conducted in the presence of at least one cytokine.

B49. The foregoing cell of B47 or B48, wherein the culturing is conducted in the presence of IL2, IL7, IL15, or any combination thereof.

B50. The foregoing cell of B47 or B48, wherein the culturing is conducted in the presence of IL7 and IL15.

C. In certain non-limiting embodiments, the presently disclosed subject matter provides for a method of modifying a cell, the method comprising: introducing into a cell a homologous recombination (HR) template nucleic acid sequence; recombining the HR template nucleic acid into an endogenous locus of the cell; and disrupting a TET2 locus of the cell.

C1. The foregoing method of C, wherein the HR template comprises: first and second homology arms homologous to first and second target nucleic acid sequences; and a TCR gene sequence positioned between the first and second homology arms.

C2. The foregoing method of C or C1, wherein the HR template comprises a first 2A-coding sequence positioned upstream of the TCR gene sequence and a second 2A-coding sequence positioned downstream of the TCR gene sequence, wherein the first and second 2A-coding sequences code for the same amino acid sequence that are codon-diverged relative to each other.

C3. The foregoing method of C1 or C2, wherein the 2A-coding sequence is a P2A-coding sequence.

C4. The foregoing method of C2 or C3, wherein a sequence coding for the amino acid sequence Gly Ser Gly is positioned immediately upstream of the 2A-coding sequences.

C5. The foregoing method of C2-C4, wherein the HR template comprises a sequence coding for a Furin cleavage site positioned upstream of the second 2A-coding sequence.

C6. The foregoing method of C1-C5, wherein the first and second homology arms are each from about 300 bases to about 2,000 bases in length.

C7. The foregoing method of C1-C6, wherein the first and second homology arms are each from about 600 bases to about 2,000 bases in length.

C8. The foregoing method of C2-C7, wherein the HR template further comprises a signal sequence positioned between the first 2A-coding sequence and the TCR gene sequence.

C9. The foregoing method of C2-C8, wherein the HR template comprises a second TCR sequence positioned between the second 2A-coding sequence and the second homology arm.

C10. The foregoing method of C9, wherein the HR template comprises: a first signal sequence positioned between the first 2A-coding sequence and the first TCR gene sequence; and a second signal sequence positioned between the second 2A-coding sequence and the second TCR gene sequence; wherein the first and the second signal sequences encode for the same amino acid sequence and are codon diverged relative to each other.

C11. The foregoing method of C8 or C10, wherein the signal sequence is a human growth hormone signal sequence.

C12. The foregoing method of C1-C11, wherein the HR template is non-viral.

C13. The foregoing method of C-C12, wherein the HR template is a circular DNA.

C14. The foregoing method of C-C13, wherein the HR template is a linear DNA.

C15. The foregoing method of C-C14, wherein the introducing occurs via electroporation.

C16. The foregoing method of C-C15, wherein the recombining comprises cleavage of the endogenous locus by a nuclease; and recombination of the HR template nucleic acid sequence into the endogenous locus by homology directed repair.

C17. The foregoing method of C16, wherein the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof.

C18. The foregoing method of C17, further comprising a gRNA.

C19. The foregoing method of C-C18, wherein the disrupting comprises introducing a substitution, a deletion, an insertion, or any combination thereof.

C20. The foregoing method of C-C19, wherein the disrupting comprises introducing a missense mutation, a nonsense mutation, a non-frameshift deletion, a frameshift deletion, a non-frameshift insertion, a frameshift insertion, or any combination thereof.

C21. The foregoing method of C-C20, wherein the disrupting results in a non-functional TET2 protein.

C22. The foregoing method of C-C21, wherein the disrupting results in knockout of the TET2 gene expression.

C23. The foregoing method of C-C22, wherein the disrupting comprises cleavage of the TET2 locus by a nuclease.

C24. The foregoing method of C23, wherein the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof.

C25. The foregoing method of C24, further comprising a gRNA.

C26. The foregoing method of C25, wherein the gRNA comprises a sequence set forth in SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, or SEQ ID NO.5.

C27. The foregoing method of C23-C26, wherein the nuclease is expressed by a vector.

C28. The foregoing method of C25-C27, wherein the gRNA is expressed by a vector.

C29. The foregoing method of C27 or C28, wherein the vector is a viral vector.

C30. The foregoing method of C27-C29, wherein the vector is a non-viral vector.

C31. The foregoing method of C-C30, wherein the disrupting of the TET2 locus enhances cell persistence.

C32. The foregoing method of C-C31, wherein the disrupting of the TET2 locus enhances memory cell differentiation.

C33. The foregoing method of C-C32, wherein the cell is a primary cell.

C34. The foregoing method of C-C32, wherein the cell is a patient-derived cell.

C35. The foregoing method of C-C32, wherein the cell is a lymphocyte.

C36. The foregoing method of C-C32, wherein the cell is a T cell.

C37. The foregoing method of C36, wherein the T cell is a patient-derived cell.

C38. The foregoing method of C-C32, wherein the cell is a young T cell.

C39. The foregoing method of C38, wherein the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+.

C40. The foregoing method of C38, wherein the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+.

C41. The foregoing method of C38, wherein the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

C42. The foregoing method of C-C41, wherein the endogenous locus is within an endogenous TCR gene.

C43. The foregoing method of C1-C42, wherein the TCR gene sequence encodes for a TCR that recognizes a tumor antigen.

C44. The foregoing method of C43, wherein the tumor antigen is a neoantigen.

C45. The foregoing method of C43, wherein the tumor antigen is a patient specific neoantigen.

C46. The foregoing method of C1-C45, wherein the TCR gene sequence is a patient specific TCR gene sequence.

C47. The foregoing method of C-C46, wherein the method further comprises culturing the cell.

C48. The foregoing method of C47, wherein the culturing is conducted in the presence of at least one cytokine.

C49. The foregoing method of C47 or C48, wherein the culturing is conducted in the presence of IL2, IL7, IL15, or any combination thereof.

