adversely affected by the introduction of DNA in their cytoplasm: high rate of apoptosis is observed when transforming cells with DNA vectors; (2) the CRISPR system requires stable expression of Cas9 in the cells, however, prolonged expression of Cas9 in living cells may lead to chromosomal defects; and (3) the specificity of current RNA-guided endonuclease is determined only by sequences comprising 11 nucleotides (N12-20NGG, where NGG represents the PAM), which makes it very difficult to identify target sequences in desired loci that are unique in the genome. Other nucleases, in addition to Cas9, are Cas12a, zinc finger nucleases (ZFN) or TAL effector nucleases (TALEN).

The present disclosure aims to provide solutions to these limitations in order to efficiently implement RNA-guided endonuclease engineering in host cells such as T cells. There is a need in the art for safer transduction techniques for Chimeric Antigen Receptor constructs that do not include transduction with viral vectors but instead can use transfection techniques. This includes increasing CAR construct transfection efficiency, while avoiding the risk of having viral genes potentially expressed by the transduced cells that are administered to a patient. The present disclosure was made to address this need in the art.

Summary

In one aspect, the present disclosure refers to a method for genetically modifying a primary human T cell at two different genetic loci, comprising introducing into a primary human T cell: a first ribonucleoprotein (RNP) comprising a first RNA-guided endonuclease and a first guide RNA; a second RNP comprising a second RNA-guided endonuclease and a second guide RNA; and a donor DNA molecule comprising at least two nucleic acid modifications;
wherein the first guide RNA comprises a target sequence designed to hybridize with a first target site at a first genetic locus in the target DNA and the donor DNA is inserted into the target DNA molecule at the first target site; further wherein the second guide RNA comprises a second target sequence designed to hybridize with a second target site at a second genetic locus in the target DNA resulting in a mutation at the second target site

In another aspect, the present disclosure refers to an engineered primary T cell comprising:
a non-native genetic construct integrated into the genome at a first genetic locus comprising a first target site of an RNA-guided nuclease and a mutation at a second genetic locus comprising a second target site of an RNA-guided nuclease, wherein the engineered primary T cell is produced by the method as described herein.

In another aspect, the present disclosure refers to a population of primary human T cells transfected with a genetic construct, wherein the cell population comprises cells having a non-native genetic construct integrated into the genome at a first genetic locus, and further have a mutation in a gene at a second genetic locus, wherein at least 25% of the cells of the population express the genetic construct and exhibit reduced expression of the gene at the second genetic locus.

In another aspect, the present disclosure refers to a method for site-specific integration of a donor DNA into a target DNA molecule, comprising:

introducing into a cell:

an RNP comprising a Cas12a endonuclease and an engineered guide RNA; and

a donor DNA molecule comprising at least two nucleic acid modifications;

wherein the guide RNA comprises a target sequence designed to hybridize with a target site in the target DNA and the donor DNA is inserted into the target DNA molecule at the target site.

In yet another aspect, the present disclosure refers to a system for targeted integration of a donor DNA into a target locus, comprising:

a Cas12a endonuclease;

a guide RNA; and

a double-stranded donor DNA molecule, wherein the donor DNA molecule includes one or more phosphorothioate bonds on a single modified strand of the double stranded DNA molecule within ten nucleotides of the 5’ terminus of the modified strand of the double stranded DNA molecule.

In yet another aspect, the present disclosure refers to a primary human T cell having a CAR or DAR construct inserted into the CD7 gene.

In yet another aspect, the present disclosure refers to a population of T cells as described herein.

The present disclosure provides an improved, safer, and commercially efficient process for developing genetically engineered and transduced cells, including cells for immunotherapy. More specifically, the disclosed process comprises introducing an RNA-guided endonuclease, a guide RNA, and a donor DNA construct into host cells, where the guide RNA is engineered to direct the cas protein with which it is complexed to a targeted site of the host genome. Cleavage of the genomic DNA at the target site by the RNA-guided endonuclease and subsequent repair of the double stranded break using the donor fragment that includes homology arms by homology-directed repair (HDR) results in integration of sequences of the donor DNA molecule positioned

between the homology arms. The method can be used to simultaneously knock out a gene at the target locus and insert or “knock in” at the disrupted locus a transgene that is provided in the donor DNA molecule. Further provided are methods for inserting a genetic construct at a first genetic locus, where insertion of the genetic construct knocks out a gene at the first locus, and simultaneously knocking out a gene at a second locus. The knockin / double knock out is achieved by introducing two RNPs into the target cell, a first RNP having a guide targeting the first genetic locus, and a second RNP having a guide targeting the second genetic locus. The two RNPs can comprise the same (e.g., Cas12a) or different (e.g., Cas12a and Cas9) cas proteins. The method can be used on any host cells, including prokaryotic and eukaryotic cells, and can be

used with mammalian cells, such as human cells. The method has advantages in ease of use, efficiency, and the ability to generate genome modifications that do not entail the use of selectable markers or viral vectors that are undesirable in many applications, including clinical applications. In some examples, the host cells are hematopoietic cells, such as, for example, T cells.

The present disclosure also provides systems for targeted integration of a donor DNA into a locus of the genome of a eukaryotic cell. Also provided are donor DNA compositions, where the donor DNA molecule includes one or more modifications to nucleotides of one donor DNA strand. The donor DNA can include homology arms flanking a sequence of interest whose integration into the host genome is desired, where the homology arms have sequences homologous to sequences occurring in the host genome on either side of the target sequence. The donor DNA in some examples is double-stranded, or substantially double-stranded. In various examples the donor DNA includes from one to ten modified nucleotides that are proximal to the 5' end of one strand of the donor DNA, for example, that occur within ten nucleotides or within five nucleotides of the 5' terminus of one strand of the donor DNA. In some examples the donor DNA has at least two types of nucleic acid modification of from one to ten nucleotides at the 5' end of one strand of the donor DNA. In some examples the donor DNA has two types of nucleic acid modification of from one to ten nucleotides at the 5' end of one strand of the donor DNA. The modification may be, for example, phosphorothioate (PS) linkages between nucleotides, or may be 2'-O-methylation of the deoxyribose of one or more nucleotides of the donor DNA molecule. For example, a donor DNA molecule can have one, two, three or four PS bonds within the first five, first six, or first seven nucleotides from the 5' end of the modified strand and can also have one, two, three or four 2'-O-methyl modified nucleotides within the first five, first six, or first seven nucleotides from the 5' end of the modified strand. In some examples the donor DNA molecule is double-stranded and one strand comprises the modifications at the 5' end. In some examples the donor DNA molecule is double-stranded and one strand has two or more modifications on any of the first ten or first five nucleotides from the 5' end and the opposite strand has a terminal 5' phosphate. In various examples, the donor DNA molecule is double-stranded and has at least two PS bonds and at least two 2'O-methyl-modified nucleotides on one strand of the donor DNA, where the PS and 2'-O methyl modifications occur within the first five nucleotides from the 5' end of the modified strand. In various examples, the donor DNA molecule is double-stranded or substantially double-stranded and has three PS bonds and three 2'O-methyl-modified nucleotides on one

strand of the donor DNA, where the PS and 2'-O methyl modifications occur within the first five nucleotides from the 5' end of the modified strand. In some examples of these examples, the opposite strand includes a terminal 5' phosphate. The donor DNA can be introduced into the cell as a double-stranded or substantially double-stranded molecule.

The present disclosure further provides a donor DNA construct designed for inserting a CAR (chimeric antigen receptor) or DAR (dimeric antigen receptor) into a host cell. CAR constructs are well-known in the art and reviewed, for example, in Zhang et al. (2017) *Biomarker Res*. 5:22. DAR constructs, that encode a two polypeptide receptor, are described for example in WO 2019/173837. Further, the present disclosure provides a host cell transduced with a CAR that lacks a viral vector or component thereof, such as sequences of a retroviral or adeno-associated viral (AAV) vector. The disclosure provides for more efficient and more cost-effective process for engineering T cells to express CAR or DAR constructs. The CAR or DAR construct can include homology arms that target the construct to a T cell receptor gene, PD-1 gene, CD7 gene, or TIM3 gene, as nonlimiting examples, for simultaneous knock-in of the CAR construct and knock out of the TCR, PD-1, TIM3, GM-CSF, CD7, or other gene.

In a further example, provided herein is a system for genome modification that comprises: at least one RNA-guided endonuclease or at least one nucleic acid molecule encoding an RNA-guide endonuclease; at least one guide RNA or at least one nucleic acid molecule encoding a guide RNA; and a donor DNA molecule, where the donor DNA molecule includes at least one nucleotide modification within twenty, within ten, or within five nucleotides of the 5' terminus. In some examples the donor DNA is double-stranded or substantially double-stranded and includes at least one, at least two, or at least three modifications on at least one, at least two, or at least three nucleotides occurring within ten or within five nucleotides of one strand of the double stranded donor molecule. The modifications can be, for example, backbone modifications such as phosphorothioate bonds and/or 2'-O methylation of the sugar of nucleotides. The donor DNA can be at least 250 nt or bp in length, and can be at least 300, 400, 500, 600, 700, 800, 900, or 1000 nt or bp in length, and in some examples can be greater than 2000 nt or bp in length, for example, may be between about 0.5 and 4 kb in length, or between about 1 kb and 3.5 kb in length, or between about 1.5 kb and about 2.8 kb in length, or between about 1.8 kb and about 3 kb in length, as nonlimiting examples. The donor DNA can have homology arms (HAs) flanking a sequence of interest to be integrated into the genome. The sequence of interest can be an expression cassette, for example, for expression a construct that includes one or more antibody or receptor domains. Homology arms can be between about 50 and about 5000 nucleotides in

length, or between about 100 and 1000 nucleotides in length, for example between about 120 and about 800 nucleotides in length, or between about 140 and about 600 nucleotides in length.

In some examples, an RNA-guide nuclease used in the systems and methods provided herein is selected from the group consisting of Cas9, Cas12a, CasX, and combinations thereof. The guide RNA can be a chimeric guide, having sequences of both crRNA and tracrRNA, or can be a crRNA, and can optionally include one or more nucleic acid modifications, including phosphorothioate (PS) oligonucleotides. Where the guide is a crRNA, and the RNA-guided endonuclease uses a tracrRNA, the system can also include a tracrRNA. For example, Cas9 can be used with a crRNA and a tracrRNA or can be used with a chimeric guide RNA (sometimes called a single guide or "sgRNA") that combines structural features of the crRNA and tracrRNA. Cas12a on the other hand naturally uses only a crRNA and has no associated tracrRNA. In various examples, the RNA-guide endonuclease, guide RNA (that can be a crRNA or a chimeric guide RNA), and, when included, tracr RNA, can be complexed as a ribonucleoprotein complex that is introduced to the cell. The donor DNA can be introduced into the target cell together with the RNP, or separately, for example, in a separate electroporation or transfection.

Also provided herein is a method for site-specific integration of a donor DNA into a target DNA molecule, where the method includes introducing into a cell: at least one RNA-guided endonuclease or a nucleic acid molecule encoding an RNA-guided endonuclease; at least one engineered guide RNA or at least one nucleic acid molecule encoding an engineered guide RNA; and a donor DNA molecule comprising at least one nucleic acid modification; where the guide RNA comprises a target sequence designed to hybridize with a target site sequence in the target DNA and the donor DNA is inserted into the target DNA molecule at the target site. The donor DNA can be, for example, at least 250 nucleotides or base pairs in length, at least 500 nucleotides or base pairs, at least 1000 nucleotides or base pairs, at least 1500 nucleotides or base pairs, at least 2000 nucleotides or base pairs, at least 2500 nucleotides or base pairs, or at least 3000 nucleotides or base pairs (bp) in length, where the donor fragment can be delivered to the cells as a double-stranded or substantially double-stranded molecule. In some examples the RNA-guided endonuclease is introduced into the cell as a protein. In some examples the guide RNA is introduced into the cell as an RNA molecule. In exemplary examples, the RNA-guided endonuclease, guide RNA, and, where employed, tracrRNA, are introduced into the cell as a ribonucleoprotein complex (RNP). In various examples the RNA-guided endonuclease

also be delivered to the cell, simultaneously with the first RNP or at a later or earlier time. In examples where a donor DNA is delivered, the methods can include insertion of a non-native genetic construct to a first genetic locus of the cell, where the first genetic locus can be disrupted by the insertion of the non-native genetic construct, and cas-mediated disruption of a second genetic locus of the cell targeted by the second RNP. Cells modified by these methods can express a non-native construct, such as but not limited to a CAR or DAR construct, and can exhibit reduced expression of endogenous genes at the first and second genetic loci. For example, the first and second genetic loci can be mutated by means of the cas proteins and complexed guide RNAs delivered to the cell. The genes at the first and second loci can be disrupted to greatly reduce or eliminate (knock out) gene expression. The donor DNA can be any donor DNA as provided herein, such as a double-stranded donor DNA having at least two nucleic acid modifications to at least one strand. The donor DNA preferably has homology arms that comprise sequences of the first genetic locus that flank the genetic construct (e.g., CAR or DAR encoding sequence).

In various examples of the systems, compositions, and methods provided herein the donor DNA includes at least two modified nucleotides, which can have the same or different modifications, and preferably occur within ten or within five nucleotides of the 5' terminus of one strand of the donor DNA. In some examples, the donor DNA is double-stranded and the one or more nucleotide modifications occur on a single strand of the donor DNA molecule. In some examples, the donor DNA is double-stranded and the one or more nucleotide modifications occur on a single strand of the donor DNA molecule within twenty, within ten, or within five nucleotides of the 5' terminus of the modified strand. In some examples, the donor DNA includes a backbone modification such as a phosphoramidite or phosphorothioate modification. In some examples, the donor DNA includes a modification of a sugar moiety of a nucleotide. In some examples, the donor DNA is double stranded and includes at least one, at least two, or at least three phosphorothioate modifications within five nucleotides of the 5' end of a single strand of the donor DNA molecule and further includes at least one, at least two, or at least three 2'-O-methylated nucleotides within five nucleotides of the 5' end of a single strand of the donor DNA molecule. In various examples the donor DNA includes homology arms flanking a DNA sequence of interest, such as, for example, an expression cassette, where the homology arms have homology to sites in the target genome on either side of the target site of the RNA-guide endonuclease. Homology arms can be from about 50 to about 2000 nt in length, and may be, for example between 100 and 1000 nt in length, or between 150

and 650 nt in length, for example, between 150 and 350 nt in length, or 150 to 200 nt in length. In various examples a donor DNA molecule has two or more nucleotide modifications on the modified strand and the opposite strand includes a terminal phosphate.

The RNA-guided endonuclease can be a cas protein and can be, as nonlimiting examples, a Cas9, Cas12a, or CasX protein. In various examples of the method, the at least one RNA-guided endonuclease and at least one RNA guide are introduced into the cell as one or more ribonucleoprotein complexes (RNPs). In various examples a first RNP is formed with a cas protein and a first guide RNA and a second RNP is formed in a separate incubation of a cas protein and a second guide RNA. The cas protein for each RNP can be the same or different. For example, a first RNP can be formed with cas9, and a second RNP can be formed with cas12a. One or more RNPs that includes a cas9 protein can in some examples further include a tracrRNA. The two RNPs, and, optionally a donor DNA, can be added to the cells for multiple site gene editing, where at least one of the edited sites optionally incorporates a DNA donor. An RNP can be introduced into a target cell by any feasible means, including electroporation or liposome transfer, for example. The donor DNA can be delivered to the cell simultaneously with the one or more RNPs, or separately.

The methods can be used to modify the genomes of eukarotic cells, including the cells of animals, including avian, fish, insect, and mammalian cells. In various examples the cells whose genomes are manipulated using the methods and systems provided herein are mammalian cells and may be human cells. Cells used in the methods provided herein can be of cell lines or can be primary cells, such as, for example, stem cells or hematopoietic cells, including T cells and NK cells.

Further included herein are engineered primary T cells, which may be human primary T cells, where the cells include a non-native genetic construct integrated into the genome at a first genetic locus that comprises a first target site of an RNA-guided nuclease and a mutation at a second genetic locus that comprises a second target site of an RNA-guided nuclease. The mutation at the second genomic locus can be, for example, a knockout mutation by means of an insertion or deletion inserted at the second target site as a result of cas nuclease activity and misrepair by the cell. The second target site may be in a gene whose reduced expression is desired. The first target site, into which the donor fragment that comprises the non-native genetic construct is inserted, may also be in a gene whose reduced expression is desired. “Target site” as used herein means a sequence adjacent to a PAM sequence recognized by an RNA-guided nuclease. Such PAM-adjacent sequences (of, for example 17-22 nucleotides in length) can be

used as target sequences in guide RNAs to direct the activity of a cas nuclease such as cas9 or cas12a to cleave the genomic DNA the target site.