C50. The foregoing method of C47 or C48, wherein the culturing is conducted in the presence of IL7 and IL15.

D. In certain non-limiting embodiments, the presently disclosed subject matter provides for a method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell of any one of A-A20 or B-B50.

D1. The foregoing method of D, wherein prior to administering the therapeutically effective amount of cells, a non-myeloablative lymphodepletion regimen is administered to the subject.

D2. The foregoing method of D or D1, wherein the cancer is a solid tumor.

D3. The foregoing method of D or D1, wherein the cancer is a liquid tumor.

D4. The foregoing method of D2, wherein the solid tumor is selected from the group consisting of melanoma, thoracic cancer, lung cancer, ovarian cancer, breast cancer, pancreatic cancer, head and neck cancer, prostate cancer, gynecological cancer, central nervous system cancer, cutaneous cancer, HPV+ cancer, esophageal cancer, thyroid cancer, gastric cancer, hepatocellular cancer, cholangiocarcinomas, renal cell cancers, testicular cancer, sarcomas, and colorectal cancer.

D5. The foregoing method of D3, wherein the liquid tumor is selected from the group consisting of follicular lymphoma, leukemia, and multiple myeloma.

E. In certain non-limiting embodiments, the presently disclosed subject matter provides for a composition comprising an effective amount of a cell of any one of A-A20 or B-B50.

E1. The foregoing composition of E, wherein the composition is a pharmaceutical composition that further comprises a pharmaceutically acceptable excipient.

E2. The foregoing composition of E or E1, wherein the composition is administered to a patient in need thereof for the treatment of cancer.

E3. The foregoing composition of E-E2, wherein the composition comprises a cryopreservation agent.

E4. The foregoing composition of E-E3, wherein the composition comprises serum albumin.

E5. The foregoing composition of E-E4, wherein the composition comprises Plasma-Lyte A, HSA, and CryoStor CS10.

F. In certain non-limiting embodiments, the presently disclosed subject matter provides for a kit comprising the cell of any one of A-A20 or B-B50, reagents for performing the method of any one of C-C50, or a composition of any one of E-E5.

F1. The foregoing kit of F, wherein the kit further comprises written instructions for treating a cancer.

EXAMPLES

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T.E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A.L. Lehninger, Biochemistry (Worth Publishers, Inc., current addition); Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pennsylvania: Mack Publishing Company,

1990); Carey and Sundberg Advanced Organic Chemistry 3rd Ed. (Plenum Press) Vols A and B(1992).

Provided herein are examples of engineering T cells to express a NeoTCR and knockout the expression of TET2 to create a personalized adoptive T cell therapy (i.e., a TET2 Product) which is composed of apheresis-derived, patient-autologous, CD8 and CD4 T cells that have been precision genome engineered to express an autologous T cell receptor targeting a neoepitope presented exclusively on the surface of the patient's tumor cells (neoTCR), wherein the TET2 Product comprises T cells with a young phenotype.

Example 1. Target Integration of the NeoTCR

Neoepitope-specific TCRs identified by the imPACT Isolation Technology described in PCT/US2020/17887 (which is herein incorporated by reference in its entirety) were used to generate homologous recombination (HR) DNA templates. These HR templates were transfected into primary human T cells in tandem with site-specific nucleases (*see* **Figures 2A-2C**, and **Figure 3**). The single-step non-viral precision genome engineering resulted in the seamless replacement of the endogenous TCR with the patient's neoepitope-specific TCR, expressed by the endogenous promoter. The TCR expressed on the surface is entirely native in sequence.

The precision of neoTCR-T cell genome engineering was evaluated by Targeted Locus Amplification (TLA) for off-target integration hot spots or translocations, and by next generation sequencing based off-target cleavage assays and found to lack evidence of unintended outcomes.

As shown in **Figures 2A-2C**, constructs containing genes of interest were inserted into endogenous loci. This was accomplished with the use of homologous repair templates containing the coding sequence of the gene of interest flanked by left and right HR arms. In addition to the HR arms, the gene of interest was sandwiched between 2A peptides, a protease cleavage site that is upstream of the 2A peptide to remove the 2A peptide from the upstream translated gene of interest, and signal sequences (**Figure 2B**). Once integrated into the genome, the gene of interested expression gene cassette was transcribed as single messenger RNA. During the translation of this gene of interest in messenger RNA, the flanking regions were unlinked from the gene of interest by the self-cleaving 2A peptide and the protease cleavage site was cleaved for the removal of the 2A peptide upstream from the translated gene of interest (**Figure 2C**). In addition to the 2A peptide and protease cleavage site, a gly-ser-gly (GSG)

linker was inserted before each 2A peptide to further enhance the separation of the gene of interest from the other elements in the expression cassette.

It was determined that P2A peptides were superior to other 2A peptides for NeoTCR and TET2 Products because of its efficient cleavage. Accordingly, two (2) P2A peptides and codon divergence were used to express the gene of interest without introducing any exogenous epitopes from remaining amino acids on either end of the gene of interest from the P2A peptide. The benefit of the gene edited cell having no exogenous epitopes (i.e., no flanking P2A peptide amino acids on either side of the gene of interest) is that immunogenicity is drastically decreased and there is less likelihood of a patient infused with a NeoTCR and TET2 Products containing the gene edited cell to have an immune reaction against the gene edited cell.

As described in PCT/US/2018/058230, NeoTCRs were integrated into the TCRα locus of T cells. Specifically, a homologous repair template containing a NeoTCR coding sequence flanked by left and right HR Arms was used. In addition, the endogenous TCRβ locus was disrupted leading to the expression of only TCR sequences encoded by the NeoTCR construct. The general strategy was applied using circular HR templates as well as with linear templates.

The neoantigen-specific TCR construct design is diagrammed in **Figures 3A and 3B**. The target TCRα locus (Cα) is shown along with the plasmid HR template, and the resulting edited sequence and downstream mRNA/protein products are shown. The target TCRα locus (endogenous TRAC) and its CRISPR Cas9 target site (horizontal stripe, cleavage site designated by arrow) are shown (**Figure 3A**). The circular plasmid HR template with the polynucleotide encoding the NeoTCR, which is located between left and right homology arms ("LHA" and "RHA" respectively), is shown (**Figure 3A**). The region of the TRAC introduced by the HR template that was codon optimized is shown (vertical stripe). The TCRβ constant domain was derived from TRBC2, which is indicated as being functionally equivalent to TRBC1. Other elements in the NeoTCR cassette include: 2A = 2A ribosome skipping element (by way of non-limiting example, the 2A peptides used in the cassette are both P2A sequences that are used in combination with codon divergence to eliminate any otherwise occurring non-endogenous epitopes in the translated product); P = protease cleavage site upstream of 2A that removes the 2A tag from the upstream TCRβ protein (by way of non-limiting example the protease cleavage site can be a furin protease cleavage site); SS = signal sequences (by way of non-limited example the protease cleavage site can be a human growth hormone signal sequence). The HR template of the NeoTCR expression gene cassette includes two flanking homology arms to direct insertion into the TCRα genomic locus targeted by the CRISPR Cas9 nuclease RNP with the TCRα guide RNA. These homology arms (LHA and RHA) flank the neoE-specific TCR

sequences of the NeoTCR expression gene cassette. While the protease cleavage site used in this example was a furin protease cleavage site, any appropriate protease cleavage site known to one of skill in the art could be used. Similarly, while HGH was the signal sequence chosen for this example, any signal sequence known to one of skill in the art could be selected based on the desired trafficking and used.

Once integrated into the genome (**Figure 2C**), the NeoTCR expression gene cassette is transcribed as a single messenger RNA from the endogenous TCRα promoter, which still includes a portion of the endogenous TCRα polypeptide from that individual T cell (**Figure 2C**). During ribosomal polypeptide translation of this single NeoTCR messenger RNA, the NeoTCR sequences are unlinked from the endogenous, CRISPR-disrupted TCRα polypeptide by self-cleavage at a P2A peptide (**Figure 2C**). The encoded NeoTCRα and NeoTCRβ polypeptides are also unlinked from each other through cleavage by the endogenous cellular human furin protease and a second self-cleaving P2A sequence motifs included in the NeoTCR expression gene cassette (**Figure 2C**). The NeoTCRα and NeoTCRβ polypeptides are separately targeted by signal leader sequences (derived from the human growth hormone, HGH) to the endoplasmic reticulum for multimer assembly and trafficking of the NeoTCR protein complexes to the T cell surface. The inclusion of the furin protease cleavage site facilitates the removal of the 2A sequence from the upstream TCRβ chain to reduce potential interference with TCRβ function. Inclusion of a gly-ser-gly linker before each 2A (not shown) further enhances the separation of the three polypeptides.

Additionally, three repeated protein sequences are codon diverged within the HR template to promote genomic stability. The two P2A are codon diverged relative to each other, as well as the two HGH signal sequences relative to each other, within the TCR gene cassette to promote stability of the introduced NeoTCR cassette sequences within the genome of the *ex vivo* engineered T cells. Similarly, the re-introduced 5' end of TRAC exon 1 (vertical stripe) reduces the likelihood of the entire cassette being lost over time through the removal of intervening sequence of two direct repeats.

In addition to NeoTCR Products, this method can be used for any TET2 Product.

Figure 3 shows the results of an In-Out PCR confirming precise target integration of the NeoE TCR cassette. Agarose gels show the results of a PCR using primers specific to the integration cassette and site generate products of the expected size only for cells treated with both nuclease and DNA template (KOKI and KOKIKO), demonstrating site-specific and precise integration.

Furthermore, Targeted Locus Amplification (TLA) was used to confirm the specificity of targeted integration. Crosslinking, ligation, and use of primers specific to the

NeoTCR insert were used to obtain sequences around the site(s) of integration. The reads mapped to the genome are binned in 10 kb intervals. Significant read depths were obtained only around the intended site the integration site on chromosome 14, showing no evidence of common off-target insertion sites.

Antibody staining for endogenous TCR and peptide-HLA staining for neoTCR reveals that the engineering results in high frequency knock-in of the NeoTCR, with some TCR- cells and few WT T cells remaining (**Figure 4A**). Knock-in is evidenced by neoTCR expression in the absence of an exogenous promoter. Engineering was carried out multiple times using the same neoTCR with similar results (**Figure 4B**). Therefore, efficient and consistent expression of the NeoTCR and knockout of the endogenous TCR in engineered T cells was achieved.

Example 2. Generation of TET2 Knockout NeoTCR-P1 T cells

Materials and Methods. T cells were transfected with gRNAs targeting the first exon of TET2 as Cas9 RNPs along with the TRA and TRB gRNA-Cas9 RNPs as described in Example 1 and in PCT/US2020/17887 (which is herein incorporated by reference in its entirety), resulting in disruption of the endogenous TET2 coding sequence and reduced TET2 protein expression (i.e., a TET2 Product).

T cell Isolation and Editing. TET2 Products were made by first isolating CD4 and CD8 T cells from leukopaks of donors and then transfecting the cells with the TCRα and TCRβ gRNAs (using the NeoTCR integration methods disclosed in **Example 1**) along with a TET2 gRNA to knockout the TET2 gene. While any gRNA that specifically binds to TET2, disrupts the TET2 gene, and knocks out the TET2 gene expression can be used, the exemplary gRNAs provided in **Table 2** were used herein. sgRNA 1 and 5 are negative strand sgRNAs and sgRNAs 2-4 are positive strand sgRNAs.