The non-native genetic construct is a genetic construct that does not naturally occur in the cells that is introduced on a donor fragment for integration using the cas-mediated methods provided herein. The engineered primary T cells can express the non-native genetic construct and can have reduced expression of the gene at the second genetic locus, and can also have reduced expression of the gene at the first genetic locus, where the gene at the first genetic locus may be disrupted by insertion of the non-native genetic construct.

The non-native genetic construct can be a genetic construct that encodes one or more polypeptides having one or more immunoglobulin domains. In some examples, the non-native genetic construct is construct that encodes a CAR or DAR. Thus, in some examples primary human T cells are provided that include a non-native genetic construct such as a CAR or DAR-encoding construct integrated into the genome, where the cells express the construct (e.g., express a CAR or DAR molecule) and may have reduced expression of a gene disrupted by insertion of the CAR or DAR (or other) construct, and where the cells can have a second site mutation that results in reduced expression of a second gene. Genes that may be disrupted by insertion of a genetic construct include, without limitation, genes encoding the TCR, TRAC, PD-1, CTL4-A, TIM3, LAG3, GM-CSF, and CD7. A CAR or DAR can be a CAR or DAR designed to bind a tumor cell surface antigen, such as but not limited to, BCMA, CD19, CD20, CD38, CD123, or any other tumor cell surface antigen. In various examples, at least 25% of a population of cells as provided herein may express a CAR, DAR, or other introduced construct and exhibit reduced expression of a gene at a second genetic locus. In various examples, at least 25% of a population of cells as provided herein may express a CAR, DAR, or other introduced construct and exhibit reduced expression of a gene at the first genetic locus into which the non-native construct have been introduced and exhibit reduced expression of a gene at the second genetic locus. The cells may be produced using the methods provided herein.

Provided in yet another example are human primary T cells having a CAR or DAR construct inserted into the CD7 gene, as demonstrated herein. A population of T cells having a CAR or DAR insertion in the CD7 gene can demonstrate CAR or DAR expression and reduced expression of CD7.

Brief Description of the Figures

Figure 2 A provides chemical drawings that show, in the right structure, a phosphorothioate (PS) modification of the bond between nucleotides as they might occur in a

primer. The nucleotides shown in the oligonucleotide on the left are attached via a (nonmodified) phosphodiester bond. **Figure 2B** provides a chemical drawing of an oligonucleotide having two PS bonds that join the 5'-most nucleotide to the next nucleotide "downstream" in the oligonucleotide, which in turn is attached to the following downstream nucleotide of the oligonucleotide by a PS bond. The 5'-most nucleotide of the oligonucleotide includes a 2' O-methyl modification.

Figure 1 A is a diagram of a CAR donor DNA construct that includes an open reading frame having a sequence encoding a single chain variable fragment (scFv), followed by the CD8a leader peptide which is then followed by a CD28 hinge-CD28 transmembrane-intracellular regions and then a CD3 zeta intracellular domain. The coding sequence is preceded by a JeT promoter (SEQ ID NO:3) and the construct includes homology arms (HAs), in this case matching sequences of the human TRAC locus, flanking the promoter plus coding sequences. shows the structure of the donor DNA construct (top) and primer design for confirming right knock in (bottom). This provides a diagram of the template DNA used for generating donor DNA. The anti-CD38A2 contains a CD38 CAR transgene with expression driven by the JeT promoter and flanked by homology arms on the 5' and 3' sides to enable targeted integration. **Figure 1B)** shows the same diagram indicating the positions of PCR primers used to confirm CAR integration by amplification with one primer located within the CAR and one primer in TRAC outside of the homology arms at both the 5' and 3' ends to generate 1371-bp and 1591-bp products, respectively, when integration is at the targeted integration site.

Figure 3A to 3E provides flow cytometry plots of PBMCs 8 days after transformation with a donor DNA that included a construct for expressing an anti-CD38 CAR and an RNP comprising a guide RNA targeting the TRAC locus. The CAR cassette was flanked by homology arms having homology to TRAC locus sequences flanking the integration target site in exon 1 of the TRAC gene. The Y axis reports cell size. Anti-CD38 construct expression is along the x axis. Negative control: no donor DNA was transformed into the target cells; No modification- the donor DNA had no chemical modifications; PS modification: three phosphorothioate bonds occurred within the 5'-most five nucleotide backbone positions; PS + 2'-OMe: in addition to phosphorothioate bonds, the three nucleotides within the 5'-most five nucleotides of the donor included 2'-OMe in addition to PS modifications; TCR KO/retroviral construct: the cells were transfected with the RNP in the absence of donor DNA to knock out the TCR gene and transduced with a retrovirus to express the anti-CD38 CAR.

Figure 3F to 3J provides the results of flow cytometry performed on the same cultures as in A) ten days after transfection.

Figure 3K to 3M provides the

results of flow cytometry performed on the culture that received the doubly-modified donor DNA and control (TRAC knockout only and TRAC knockout with retroviral transduction) twenty days after transfection.

Figure 4 shows a gel of PCR products showing integration of the donor DNA at the targeted TRAC (Exon1) site. Primary human T cells were electroporated with TRAC RNP only or together with ssDNA. PCR was used to confirm the presence of the anti-CD38A2 CAR transgene integrated in the TRAC locus two weeks post-electroporation (lanes 3 and 6, depicting products from 5' and 3' integration regions). No bands were observed in non-transformed ATCs (lanes 1 and 4) or T cells that were transformed with the TRAC exon 1 targeting RNP but did not receive the donor DNA (lanes 2 and 5).

Figure 5 is a graph showing cytotoxicity assay results with Activated T cells (ATCs, stars) as a control, TCR knock out ATC, anti-CD38A2 retrovirus transduced CART cells RV CART, black line), TRAC knock out retrovirus transduced CART cells (dots), TRAC knock out together with phosphorothioate modified ss donor DNA knock in (dashes), TRAC knock out together with phosphorothioate and 2' O-Methyl modified ssDNA knock in (dashes and dots). The plot provides the percent cytotoxicity toward GFP-labeled RPMI8226 CD38-expressing cells after correcting for the cytotoxicity observed toward RPE-labeled K562 cells that do not express CD38.

Figure 6A to 6C provides graphs of the results of cytokine secretion assays using anti-CD38 CART cells and controls co-cultured with K52 or RPM18226 cells. The T cell cultures tested are as provided in Figure 5.

Figure 7A to 7D provides the results of testing donor DNAs having homology arms (HAs) of different lengths. Cultures were assessed by flow cytometry for loss of TCR (CD3) expression (Y axis) and anti-CD38 expression (X axis).

Figure 8A to 8C provides the results of testing double stranded donor DNAs modified by the addition of three PS bonds and three 2'O methyl nucleotides proximal to the 5' end of one strand of the donor DNA molecule. Cultures were assessed by flow cytometry for loss of TCR expression (Y axis) and anti-CD38 expression (X axis).

Figure 9A to 9B provides the results of flow cytometry on cells transfected with a ds PS and 2'-OMe- modified donor DNA that included a cassette for expressing an anti-CD19 CAR. The donor was directed to the TRAC exon 1 locus by cotransfection with an RNP. TCR expression is determined on the Y axis and anti-CD19 CAR expression on the Y axis.

Figure 10A to 10B provides the results of flow cytometry on cells transfected with a ds PS and 2'-OMe- modified donor DNA that included a cassette for expressing an anti-BCMA CAR. The donor was directed to the TRAC exon 1 locus by cotransfection with an RNP. TCR expression is determined on the Y axis and anti-BCMA CAR expression on the Y axis.

Figure 11A to 11C provides the results of flow cytometry on cells transfected with a ds PS and 2'-OMe- modified donor DNA that included a cassette for expressing an anti-CD38 CAR. The donor was directed to the TRAC exon 3 locus by cotransfection with an RNP. TCR expression is determined on the Y axis and anti-CD38 CAR expression on the Y axis.

Figure 12A to 12D provides the results of flow cytometry on cells transfected with a ds PS and 2'-OMe- modified donor DNA that included a cassette for expressing an anti-CD19 CAR. In one culture, the donor had homology arms derived from TRAC exon 3 was directed to the TRAC exon 3 locus by cotransfection with an RNP having an exon 3 guide RNA (Figure 12B). In another culture, the donor had homology arms derived from TRAC exon 1 was directed to the TRAC exon 1 locus by cotransfection with an RNP having an exon 1 guide RNA (Figure 12C). TCR expression is determined on the Y axis and anti-CD19 CAR expression on the Y axis.