Table 2. Exemplary Primers for Regions Flanking the gRNA

sgRNA 1	CCTCCCATTTGCCAGACAGAACC	SEQ ID NO: 1	**Figure 5A**
sgRNA 2	TTAAGGGAAGTGAAAATAGAGGG	SEQ ID NO: 2	**Figure 5B**
sgRNA 3	GGAATGACATACAGACTGCAGGG	SEQ ID NO: 3	**Figure 5C**
sgRNA 4	GATAGAACCAACCATGTTGAGGG	SEQ ID NO: 4	**Figure 5D**
sgRNA 5	CCAACCATGTTGAGGGCAACAGA	SEQ ID NO: 5	**Figure 5E**

gRNA Selection and Reactions. TET2 gRNA-Cas9 RNP edited CD4 and CD8 T cells described above (i.e., TET2 Products) were lysed and DNA was extracted. Regions

flanking the gRNA target sites were amplified via PCR using the exemplary primers provided in **Table 3**. Alternatively, redesigned primers with more specificity or alternate primers designed to accomplish the amplification could also be used. PCR amplicons were then incubated with T7 endonuclease I (NEB) and resulting products were run on agarose gels (**Figures 6A** and **6B**).

Table 3. Exemplary Primers for Regions Flanking the gRNA

TET2_1 Fwd	GATCAGGAGGAGGCACAGTG	SEQ ID NO: 6
TET2_1 Rev	AGGAGCCCAGAGAGAGAAGG	SEQ ID NO: 7
TET2_2 Fwd	GTTTCTGCCTCTTCCGTGGA	SEQ ID NO: 8
TET2_2 Rev	TGTTGGGGGCACAAGATCTC	SEQ ID NO: 9

The TET2_1 Fwd primer (SEQ ID NO:6) was used for analyzing sgRNA indels for sgRNA 1 (SEQ ID NO:1), 4 (SEQ ID NO:4), and 5 (SEQ ID NO:5). The expected product size for this reaction is 849 bp.

The TET2_1 Rev primer (SEQ ID NO:7) was used for analyzing sgRNA indels for sgRNA 1 (SEQ ID NO:1), 4 (SEQ ID NO:4), and 5 (SEQ ID NO:5). The expected product size for this reaction is 849 bp.

The TET2_2 Fwd primer (SEQ ID NO:8) was used for analyzing sgRNA indels for sgRNA 2 (SEQ ID NO:2) and 3 (SEQ ID NO:3). The expected product size for this reaction is 1032 bp.

The TET2_2 Rev primer (SEQ ID NO:9) was used for analyzing sgRNA indels for sgRNA (SEQ ID NO:2) and 3 (SEQ ID NO:3). The expected product size for this reaction is 1032 bp.

The optimal gRNA was determined by assessing the TET2 knock out (KO) via a T7 endonuclease I (T7EI) assay to detect non-perfectly annealed PCR products. Further characterization of loss of gene expression was determined by qPCR quantification of TET2 mRNA and Western blot for TET2 protein in neoTCR T cells nucleofected with TET2 gRNA. T cells untreated with TET2 gRNA were used as a negative control for TET2 gene and protein expression. The T7EI assay quantified the gRNA targeting efficiency by measuring the fraction of the PCR product that was cleaved by the T7EI enzyme. TET2 mRNA transcript abundance was determined by qPCR. TET2 protein abundance was determined via Western blot using an anti-TET2 antibody. Successful knock out of the TET2 gene resulted in reduced mRNA and protein levels in the TET2 Products compared to that of the NeoTCR Products.

crispr-dav (https://github.com/pinetree1/crispr-dav/blob/master/Install-and-Run.md#description-of-files-created-by-prepare_runpl-script) analysis was performed to

identify the most efficient gRNA for generating outframe indels. Coverage of the PCR amplicons was robust for all amplicons surrounding the guide site. *See*, **Figures 7A-7E**.

Five distinct guide RNAs were designed to target the first coding exon of TET2 and robustly edit and lead to >70% out of frame indels as assessed by the T7E1 cutting efficiency assay as well as PCR amplicon DNA transposon-based sequencing (**Figures 8A** and **8B**).

To confirm loss of TET2 activity, intracellular staining of 5-hmC using a polyclonal anti-5hmC antibody (Active Motif; 1 ug/mL; Cat No 39791) was performed. Specifically, the 5-hmC Flow Cytometry experiment was performed as follows: 1) 200K T cells from WT and each gRNA nucleofected sample were stimulated for 1.5h on a plate coated PACT035 comPACT, 2) samples were then harvest and stained for Live/Dead and then fixed with BD ICS Fix/Perm followed by 2 washes and resuspension in Staining Buffer, 3) the cells were permeabilized for 15min in Perm Buffer, 4) the cells were treated with DNaseI (300ug/mL) in Perm Buffer for 3hr at 37C (wanted to treat for 1hr), 5) the cells were washed 2X with Perm Buffer, 6) the cells were stained with anti-5-hmC (1ug/mL) in Perm Buffer for 30min at 4C, 7) the cells were washed 2X with Perm Buffer, 8) the cells were stained with anti-Rabbit IgG-AF647 (5uL/50uL) for 30min at 4C, 9) the cells were 2X with Perm Buffer, and 10) the cells were resuspend in 200uL for processing with a flow cytometer.

The 5-hmC staining showed that there were no appreciable differences in 5-hmC state of resting TET2 Products v. stimulated TET2 Products. No appreciable reduction in 5-hmC was observed in the absence of TET2 with or without 1.5h stimulation with cognate comPACT plate coating. *See* **Figure 9**.

To further characterize the effects of TET2 disruption, ATAC-seq (as described in Buenrostro et al., 2013, N. Methods, 10(12) 1213-1218) was performed on the TET2 Products to determine the genome-wide chromatin state of the cells.

TCF7 and TBET staining of the resting TET2 Products was also performed (**Figure 10**). It was shown that knocking out TET2 had no effect on the expression of the TCF7 and TBET transcription factors.

The ability of TET2 Products to produce IFNγ was tested (**Figure 11A**). It was determined that knocking out TET2 reduced the frequency of IFNγ producing T cells compared to NeoTCR Products.

TET2 Products were tested to determine if the number of CD107a+ cells would be affected by the knockout (**Figure 11B**). It was determined that knocking out TET2 reduced the frequency of CD107a+ T cells compared to NeoTCR Products.

Multiple rounds of IncuCyte experiments were performed using TET2 Products (the "PACT-035-TCR089" cells) and NeoTCR Products (the "PACT035-TCR089 TET2 gRNA3"

cells) (**Figures 12A** and **12B**). In these experiments, 14 day post gene editing cells and > 30 day post gene editing cells from the TET2 Products and NeoTCR Products were used. It was observed that the >30 day TET2 Product cells were more effective killers than the >30 day NeoTCR Product cells (data not shown), however, there were no differences between 14 day cells of both products (**Figures 12A** and **12B**). In both cases, controls were performed and the TET2 Product cells and NeoTCR Product cells did not kill SW620 cells lacking the COX6C R20Q mutation. These IncuCyte experiments demonstrate that the number of days post gene editing of cells to engineer a TET2 deficiency affects the phenotype of the cells.

TET2 disruption enhances CD8 memory cell differentiation (Carty et al., 2018; Fraietta et al., 2018, Nature, 558(7709), 307–312). The phenotypic state of the edited cells was assessed via flow cytometry after staining with antibodies against CCR7 and CD27. Based on these experiments, it was shown that TET2 Products are enriched for expression of CCR7 and CD27 as compared to control T cells and NeoTCR Products indicating that TET2 Products comprise Tcm T cells.

CD27 is a member of the TNF receptor family. It is also constitutively expressed on central memory T (Tcm) and naive T cells (Tn). To determine if the TET2 Product cells had a memory-like phenotype, the TET2 Product cells were stimulated for 7 days via cognate comPACT coating. This stimulation revealed an increased frequency of cells expressing CD27 (**Figure 14B**) and CCR7 (**Figure 14A**) among TET2 Product cells. In other words, the TET2 Product cells were shown to have a memory-like phenotype.

Cytokine and granzyme assays were performed on the TET2 Products. Specifically, intracellular staining and detection via flow cytometry and neoantigen-specific killing assays were performed. The results of these experiments showed that TET2 Products produce higher levels of effector cytokines and release more cytotoxic granules than cells that express TET2 (e.g., NeoTCR Products).

Lastly, the expression of TOX and TCF7 were characterized in NeoTCR Product cells and TET2 Product cells. It was shown that both TOX and TCF7 expression were unchanged between NeoTCR Product cells and TET2 Product cells at 1) resting state, 2) 7 days following stimulation, and 3) 9 days following stimulation. *See* **Figures 15A-15D**).

Example 3. Methods and Materials used for the generation of TET2 Products

Materials and Methods. T cells were transfected with gRNAs targeting the first exon of TET2 as Cas9 RNPs along with the TRA and TRB gRNA-Cas9 RNPs as described in Example 1 and in PCT/US2020/17887 (which is herein incorporated by reference in its entirety),

resulting in disruption of the endogenous TET2 coding sequence and reduced TET2 protein expression.

T cell Isolation and Editing. CD4 and CD8 T cells were isolated from healthy donor PBMCs. By means of example, isolation of such cells can be achieved using the Miltenyi Prodigy or Miltenyi MACS separation columns according to the manufacturers' instructions. Positively-selected CD4 and CD8 T cells (for example, using Miltenyi antibodies and isolation column) were used fresh or cryopreserved in 1% human serum albumin, 49% plasmalyte (Baxter), and 50% CS10 (Sigma). Cryopreserved cells were thawed, washed in TexMACS (Miltenyi) + 10% human AB serum (Valley Biomedical), and seeded at a density of 2 x 106 cells per mL in TexMACS + 3% human AB serum (culture medium). One day after thawing, or immediately if used fresh, the cells were washed and re-seeded at a density of 1.46 x 106 cells per mL in culture medium + 12.5 ng/mL IL7 + 12.5 ng/mL IL15 + 1:17.5 ratio of TransACT T cell activation reagent (all reagents from Miltenyi) by volume. Two days after activation, T cells were electroporated with a plasmid comprising the NeoTCR of interest.

comPACT and comPACT-Dextramer preparation. Neoantigen-specific peptide-HLA complex polypeptides (i.e., each a "comPACT") were prepared according to the method as described in PCT/US2019/025415, hereby incorporated by reference in its entirety. A comPACT-dextramer complex was made for the labeling of neoTCR expressing T cells. Biotinylated comPACT protein was incubated with a streptavidin-conjugated fluorophore for 10 min at room temperature (RT). Biotin-40-dextran (NANOCS) was added to the mixture and incubated at RT for an additional 10 minutes. The comPACT-Dextramers were stored at 4ºC.

Confirmation of comPACT binding to neoTCR edited T cells. T cells were stained for flow cytometry on the indicated days. Cells were first stained with viability dye for 20 minutes at 4ºC, then washed and stained with the comPACT-dextramer for 10 minutes at 4ºC. Surface antibodies (anti-CD8α, anti-CD8β, anti-CD4) were added to the suspension of cells and comPACT-dextramer, and the cells were incubated for an additional 20 minutes at 4ºC. Cells were then washed and fixed in intracellular fixation buffer (BD Biosciences). All cells were acquired on an Attune NxT Flow Cytometer (ThermoFisher Scientific) and data was analyzed with either FCS Express or FlowJo.

Cytometric Bead Array (CBA). Streptavidin coated plates (Eagle Biosciences) were washed 3 times with wash buffer (PBS supplemented with 1% BSA and 0.05% tween20) and then coated with comPACTs at different concentrations ranging from 100-0.01 ng/well. Wells with no comPACTs and wells coated with mismatched comPACTs as used as controls. The plates were incubated for 2 hr at room temperature, washed three times with wash buffer, and then washed three times with TexMACS supplemented with 3% human AB serum to remove the

tween20. T cells were given two washes with TexMACS supplemented with 3% human AB serum and resuspended at 1 million cells/mL in TexMACS supplemented with 3% human AB serum and 1X penicillin-streptomycin solution. T cells were plated onto the comPACT coated plate at 100 µL/well and incubated at 37°C, 5% CO2. After 24h the supernatant was collected, and the cytokine concentrations were analyzed using the BD Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II (Catalog No. 551809) following the manufacturer's protocol. Capture beads were mixed with culture supernatant, incubated with the detection reagent for 3 hr at RT protected from light, washed, and resuspended in wash buffer. Samples were assayed on an Attune NxT Flow Cytometer and data analyzed with FlowJo. The EC50 represents the concentration of cognate comPACT that elicits 50% of the maximum response and was calculated utilizing a least-squares fit of IFNγ secretion over a range of comPACT concentrations.