Figure 13A to 13D provides the results of flow cytometry on cells transfected with a ds PS and 2'-OMe- modified donor DNA that included a cassette for expressing an anti-C38 CAR and homology arms derived from the TRAC gene or the PD-1 gene. In one culture, the donor had homology arms derived from TRAC exon 1 was directed to the TRAC exon 1 locus by cotransfection with an RNP having an exon 1 guide RNA (Figure 13D). In another culture, the donor had homology arms derived from the PD-1 locus and was directed to the PD-1 gene by cotransfection with an RNP having a PD-1 gene guide RNA (Figure 13C). TCR expression is determined on the Y axis and anti-CD38 or PD-1 expression on the Y axis.

Figure 14 provides the results of cytotoxicity assays using T cell cultures that were transfected with doubly modified (PS and 2'-OMe) donor fragment that included and anti-CD38 CAR construct and PD-1 gene-derived homology arms was targeted to the PD-1 gene by an RNP that included a guide RNA having a target sequence from the PD-1 gene.

Figure 15A to 15E provides the results of flow cytometry of cells transfected with a donor DNA comprising an anti-CD38 DAR construct along with an RNP comprising the Cas9 protein (Figure 15D) or a Cas12a RNP (Figure 15E). T cell receptor expression is depicted on the Y axis and expression of the anti-CD38 DAR construct on the Y axis. Panels 2 and 3 provide the results of transfecting T cells with an RNP that included the Cas9 protein and Cas12a protein, respectively, in the absence of a donor fragment.

Figure 16 provides a graph of the results of cytotoxicity assays using T cells transfected with an anti-CD38 DAR construct and either a Cas9 RNP or a Cas12a RNP.

Figure 18A to 18B provides the results of flow cytometry of nonmodified activated T cells (ATC) (Figure 18A), or T cells transfected with a Cas12a RNP targeting the Tim3 gene along with a donor DNA that included an anti-CD38 DAR construct (Figure 18B).

Figure 17 is a table providing the genome location and rate of off-target mutations generated during insertion of the anti-CD38 CAR into the TRAC locus with a Cas9 RNP.

Figure 19A to 19H is flow cytometry data of T cells transfected with a Cas9 RNP targeting the TRAC locus and an anti-CD38 DAR donor DNA as well as T cells transfected with a Cas9 RNP targeting the GM-CSF gene in addition to a Cas9 RNP targeting the TRAC locus and an anti-CD38 DAR donor DNA for insertion into the TRAC locus. Also shown, in panels 19E and 19H, is flow cytometry data of T cells transfected with a Cas12a RNP targeting the GM-CSF gene in addition to a Cas12a RNP targeting the TRAC locus and an anti-CD38 DAR donor DNA for insertion into the TRAC locus.

Figure 20A provides flow cytometry data of T cells transfected with a Cas12a RNP targeting the TRAC locus but no donor DNA and Figure 20**B** provides flow cytometry data of T cells transfected with a Cas12a RNP targeting the TRAC locus and an anti-CD20 DAR construct donor DNA.

Figure 21 is a graph of the percent cytotoxicity of T cells transfected with the anti-CD20 DAR construct and the anti-CEA DAR construct as a function of effector : target cell ratio. The target cells in the assay were Daudi cells.

Figure 22A and 22B is a bar chart showing the amount of **Figure 22A** interferon gamma and **Figure 22B** GMCSF secreted by T cells transfected with the anti-CD20 DAR construct and an anti-CD19 CAR construct after antigen stimulation. T cells were stimulated with K562 cells or Daudi cells. Only Daudi cell stimulation resulted in a significant response as indicated by the bars. No cytokine release was detected from unstimulated cells.

Figure 23A to 23D provides the results of flow cytometry that assessed the expression of the T cell receptor (CD3) and the anti-CEA CAR construct by engineered T cells: Figure 23**A**) T cells transfected with an RNP targeting the TRAC locus but no donor fragment assessed for CD3 (TCR) expression and anti-CEA CAR expression; Figure 23**B**) T cells transfected with an RNP targeting the TRAC locus and a donor fragment that included the anti-CEA CAR construct assessed for CD3 (TCR) expression and anti-CEA CAR expression; Figure 23**C**) T cells transfected with an RNP targeting the TRAC locus but no donor fragment assessed for CD7 expression and anti-CEA CAR

expression; and Figure 23**D**) T cells transfected with an RNP targeting the CD7 locus and a donor fragment that included the anti-CEA CAR construct assessed for CD7 expression and anti-CEA CAR expression.

Figure 24 provides the results of cytotoxicity assays using T cells transfected with the anti-CEA CAR construct targeted to the CD7 locus, T cells transfected with the anti-CEA CAR construct targeted to the TRAC locus; and T cells knocked out at the TRAC locus. The X axis provides effector to target cell ratios in the assays.

Figure 25 provides a bar graph of IFNγ secretion by T cells transfected with the anti-CEA CAR construct targeted to the TRAC locus and T cells transfected with the anti-CEA CAR construct targeted to the CD7 locus.

Detailed Description

Definitions

Unless specifically indicated otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art. In addition, any method or material similar or equivalent to a method or material described herein can be used in the practice of the present disclosure. All publications cited herein are incorporated by reference in their entireties.

The terms "a," "an," or "the" as used herein not only include aspects with one member, but also include aspects with more than one member. For instance, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the agent" includes reference to one or more agents known to those skilled in the art, and so forth.

The term "about" in relation to a reference numerical value can include a range of values plus or minus 10% from that value. For example, the amount "about 10" includes amounts from 9 to 11, including the reference numbers of 9, 10, and 11. The term "about" in relation to a reference numerical value can also include a range of values plus or minus 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% from that value.

The term "primary cell" refers to a cell isolated directly from a multicellular organism. Primary cells typically have undergone very few population doublings and are therefore more representative of the main functional component of the tissue from which they are derived in comparison to continuous (tumor or artificially immortalized) cell lines. In some cases, primary cells are cells that have been isolated and then used immediately. In other cases, primary cells cannot divide indefinitely and thus cannot be cultured for long periods of time *in vitro*.

DNA were transduced with a retrovirus that included a retroviral vector having the same anti-CD38A2 expression cassette (SEQ ID NO:2) that was used to make the donor fragment employed in CRISPR/Cas9 targeting.

Figure 3A to 3E shows that 8 days after transfection no expression of an anti-CD38 construct was detected in cells transfected with the RNP (for knocking out the TRAC gene) in the absence of a donor fragment for expression of the anti-CD38 CAR (Figure 3A). On the other hand, PBMCs that had a TRAC knockout and were subsequently transduced with a retrovirus that included a construct for expressing the anti-CD38 CAR did show expression of the anti-CD38 CAR in about 70% of the cells 8 days after transfection (**Figure 3E**). For cultures transformed with anti-CD38 CAR ss donor DNA in addition to an RNP targeting exon 1 of the TRAC gene, approximately 12% of the population that received the ss donor DNA having no chemical modifications and approximately 13% of cultures that were transduced with ss donor DNA having only PS backbone modifications on nucleotides near the 5'-end of the donor DNA (introduced by using a PCR primer having PS bonds between nucleotides 1 and 2, 2 and 3, and 3 and 4, numbering from the 5' end) demonstrated expression of the anti-CD38 construct. Adding methyl groups to the 2' oxygen of the three nucleotides at the second, third, and fourth nucleotides from the 5'-end of the donor fragment strand that also included PS modifications (by using the primer of SEQ ID NO:8 that included these modifications to generate the donor DNA by PCR) resulted in significantly higher expression of the anti-CD38 CAR in the transfected population, where expression of the anti-CD38 CAR was seen in approximately 20% of the cells that received the 'double modified' (2'-O-methyl and PS) single-stranded donor fragment at 8 days. Notably, chemical modifications of the donor DNA did not affect viability of the transfected cultures.