Intracellular Staining. T cells were stained for flow cytometry on the indicated days. T cells were first stained with viability dye for 20 minutes at 4°C, then washed and incubated with surface antibodies (anti-CD8α, anti-CD8β, anti-CD4) for an additional 20 minutes at 4°C. T cells were then washed and permeabilized for intracellular staining. T cells were stained with anti-2A peptide or with anti-IFNγ, anti-TNF, or anti-IL2 in permeabilization buffer for 20 minutes at 4°C. T cells were fixed in intracellular fixation buffer (BD Biosciences). Samples were assayed on an Attune NxT Flow Cytometer (ThermoFisher Scientific) and data analyzed with either FCS Express or FlowJo.

T cell Proliferation Assay. Edited CD4 and CD8 T cells were labeled with the e450 proliferation dye (eBioscience) according to the manufacturer's instructions. Labeled cells were stimulated on comPACT coated plates with a range of concentrations as described above. T cells were harvested over 48-96 hours and analyzed for proliferation as measured by dilution of the e450 dye.

T cell Killing Assay. HLA-matched cell lines were pulsed with the cognate neoantigen peptide or mismatched peptide for 1h at 37°C, 5% CO2. The cells were washed 3 times with media to remove any unbound peptide and then co-cultured with edited CD4 and CD8 T cells that are labeled with the e450 proliferation dye described above. Co-cultures were incubated for 48h at 37°C with 5% CO2 before harvest. Cells were washed and stained with a fixable viability dye to determine killing efficiency. The e450 proliferation dye is used to distinguish edited T cells from target cells.

While the present invention has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should

be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the invention.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.

WHAT IS CLAIMED IS:

1. A cell, comprising:
    a. an exogenous T cell receptor (TCR); and
    b. a gene disruption of a TET2 locus.
2. The cell of claim 1, wherein the gene disruption comprises a substitution, a deletion, an insertion, or any combination thereof.
3. The cell of claim 1 or 2, wherein the gene disruption comprises a missense mutation, a nonsense mutation, a non-frameshift deletion, a frameshift deletion, a non-frameshift insertion, a frameshift insertion, or any combination thereof.
4. The cell of any one of claims 1-3, wherein the gene disruption of the TET2 locus results in a non-functional TET2 protein.
5. The cell of any one of claims 1-3, wherein the gene disruption of the TET2 locus results in knockout of the TET2 gene expression.
6. The cell of any one of claims 1-5, comprising a gRNA and a Cas9 nuclease.
7. The cell of claim 6, wherein the gRNA comprises a nucleotide sequence set forth in SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, or SEQ ID NO.5.
8. The cell of any one of claims 1-7, wherein the gene disruption of the TET2 locus enhances cell persistence.
9. The cell of any one of claims 1-8, wherein the gene disruption of the TET2 locus enhances memory cell differentiation.
10. The cell of any one of claims 1-9, wherein the cell is a primary cell.
11. The cell of any one of claims 1-10, wherein the cell is a patient-derived cell.
12. The cell of any one of claims 1-11, wherein the cell is a lymphocyte.
13. The cell of any one of claims 1-12, wherein the cell is a T cell.
14. The cell of claim 13, wherein the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+.
15. The cell of claim 13, wherein the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+.
16. The cell of claim 13, wherein the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.
17. The cell of any one of claims 1-16, wherein the exogenous TCR is a patient-derived TCR.

18. The cell of any one of claims 1-17, wherein the exogenous TCR comprises a signal sequence, a first and second 2A sequence, and a TCR polypeptide sequence.
19. The cell of any one of claims 1-18, wherein the exogenous TCR recognizes a cancer antigen.
20. The cell of claim 19, wherein the cancer antigen is a neoantigen.
21. The cell of claim 19, wherein the cancer antigen is a patient specific antigen.
22. A cell modified by a process, the process comprising:
    a. introducing into a cell a homologous recombination (HR) template nucleic acid sequence;
    b. recombining the HR template nucleic acid into an endogenous locus of the cell; and
    c. disrupting a TET2 locus of the cell.
23. The cell of claim 22, wherein the HR template comprises:
    a. first and second homology arms homologous to first and second target nucleic acid sequences; and
    b. a TCR gene sequence positioned between the first and second homology arms.
24. The cell of claim 22 or 23, wherein the HR template comprises a first 2A-coding sequence positioned upstream of the TCR gene sequence and a second 2A-coding sequence positioned downstream of the TCR gene sequence, wherein the first and second 2A-coding sequences code for the same amino acid sequence that are codon-diverged relative to each other.
25. The cell of claim 24, wherein the 2A-coding sequence is a P2A-coding sequence.
26. The cell of claim 24 or 25, wherein a sequence coding for the amino acid sequence Gly Ser Gly is positioned immediately upstream of the 2A-coding sequences.
27. The cell of any one of claims 22-26, wherein the HR template comprises a sequence coding for a Furin cleavage site positioned upstream of the second 2A-coding sequence.
28. The cell of any one of claims 23-27, wherein the first and second homology arms are each from about 300 bases to about 2,000 bases in length.
29. The cell of any one of claims 23-28, wherein the first and second homology arms are each from about 600 bases to about 2,000 bases in length.

30. The cell of any one of claims 24-29, wherein the HR template further comprises a signal sequence positioned between the first 2A-coding sequence and the TCR gene sequence.
31. The cell of any one of claims 24-30, wherein the HR template comprises a second TCR sequence positioned between the second 2A-coding sequence and the second homology arm.
32. The cell of claim 31, wherein the HR template comprises:
    a. a first signal sequence positioned between the first 2A-coding sequence and the first TCR gene sequence; and
    b. a second signal sequence positioned between the second 2A-coding sequence and the second TCR gene sequence;
    c. wherein the first and the second signal sequences encode for the same amino acid sequence and are codon diverged relative to each other.
33. The cell of claim 30 or 32, wherein the signal sequence is a human growth hormone signal sequence.
34. The cell of any one of claims 22-33, wherein the HR template is non-viral.
35. The cell of any one of claims 22-34, wherein the HR template is a circular DNA.
36. The cell of any one of claims 22-34, wherein the HR template is a linear DNA.
37. The cell of any one of claims 22-36, wherein the introducing occurs via electroporation.
38. The cell of any one of claims 22-37, wherein the recombining comprises:
    a. cleavage of the endogenous locus by a nuclease; and
    b. recombination of the HR template nucleic acid sequence into the endogenous locus by homology directed repair.
39. The cell of claim 38, wherein the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof.
40. The cell of claim 39, further comprising a gRNA.
41. The cell of any one of claims 22-40, wherein the disrupting comprises introducing a substitution, a deletion, an insertion, or any combination thereof.
42. The cell of any one of claims 22-41, wherein the disrupting comprises introducing a missense mutation, a nonsense mutation, a non-frameshift deletion, a frameshift deletion, a non-frameshift insertion, a frameshift insertion, or any combination thereof.

43. The cell of any one of claims 22-43, wherein the disrupting results in a non-functional TET2 protein.
44. The cell of any one of claims 22-44, wherein the disrupting results in knockout of the TET2 gene expression.
45. The cell of any one of claims 22-44, wherein the disrupting comprises cleavage of the TET2 locus by a nuclease.
46. The cell of claim 45, wherein the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof.
47. The cell of claim 46, further comprising a gRNA.
48. The cell of claim 47, wherein the gRNA comprises a sequence set forth in SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, or SEQ ID NO.5.
49. The cell of claim 45 or 46, wherein the nuclease is expressed by a vector.
50. The cell of claim 47 or 48, wherein the gRNA is expressed by a vector.
51. The cell of claim 49 or 50, wherein the vector is a viral vector.
52. The cell of claim 49 or 50, wherein the vector is a non-viral vector.
53. The cell of any one of claims 22-52, wherein the disrupting of the TET2 locus enhances cell persistence.
54. The cell of any one of claims 22-53, wherein the disrupting of the TET2 locus enhances memory cell differentiation.
55. The cell of any one of claims 22-54, wherein the cell is a primary cell.
56. The cell of any one of claims 22-55, wherein the cell is a patient-derived cell.
57. The cell of any one of claims 22-56, wherein the cell is a lymphocyte.
58. The cell of any one of claims 22-57, wherein the cell is a T cell.
59. The cell of claim 58, wherein the T cell is a patient-derived cell.
60. The cell of any one of claims 22-59, wherein the cell is a young T cell.
61. The cell of claim 60, wherein the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+.
62. The cell of claim 60, wherein the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+.
63. The cell of claim 60, wherein the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.
64. The cell of any one of claims 22-63, wherein the endogenous locus is within an endogenous TCR gene.

65. The cell of any one of claims 23-64, wherein the TCR gene sequence encodes for a TCR that recognizes a tumor antigen.
66. The cell of claim 65, wherein the tumor antigen is a neoantigen.
67. The cell of claim 65, wherein the tumor antigen is a patient specific neoantigen.
68. The cell of any one of claims 23-67, wherein the TCR gene sequence is a patient specific TCR gene sequence.
69. The cell of any one of claims 22-68, wherein the process further comprises culturing the cell.
70. The cell of claim 69, wherein the culturing is conducted in the presence of at least one cytokine.
71. The cell of claim 69 or 70, wherein the culturing is conducted in the presence of IL2, IL7, IL15, or any combination thereof.
72. The cell of claim 69 or 70, wherein the culturing is conducted in the presence of IL7 and IL15.
73. A method of modifying a cell, the method comprising:
    a. introducing into a cell a homologous recombination (HR) template nucleic acid sequence;
    b. recombining the HR template nucleic acid into an endogenous locus of the cell; and
    c. disrupting a TET2 locus of the cell.
74. The method of claim 73, wherein the HR template comprises:
    a. first and second homology arms homologous to first and second target nucleic acid sequences; and
    b. a TCR gene sequence positioned between the first and second homology arms.
75. The method of claim 73 or 74, wherein the HR template comprises a first 2A-coding sequence positioned upstream of the TCR gene sequence and a second 2A-coding sequence positioned downstream of the TCR gene sequence, wherein the first and second 2A-coding sequences code for the same amino acid sequence that are codon-diverged relative to each other.
76. The method of claim 74 or 75, wherein the 2A-coding sequence is a P2A-coding sequence.

77. The method of any one of claims 74-76, wherein a sequence coding for the amino acid sequence Gly Ser Gly is positioned immediately upstream of the 2A-coding sequences.
78. The method of any one of claims 75-77, wherein the HR template comprises a sequence coding for a Furin cleavage site positioned upstream of the second 2A-coding sequence.
79. The method of any one of claims 74-78, wherein the first and second homology arms are each from about 300 bases to about 2,000 bases in length.
80. The method of any one of claims 74-79, wherein the first and second homology arms are each from about 600 bases to about 2,000 bases in length.
81. The method of any one of claims 75-80, wherein the HR template further comprises a signal sequence positioned between the first 2A-coding sequence and the TCR gene sequence.
82. The method of any one of claims 75-81, wherein the HR template comprises a second TCR sequence positioned between the second 2A-coding sequence and the second homology arm.
83. The method of claim 82, wherein the HR template comprises:
    a. a first signal sequence positioned between the first 2A-coding sequence and the first TCR gene sequence; and
    b. a second signal sequence positioned between the second 2A-coding sequence and the second TCR gene sequence;
    wherein the first and the second signal sequences encode for the same amino acid sequence and are codon diverged relative to each other.
84. The method of claim 81 or 83, wherein the signal sequence is a human growth hormone signal sequence.
85. The method of any one of claims 73-84, wherein the HR template is non-viral.
86. The method of any one of claims 73-85, wherein the HR template is a circular DNA.
87. The method of any one of claims 73-85, wherein the HR template is a linear DNA.
88. The method of any one of claims 73-87, wherein the introducing occurs via electroporation.
89. The method of any one of claims 73-88, wherein the recombining comprises:
    a. cleavage of the endogenous locus by a nuclease; and

b. recombination of the HR template nucleic acid sequence into the endogenous locus by homology directed repair.