Increased expression of the anti-CD38 CAR was observed over time in cultures that had been transfected with anti-CD38 CAR donor fragments plus RNPs targeting the TRAC gene, while the opposite was true of cultures transduced by a retrovirus. At 10 days post-transfection (**Figure 3F to J**), flow cytometry demonstrated that for all of the cultures - that were all transfected with the TRAC-targeting RNP - at least 80% of the cells did not express the TCR. Moreover, in cultures transfected with an anti-CD38 CAR donor fragment in addition to the TRAC-targeting RNP, at least 42% of the cells that did not express the TCR expressed the anti-CD38 construct (**Figure 3G to 3I**) at 10 days post-transfection. For cultures transfected with an anti-CD38 CAR donor fragment with both PS and 2'-O-methyl groups on 5'-proximal nucleotides, 57% of the cells were expressing the anti-CD38 construct by day ten (**Figure 3I**), the highest

percentage of any test culture. At the same time, the expression of the anti-CD38 CAR in cultures that had been transduced with the retrovirus dropped to about half of what had been seen at 8 days, to approximately 34% of the cells on day ten post-transduction (**Figure 3B**, panel 5). Analysis of the culture transfected with doubly modified ss donor and the retrovirus-transduced culture at day 20 (**Figure 3C**) showed that expression of the anti-CD38 construct in the cultures had stabilized, with the Cas9-modified culture that had been transfected with a ss donor having both PS and 2'-O-methyl modifications at the 5' end demonstrating 54% of the TCR-negative cells were expressing the construct (middle panel) and the culture that had been transduced with a retrovirus demonstrating 31% of the TCR-negative cells were expressing the construct (right panel).

To confirm the occurrence of homology directed repair (HDR) at the targeted locus in Exon 1 of the TRAC gene, PCR was performed on DNA isolated from cultures to verify that the donor fragment had inserted into the TRAC site targeted by the guide RNA. Genomic DNA was amplified from non-transfected activated T cells (ATCs), TRAC knockout cells that were transformed with the RNP that included the TRAC Exon 1 guide RNA, and from T cells transfected with the RNP plus phosphorothioate and 2' O-Methyl modified donor DNA to detect targeted insertion of an anti-CD38 CAR transgene into the TRAC locus. To confirm the position of the donor DNA in the genome, oligonucleotide primers were targeted to sequences outside of the TRAC homology arms but adjacent to (outside of) the homology arm sequences in the genome. A total of 1 x 10^5 cells were resuspended in 30 µL of Quick Extraction solution (Epicenter) to extract the genomic DNA. The cell lysate was incubated at 65ºC for 5 min and then at 95ºC for 2 min and stored at -20ºC. The concentration of genomic DNA was determined by NanoDrop (Denovix). Genomic regions containing the TRAC target sites were PCR-amplified using the following primer sets: 5' PCR forward primer on TRAC: CCTGCTTTCTGAGGGTGAAG (SEQ ID NO:10), 5' PCR Reverse primer on CAR: CTTTCGACCAACTGGACCTG (SEQ ID NO:11); 3' Forward primer on CAR: CGTTCTGGGTACTCGTGGTT (SEQ ID NO:12), 3' Reverse primer on TRAC: GAGAGCCCTTCCCTGACTTT (SEQ ID NO:13**)** (see **Figure 1B**). Both primer sets were designed to avoid amplifying the HDR templates by annealing outside of the homology arms.

The concentration of genomic DNA was determined by NanoDrop (Denovix). Both primer sets were designed such that one primer of the pair annealed to a site in the genome outside of the homology arm, and the other primer of the pair annealed to a site within the coding region of the construct (*i.e.*, not in a homology arm). The PCR contained 400 ng of genomic

the highest effector to target ratios, TRAC knockout cells showed virtually no killing regardless of effector-to-target cell ratio. The anti-CD38A2 CART cells however exhibited potent and specific killing activity of CD38 positive cells - RPMI8226, but not CD38 negative cells – K562 (**Figure 5**). T cells that had integrated the chemically modified donor that included the anti-CD38 CAR cassette demonstrated cytotoxicity toward target cells similarly to that of cells transduced with retrovirus that included the anti-CD38 CAR construct.

The transfected activated T cells (ATCs) were also tested for cytokine secretion (**Figure 6A to 6C**). T cells were starved in IL-2 free medium overnight. Anti-CD38 CAR-T cells or ATC controls were then co-cultured with CD38 negative K562 or CD38 positive RPMI8226 cells. The incubation effector to target cell ratio was 2:1. After overnight incubation, the cells were centrifuged to collect the supernatants for quantitating cytokine IL-2, IFN-gamma and TNF alpha (Affymetrix eBioscience) according to the manufacturer's instructions. The gene-edited TCR knockout anti-CD38A2 CART cells also released similar amount of IFN-γ and other pro-inflammatory cytokines when co-cultured with CD38 positive tumor cells (RPMI8226) but not CD38 negative cells (K562).

In summary, *in vitro* cellular functional studies did not reveal any notable differences between TRAC-site-specific integrated anti-CD38A2 CAR achieved by this novel and efficient process and virus-mediated randomly integrated anti-CD38A2 CAR, in terms of both specific killing assay (**Figure 5**) and cytokine secretion assay (**Figure 6A to 6C**).

Example 2. Reducing length of homology arms of donor DNAs

For knock-in of the anti-CD38 CAR construct, donor fragments having homology arms (HAs) of different lengths were produced and tested. The pAAV-TRAC-anti-CD38 construct described in Example 1 that included the anti-CD38 cassette plus TRAC exon 1 homology arms of 660 and 650 nts (SEQ ID NO:4) was used as the template. A first set of primers, SEQ ID NO:8 and SEQ ID NO:9, was used to generate a donor fragment having homology arms of 660 nt and 650 nt from this template as provided in Example 1. A second set of primers, SEQ ID NO:14 and SEQ ID NO:15, was used to generate a donor fragment having homology arms of approximately 350 nt (375 and 321 nucleotides), where the primer of SEQ ID NO:14 had PS linkages between the between first and second, second and third, and third and fourth nucleotides from the 5' terminus and had 2'-O-methyl-modified nucleotides at positions 2, 3, and 5. A third set of primers, SEQ ID NO:18 and SEQ ID NO:19, was used to generate a donor fragment having homology arms of approximately 165 nt (171 and 161 nts), where the primer of SEQ ID

NO:18 had PS linkages between the between first and second, third and fourth, and fourth and fifth nucleosides from the 5' terminus and had 2'-O-methyl-modified nucleotides at positions 3, 4, and 5. In each case, the forward primer (SEQ ID Nos: 8, 14, and 18) was designed to have three PS linkages within the 5'terminal-most five nucleotides (for example, between any of the first and second, second and third, third and fourth, and fourth and fifth nucleosides from the 5' terminus of the primer, and three 2'-O-methyl groups occurring in any of the five 5'terminal-most nucleotides. In each case, the reverse primer (SEQ ID Nos: 9, 15, and 17) had a 5' terminal phosphate (see **Table 1**).

Each of the primer sets was used with the pAAV CD38 DAR construct as a template to generate a donor DNA molecule having multiple PS and 2'-O methyl modifications proximal to the 5'end of one strand of the donor and a 5' phosphate at the 5' terminus of the opposite strand of the donor. RNPs were assembled to include tracr and crRNAs as described in Example 1, where the crRNA included the target sequence of SEQ ID NO:1, a sequence found in exon 1 of the TRAC gene. When synthesizing donor DNA by PCR, the nuclease reaction to generate the single stranded donor fragment and subsequent purification of the single stranded DNA are time consuming and typically result in significant losses in the yield of donor fragment for transfections. In addition, the nuclease reaction can be difficult to control so that the ends of the donor fragments can be degraded. In further experiments testing the efficiency of directed gene knockouts and antibody construct knock-ins, double-stranded donor DNAs were tested in transfections to eliminate the nuclease digestion and single-strand purification of the PCR-synthesized donor.

The double-stranded donor molecules, having homology arms of approximately 665, 350, and 165 base pairs in length, were independently transfected into activated T cells as described in Example 1 except that donor fragments and RNPs were transfected in the same electroporation under conditions for electroporating the RNP (using a Neon® Transfection System (ThermoFisher Scientific) 1700 V, 20 ms pulse width, 1 pulse). As a control, activated T cells were transfected with the RNP in the absence of a donor fragment, which should result in knockout of the targeted TRAC locus, but without donor DNA insertion. To test for expression of the T cell receptor and the anti-CD38 CAR construct, flow cytometry was performed as provided in Example 1. **Figure 7A to 7D** shows that, as expected, the T cell culture transfected with the RNP only had low levels of expression of the T cell receptor and also demonstrated no expression of the anti-CD38 CAR. T cells transfected with the RNP plus donor DNAs having homology arms of different sizes however show low levels of T cell receptor expression and

flow cytometry was performed essentially as provided in Example 1. The results, shown in **Figure 8A to 8C**, show that transfection with the RNP and a modified double stranded donor resulted in greater than 50% of the cells expressing the anti-CD38 CAR while demonstrating no TCR expression, at least twice the percentage of TCR negative cells expressing the anti-CD38 construct as observed in the culture transfected with the RNP and the unmodified double-stranded donor (22%).