90. The method of claim 89, wherein the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof.

91. The method of claim 90, further comprising a gRNA.

92. The method of any one of claims 73-91, wherein the disrupting comprises introducing a substitution, a deletion, an insertion, or any combination thereof.

93. The method of any one of claims 73-92, wherein the disrupting comprises introducing a missense mutation, a nonsense mutation, a non-frameshift deletion, a frameshift deletion, a non-frameshift insertion, a frameshift insertion, or any combination thereof.

94. The method of any one of claims 73-93, wherein the disrupting results in a non-functional TET2 protein.

95. The method of any one of claims 73-94, wherein the disrupting results in knockout of the TET2 gene expression.

96. The method of any one of claims 73-95, wherein the disrupting comprises cleavage of the TET2 locus by a nuclease.

97. The method of claim 96, wherein the nuclease is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease, or derivative thereof.

98. The method of claim 97, further comprising a gRNA.

99. The method of claim 98, wherein the gRNA comprises a sequence set forth in SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, or SEQ ID NO.5.

100. The method of claim 96 or 97, wherein the nuclease is expressed by a vector.

101. The method of claim 98 or 99, wherein the gRNA is expressed by a vector.

102. The method of claim 96 or 97, wherein the vector is a viral vector.

103. The method of claim 96 or 97, wherein the vector is a non-viral vector.

104. The method of any one of claims 73-103, wherein the disrupting of the TET2 locus enhances cell persistence.

105. The method of any one of claims 73-104, wherein the disrupting of the TET2 locus enhances memory cell differentiation.

106. The method of any one of claims 73-105, wherein the cell is a primary cell.

107. The method of any one of claims 73-106, wherein the cell is a patient-derived cell.

108. The method of any one of claims 73-107, wherein the cell is a lymphocyte.

109. The method of any one of claims 73-108, wherein the cell is a T cell.

110. The method of claim 109, wherein the T cell is a patient-derived cell.

111. The method of any one of claims 73-103, wherein the cell is a young T cell.

112. The method of claim 111, wherein the cell is CD45RA+, CD62L+, CD28+, CD95-, CCR7+, and CD27+.

113. The method of claim 111, wherein the cell is CD45RA+, CD62L+, CD28+, CD95+, CD27+, CCR7+.

114. The method of claim 111, wherein the cell is CD45RO+, CD62L+, CD28+, CD95+, CCR7+, CD27+, CD127+.

115. The method of any one of claims 73-114, wherein the endogenous locus is within an endogenous TCR gene.

116. The method of any one of claims 73-115, wherein the TCR gene sequence encodes for a TCR that recognizes a tumor antigen.

117. The method of claim 116, wherein the tumor antigen is a neoantigen.

118. The method of claim 116, wherein the tumor antigen is a patient specific neoantigen.

119. The method of any one of claims 73-118, wherein the TCR gene sequence is a patient specific TCR gene sequence.

120. The method of any one of claims 73-119, wherein the method further comprises culturing the cell.

121. The method of claim 120, wherein the culturing is conducted in the presence of at least one cytokine.

122. The method of claim 120 or 121, wherein the culturing is conducted in the presence of IL2, IL7, IL15, or any combination thereof.

123. The method of claim 120 or 121, wherein the culturing is conducted in the presence of IL7 and IL15.

124. A method of treating cancer in a subject in need thereof, the method comprising administering a therapeutically effective amount of a cell of any one of claims 1-72.

125. The method of claim 124, wherein prior to administering the therapeutically effective amount of cells, a non-myeloablative lymphodepletion regimen is administered to the subject.

126. The method of claim 124 or 125, wherein the cancer is a solid tumor.

127. The method of claim 124 or 125, wherein the cancer is a liquid tumor.

128. The method of claim 126, wherein the solid tumor is selected from the group consisting of melanoma, thoracic cancer, lung cancer, ovarian cancer, breast cancer, pancreatic cancer, head and neck cancer, prostate cancer, gynecological cancer, central nervous system cancer, cutaneous cancer, HPV+ cancer, esophageal cancer, thyroid cancer, gastric cancer, hepatocellular cancer, cholangiocarcinomas, renal cell cancers, testicular cancer, sarcomas, and colorectal cancer.

129. The method of claim 127, wherein the liquid tumor is selected from the group consisting of follicular lymphoma, leukemia, and multiple myeloma.

130. A composition comprising an effective amount of a cell of any one of claims 1-72.

131. The composition of claim 130, wherein the composition is a pharmaceutical composition that further comprises a pharmaceutically acceptable excipient.

132. The pharmaceutical composition of claim 130 or 131, wherein the composition is administered to a patient in need thereof for the treatment of cancer.

133. The composition of any one of claims 130-132, wherein the composition comprises a cryopreservation agent.

134. The composition of any one of claims 130-133, wherein the composition comprises serum albumin.

135. The composition of any one of claims 130-134, wherein the composition comprises Plasma-Lyte A, HSA, and CryoStor CS10.

136. A kit comprising the cell of any one of claims 1-72, reagents for performing the method of any one of claims 73-124, or a composition of any one of claims 130-135.

137. The kit of claim 136, wherein the kit further comprises written instructions for treating a cancer.