Example 4. HDR-mediated knock-in of anti-CD19 and anti-BCMA CAR constructs with simultaneous TCR knockout

Additional donor DNAs that included anti-CD19 CAR and anti-BCMA CAR expression constructs were also tested for insertion into the TRAC locus.

An anti-CD19 CAR construct that included an anti-CD19 CAR cassette (SEQ ID NO:22) that included the Jet promoter (SEQ ID NO:3) and intron, an anti-CD19 CAR construct, and an SV40 polyA sequence was made essentially as described for the anti-CD38 CAR pAAV construct described in Example 1 and was cloned in a vector flanked by the TRAC gene exon 1 homology arms (HAs) of SEQ ID NO:20 and SEQ ID NO:21. The anti-CD19 CAR with HAs pAAV construct was used as a template in PCR reactions as provided in Example 1 using the primers provided as SEQ ID NO:18 and SEQ ID NO:19 that result in the production of modified donor DNA having HAs of approximately 170 and 160 nucleotides (see **Table 1**). The forward primer (SEQ ID NO:18) had three PS bonds between the first and second, third and fourth, and fourth and fifth nucleosides and three 2'-O-methyl modifications at nucleotides 3, 4, and 5 when numbering from the 5'-terminus of the primer and the reverse primer (SEQ ID NO:19) had a 5'-terminal phosphate (**Table 1**). The resulting double-stranded donor DNA was therefore synthesized to have the corresponding modifications, a first strand with three PS and three 2'-O-methyl modifications within five nucleotides of the 5'-terminus, and a second strand with a 5'-terminal phosphate.

The double-stranded chemically modified donor fragment having the sequence of SEQ ID NO:38 with the nucleotide modifications of primers SEQ ID NO:18 and SEQ ID NO:19 described above incorporated was used to transfect cells along with an RNP that was produced according to the methods provided in Example 1, where the crRNA of the RNP included the target sequence of SEQ ID NO:1, targeting exon 1 of the TRAC gene. As a control, activated T cells were transfected with the RNP in the absence of a donor fragment, which should result in knockout of the targeted TRAC locus without construct insertion. Flow cytometry was performed essentially as described in Example 1 to evaluate the efficiency of introducing a

different construct into the TRAC locus, except that anti-CD19 CAR expression was detected by CD19-Fc (Speed Biosystem) followed by APC anti-human IgG Fcγ (Jackson Immunoresearch). The results are shown in **Figure 9A to 9B**, where it can be seen that the anti-CD19 CAR was expressed in the absence of T cell receptor expression in approximately 42% of the cells in the culture.

An anti-BCMA CAR construct was made through replacing the anti-CD38 CAR with an anti-BCMA CAR based on the anti-CD38 CAR pAAV construct described in Example 1. The anti-BCMA CAR fragment was synthesized by IDT. The sequence of the insert is provided as SEQ ID NO:23. The anti-BCMA CAR construct was used as a template in PCR reactions as set forth in Example 1 using the primers provided as SEQ ID NO:18 and SEQ ID NO:19 that result in the production of donor DNA having TRAC Exon 1 locus HAs of approximately 160-170 nucleotides (see **Table 1**). The forward primer (SEQ ID NO:18) had three PS and three 2'-O-methyl modifications within five nucleotides of the 5'-terminus of the primer. The reverse primer (SEQ ID NO:19) had a 5'-terminal phosphate. The resulting double-stranded donor DNA was therefore synthesized to have a first strand with three PS and three 2'-O-methyl modifications within five nucleotides of the 5'-terminus, and a second strand with a 5'-terminal phosphate.

The double-stranded donor fragment having the sequence of SEQ ID NO:37, having modified nucleotides by incorporation of chemically modified primers as provided above, was used to transfect cells along with an RNP that was produced according to the methods provided in Example 1, where the crRNA of the RNP included the target sequence of SEQ ID NO:1, targeting exon 1 of the TRAC gene. As a control, activated T cells were transfected with the RNP in the absence of a donor fragment, which should result in knockout of the targeted TRAC locus without construct insertion. Flow cytometry was performed as described in Example 1 to evaluate the efficiency of introducing a different construct into the TRAC locus, except that anti-BCMA CAR expression was detected by PE or APC conjugated BCMA-Fc (R&D). The results are shown in **Figure 10A to 10B**, where it can be seen that the anti-BCMA CAR was expressed in the absence of T cell receptor expression in approximately 66% of the cells in the culture.

Example 5. HDR mediated knock-in targeting TRAC Exon 3

To test the efficiency of inserting donor DNAs into additional loci using the methods for donor insertion provided herein, an anti-CD38 CAR construct was made for producing a donor DNA having HAs from Exon 3 of the TRAC gene. In this case, the construct was produced essentially as described in Example 1 for the TRAC exon 1 targeting construct, except that the

HAs (5’ HA SEQ ID NO:24 (183 nt) and 3’ HA SEQ ID NO:25 (140 nt)) were sequences surrounding the exon3 target site (SEQ ID NO:26). The sequence of the insert of the pAAV construct that was then produced as a donor DNA with TRAC gene exon 3 homology arms is provided as SEQ ID NO:27. To generate the donor fragment, the forward primer (SEQ ID NO:28) included PS linkages between first and second, second and third, and third and fourth nucleosides and 2’-O-methyl modifications on the second, fourth, and fifth positions from the 5’-terminus, and the reverse primer (SEQ ID NO:29) had a 5’-terminal phosphate. The resulting double-stranded donor DNA that incorporated the primers had a first strand with corresponding PS and 2’-O-methyl modifications on the 5’-terminal most nucleotides, and a second strand having a 5’-terminal phosphate.

The double-stranded donor fragment having modified nucleotides by incorporation of the primers above and having the sequence of SEQ ID NO:27 was used to transfect cells along with an RNP that was produced according to the methods provided in Example 1, where the crRNA included the target sequence of SEQ ID NO:26, targeting exon 3 of the TRAC gene. As a control, activated T cells were transfected with the RNP in the absence of a donor fragment, which should result in knockout of the targeted TRAC locus without construct insertion. A further control was non-transfected activated T cells (ATCs). Flow cytometry was performed essentially as described in Example 1. The results are shown in **Figure 11A to 11C**, where it can be seen that transfection with the RNP or the RNP plus donor DNA result in greater than 80% of cells across the culture losing TCR expression. Further, anti-CD38 CAR was expressed in the absence of T cell receptor expression in approximately 42% of the cells in the culture that was transfected with the targeting RNP plus the donor DNA with HAs derived from the TRAC gene exon 3.

PCR products were generated using primers designed to diagnose the insertion locus (see **Figure 1B**): 5’- CTCCTGAATCCCTCTCACCA-3’ (SEQ ID NO:64, forward primer for sequencing across 5’ homology arm of anti-CD38 CAR in the TRAC exon 3 locus) and 5’-GCGGATCCAGCTCATGTAGT-3’ (SEQ ID NO:65, reverse primer for sequencing across 5’ homology arm of anti-CD38 CAR in TRAC exon 3 locus) and for the opposite junction, 5’-CGTTCTGGGTACTCGTGGTT -3’ (SEQ ID NO:66, forward primer for sequencing across 3’ homology arm of anti-CD38 CAR in TRAC exon 3 locus) and 5’-GGAGCACAGGCTGTCTTACA-3’ (SEQ ID NO:67, reverse primer for sequencing across 3’ homology arm of anti-CD38 CAR in TRAC exon 3 locus). The resulting PCR products were sequenced. The PCR product sequences (e.g., SEQ ID NO:41 and SEQ ID NO:42) included sequences adjacent to the homology arm in the genome, the homology arm present in the donor

fragment, and portions of the anti-CD38 CAR in a single PCR product, demonstrating the expected insertion.

Figure 12A to 12D compares targeting of the anti-CD19 CAR to exon 3 and exon 1 of the TRAC gene. The anti-CD19 CAR donor DNA directed to exon 3 is synthesized to include the anti-CD19 CAR cassette (SEQ ID NO:22) as set forth in the Examples above, where the anti-CD19 expression cassette is flanked by sequences from the exon 3 locus (SEQ ID NO:24 and SEQ ID NO:25) as set forth above. The anti-CD19 CAR donor directed to exon 1 (having the sequence of SEQ ID NO:38) is provided in Example 4. Each of these constructs – one having the anti-CD19 CAR cassette (SEQ ID NO:22) flanked by TRAC exon 1 Has (SEQ ID NO:18 and SEQ ID NO:19), and the other having the anti-CD19 CAR cassette (SEQ ID NO:22) flanked by TRAC exon 3 Has (SEQ ID NO:24 and SEQ ID NO:25), was used to produce donor fragment using modified forward primers having PS and 2'-O-methyl modifications on the three 5'-terminal most nucleotides. The reverse primers had 5'-terminal phosphates. The primers for producing the anti-CD19 CAR donor flanked by exon 1 Has were SEQ ID NO:18 and SEQ ID NO:19, where the SEQ ID NO:18 primer included PS linkages between first and second, thIrd and fourth, and fourth and fifth nucleosides and 2'-O methyl groups at position 3, position 4, and position 5 from the 5' end. The primers for producing the anti-CD19 CAR donor flanked by exon 3 hAs were SEQ ID NO:28 and SEQ ID NO:29, where the SEQ ID NO:28 primer had PS linkages between the first and second, second and third, and third and fourth nucleosides from the 5' end and 2'-O-methyl groups at position 2, position 4, and position 5 from the 5' end. The resulting double-stranded donor DNAs thus had a first strand with corresponding PS and 2'-O-methyl modifications on the 5'-terminal end nucleotides, and a second strand having a 5'-terminal phosphate.

The donor fragments were independently transfected into activated T cells with RNPs. RNPs were produced as described in Example 1, where the target sequence of the crRNA for targeting TRAC gene exon 1was SEQ ID NO:1, and the target sequence of the crRNA for targeting TRAC gene exon 3was SEQ ID NO:26. As can be seen in **Figure 12A to 12D**, approximately 41% of the culture that was transfected with an RNP targeting exon 3 of the TRAC gene and a donor fragment for expressing the anti-CD19 CAR were both TCR negative and positive for anti-CD19 CAR, while approximately 20% of the culture that was transfected with an RNP targeting exon 1 of the TRAC gene and a donor fragment for expressing the anti-CD19 CAR were both TCR negative and positive for anti-CD19 CAR. T cell cultures transduced with a

retrovirus including the anti-CD19 CAR expression cassette demonstrated a higher percentage of anti-CD19 CAR expressing cells, but these cells did not have a TCR knockout.

Example 6. HDR mediated knock-in targeting the PD-1 gene

The PD-1 locus was also targeted with a CAR construct. In this case the anti-CD38 CAR cassette (SEQ ID NO:2) was juxtaposed with homology arms (SEQ ID NO:30 and SEQ ID NO:31) having sequences of the PD-1 locus that surround a target site (SEQ ID NO:32) using the methods essentially as described in Example 1 to provide a template for producing donor DNA.

Donor DNA was produced essentially as described in Example 1, using a forward primer (SEQ ID NO:34) that included a 5' phosphate and a reverse primer that included phosphorothioate linkages between first and second, second and third, and third and fourth nucleosides from the 5' end as well as 2'-O-methyl groups on the first, second, and fourth nucleosides from the 5' end (SEQ ID NO:35), see **Table 1**.

The double-stranded chemically modified donor fragment (SEQ ID NO:33) was used to transfect cells along with an RNP produced according to the methods provided in Example 1, where the crRNA included the target sequence of SEQ ID NO:32, targeting the PD-1 gene. As a control, activated T cells were transfected with the RNP in the absence of a donor fragment, which generates a knockout of the targeted TRAC locus without CAR construct insertion. A further control was non-transfected activated T cells (ATCs). Flow cytometry was performed essentially as described in Example 1, where an additional control of nontransfected activated T cells (ATCs) was included. A BV421-conjugated antibody to PD-1 (EH12.2H7, BioLegend) was used to detect PD-1 expression.

The results are shown in **Figure 13A to 13D**, where it can be seen the percentage of cells expressing PD-1 dropped from approximately 19% in ATCs to approximately 4% in the cells of cultures transfected with the RNP targeting the PD-1 locus (PD-1 RNP). The anti-CD38 CAR was expressed in the absence of T cell receptor expression in approximately 27% of the cells in the culture that was transfected with the PD-1 targeting RNP plus a donor with HAs having homology to the PD-1 locus. As a comparison, about 32% of cells of a culture transfected with an RNP targeting exon 1 of the TCR and an anti-CD38 CAR donor fragment with HAs having homology to sequences of exon 1 of the TRAC gene.

Sequencing of PCR products produced using primers to diagnose the insertion locus (see **Figure 1B**) provided sequences demonstrating the anti-CD38 CAR donor fragment integrated into the PD-1 gene. To obtain the junction sequences, a total of 1 x 10^7 cells were resuspended in

500 µl of Quick Extraction solution (Epicenter) to extract the genomic DNA. The cell lysate was incubated at 65ºC for 5 min and then at 95ºC for 2 min and stored at -20ºC. The concentration of genomic DNA was determined by NanoDrop (Denovix). Genomic regions, containing the target sites, were PCR-amplified. Primer sets for both the 5' junction and 3' junction were designed to anneal outside of the homology arms. PCR products were generated using primers designed to diagnose the insertion locus (see **Figure 1B**): 5'- GTGTGAGGCCATCCACAAG-3' (SEQ ID NO:68, forward primer for sequencing across 5' homology arm of anti-CD38 CAR in the TRAC exon 3 locus) and 5'- ACACACTTGCGACCCATTC-3' (SEQ ID NO:69, reverse primer for sequencing across 5' homology arm of anti-CD38 CAR in TRAC exon 3 locus) and for the opposite junction, 5'- CGTTCTGGGTACTCGTGGTT -3' (SEQ ID NO:70, forward primer for sequencing across 3' homology arm of anti-CD38 CAR in TRAC exon 3 locus) and 5'-GGGACTGTCTTAGGCTTGG-3' (SEQ ID NO:71, reverse primer for sequencing across 3' homology arm of anti-CD38 CAR in TRAC exon 3 locus).

The PCR contained 400 ng of genomic DNA and Q5 High Fidelity 2X PCR mix (New England Biolabs). The thermocycler setting consisted of one cycle of 98ºC for 2 min, 35 cycles of 98ºC for 10s, 65ºC for 15s, 72ºC for 45s and one cycle of 72ºC for 10min. The PCR product were purified on 1% agarose gel containing SYBR Safe (Life Technologies). The PCR product were eluted from the agarose gel using NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG). The PCR product was submitted for Sanger sequencing (Genewiz). The PCR product sequences included sequences adjacent to the homology arm in the genome, the homology arm present in the donor fragment, and portions of the anti-CD38 CAR in a single PCR product, demonstrating the expected insertion.

Figure 14 provides the results of cytotoxicity assays that were performed using PBMCs and isolated T cells from cultures transfected with the anti-CD38 CAR donor fragment and an RNP targeting the PD-1 locus ("PD-1 KOKI PBMC" and "PD-1 KOKI Tcell" respectively) to determine the functionality of cells expressing the anti-CD38 CAR and knocked out in the PD-1 gene. These modified cells showed a high level of cytotoxicity toward target cells in the assay with respect to control cells that had a PD-1 gene knockout but did not receive a CAR construct ("PD-1 KO") and control cells that had a TRAC gene knockout but did not receive a CAR construct ("TRAC-1 KO") and were outperformed somewhat by cells that were transfected the anti-CD38 CAR donor fragment and an RNP targeting the TRAC locus ("TRAC KOKI"), likely due to the lower efficiency of donor CAR construct integration at the PD-1 site that was observed (**Figure 13A to 13D**).

target site (SEQ ID NO:57) were analyzed by flow cytometry alongside the knockout controls as described in Example 1 (**Figure 15A to 15E**). Only about 32% of the cell population that was transfected with a Cas9 RNP targeting the TRAC gene in the absence of a donor fragment and about 22% of the cell population that was transfected with a Cas12a RNP targeting the TRAC gene in the absence of a donor fragment ("TRAC KO") expressed the TCR. By comparison, essentially all nonmodified activated T cells (activated T cells "ATC"), shown **Figure 15A**, expressed the TCR. As expected, none of the cells that did not receive donor DNA were positive for the anti-CD38 constructs (**Figure 15B and C**). On the other hand, significant percentages of cell populations transfected with an anti-CD38 DAR construct donor DNA in addition to a Cas9 or Cas12a RNP demonstrated expression of the DAR constructs: approximately 54% of the population transfected with the anti-CD38 DAR construct donor DNA along with a Cas9 RNP (**Figure 15D**) expressed anti-CD38 DAR, as did approximately 77% of the population of cells transfected with the anti-CD38 DAR construct donor DNA along with a Cas12a RNP (**Figure 15E**).

The insertion of the anti-CD38 DAR construct into the Cas9 target site of exon 1 of the TRAC gene, and insertion of the anti-CD38 DAR construct into the Cas12a target site of exon 1 of the TRAC gene were both confirmed by PCR performed on genomic DNA isolated from both transfected cell populations and sequencing of the junction fragments. For Cas9-mediated insertion, PCR of the 5' homology arm region used SEQ ID NO:72 as the forward primer and SEQ ID NO:73 as the reverse primer. PCR of the 3' homology arm region used SEQ ID NO:74 as the forward primer and SEQ ID NO:75 as the reverse primer. Sequencing of the resulting PCR fragments demonstrated that the anti-CD38 DAR construct had inserted in the targeted Cas9 target site. For Cas12a-mediated insertion, PCR of the 5' homology arm region used SEQ ID NO:76 as the forward primer and SEQ ID NO:77 as the reverse primer. PCR of the 3' homology arm region used SEQ ID NO:78 as the forward primer and SEQ ID NO:79 as the reverse primer. Sequencing of the resulting PCR fragments demonstrated that the anti-CD38 DAR construct had inserted in the targeted Cas12a target site.

The results of cytotoxicity assays with the transfected populations co-cultured with RPMI8226 cells is provided in **Figure 16** and demonstrated that T cells transfected with the DAR construct by using either a Cas9 or Cas12a system had the expected physiological behavior. Cells transfected with the Cas9 RNP plus anti-CD38 DAR construct donor DNA (SEQ ID NO:49 and the Cas12a RNP plus anti-CD38 DAR construct donor DNA (SEQ ID NO:57 both showed specific killing of RPMI88226 cells that was virtually identical and dramatically

UTR5	0
Splicing	0
ncRNA exonic	0
ncRNA intronic	1
ncRNA splicing	0
Upstream	0
Downstream	0
Intergenic	2
Total	**4**

Example 9. Targeted insertion of an anti-CD38 dimeric antibody receptor (DAR) construct into the TIM3 locus with Cas12a.

In further experiments, the anti-CD38 DAR construct was cloned between flanking sequences derived from the Tim-3 locus for simultaneously knocking out the Tim-3 gene, which may play a role in T cell exhaustion, and knocking in the anti-CD38 DAR using Cas12a. The anti-CD38 DAR construct (SEQ ID NO:48) was cloned between DNA sequences (5' flanking sequence, SEQ ID NO:58; 3' flanking sequence, SEQ ID NO:59) derived from the TIM3 gene locus and occurring on either side of a Cas12a target site (SEQ ID NO:60), which was immediately downstream of a Cas12a PAM sequence. The cloned DAR construct plus flanking sequences was used as a template for PCR reactions that used the forward primer 5'-p-TGGAATACAGAGCGGAGGTC (SEQ ID NO:60) and the reverse primer modified to include 2'-O-methyl groups on the first, second and third nucleotides from the 5' end, and having PS bonds between the first and second, second and third, and third and fourth nucleotides from the 5' end: mG*mC*mA*TGCAAATGTCCACTCAC (SEQ ID NO:61) to generate a donor DNA molecule (SEQ ID NO:62) that incorporated the modifications of the reverse primer (SEQ ID NO:61) into the 5' end of one strand.

Transfection of T cells was done as performed in Example 1, except that in the Cas12a transfections the Cas12a protein was complexed with AltR crRNA and no tracr RNA was used. Donor fragment was electroporated along with the Cas12a RNP. As a control, a transfection was also done with the RNP in the absence of the donor DNA (TRAC knockout control).

The results of flow cytometry analysis of T cell populations transfected with the DAR construct targeted to the Tim-3 locus are provided in **Figure 18A to 18B**. Non-transformed activated T cells (ATC), included as a control, demonstrated expression of the Tim-3 gene by approximately

the TRAC-targeting RNP and anti-CD38 donor fragment; 2) the GM-CSF-targeting RNP in addition to the TRAC-targeting RNP and the anti-CD38 donor fragment; and, 3) for the TRAC knockout only control, the TRAC-targeting RNP only.

Flow cytometry was performed essentially as described in Example 1 to evaluate the efficiency of introducing a different construct into the TRAC locus, where intracellular GM-CSF was detected using eBioscience™ Intracellular Fixation & Permeabilization Buffer Set, (ThermoFisher, 88-8824-00) and stained with PE-GM-CSF antibody [BioLegend, 502306]. CD3 (T cell receptor) was detected with anti-CD3-BV421 antibody SK7 (BioLegend), and anti-CD38 DAR expression was detected with PE conjugated anti-CD38-Fc protein (Chimerigen Laboratories, Allston, MA).

Where cultures were stimulated to induce GM-CSF expression, the cultures were treated for six hours with Cell Activation Cocktail (BioLegend, 423304) which contains phorbol-12-myristate 13-acetate (PMA, 40.5 µM), ionomycin (669.3 µM), and Brefeldin A (2.5 mg/ml) in DMSO.

Figure 19A to 19H shows in Figure 19A and Figure 19B that only approximately 2% of T cells transfected with the TRAC-targeting RNP and anti-CD38 DAR donor construct express GM-CSF when not stimulated with PMA and iononmycin, whereas stimulation of the transfected cells with these drugs results in approximately 53% of the cells producing GM-CSF. On the other hand, as seen in Figure 19C, only approximately 29% of T cells transfected with the GM-CSF targeting RNP in addition to the TRAC-targeting RNP and anti-CD38 DAR donor construct produce GM-CSF on stimulation, evidence that simultaneous knock-out of a second gene occurs at a frequency estimated as 45% (52.78-29.07/52.78) in this case.

The same cell populations were analyzed for T cell receptor and anti-CD38 DAR expression in **Figure 19E to 19G**. Figure 19D shows that when transfected with the TRAC targeting RNP in the absence of the anti-CD38 DAR donor fragment, nearly 80% (78.57%) of the cells did not express the T cell receptor and as expected, no expression of the anti-CD38 DAR construct was detected. In contrast, when cells were transfected with the TRAC targeting RNP along with the anti-CD38 DAR donor fragment, approximately 70% of the cell population demonstrated expression of the anti-CD38 DAR in the absence of expression of the native T cell receptor (Figure 19E). Finally, addition of a second gene targeting RNP, the GM-CSF targeting RNP, did not drastically lower the TCR knockout/knockin rate, where approximately 50% of the cells transfected with both RNPs (anti-TRAC and anti-GM-CSF) plus the anti-CD38 DAR construct expressed the anti-CD38 DAR construct while failing to express the endogenous T cell

receptor (Figure 19F). In these cultures a high proportion (~45%) of the population are calculated to be GM-CSF knockouts resulting from the inclusion of the anti-GM-CSF RNP to the transfection.

Example 11. Targeted insertion of an anti-CD38 dimeric antibody receptor (DAR) construct into the TRAC locus with second site knockout of the GM-CSF gene using Cas12a.

The double knock out of and concomitant knock in of the anti-CD38 DAR into the TRAC locus was also attempted using two Cas12a RNPs, each of which included a different guide RNA (crRNA).

The anti-CD38 DAR donor fragment was produced as described in Examples 7 and 10, above, using a forward primer that included three PS bonds between the first and second, third and fourth, and fourth and fifth nucleotides and three 2'-O-methyl modifications at nucleotides 3, 4, and 5 when numbering from the 5'-terminus of the primer (SEQ ID NO:18), and a reverse primer that included a 5' terminal phosphate (SEQ ID NO:19) (**Table 1**). The resulting PCR product (SEQ ID NO:49) included the homology arms of approximately 170 bp and 160 bp (SEQ ID NO:20 and SEQ ID NO:21) flanking the anti-CD38 DAR-encoding construct (SEQ ID NO:48) and had the primer modifications of SEQ ID NO:18 incorporated into the first strand and a 5' terminal phosphate but no introduced chemical modifications added to the opposite, or second, strand.

The guide RNA for knocking out the TRAC locus was a crRNA engineered for Cas12a (Cpf1) for targeting exon 1 of the TRAC gene and included the target sequence of SEQ ID NO:26. The guide RNA for knocking out the TRAC gene was a crRNA engineered for Cas12a (Cpf1) for targeting exon 1 of the TRAC gene and included the target sequence of SEQ ID NO:52. The guide RNA for knocking out the GM-CSF gene was a crRNA engineered for Cas12a (Cpf1) that included the target sequence of SEQ ID NO:80. Both Cas12a crRNAs were synthesized by IDT (Coralville, IA).

Two RNPs were produced with a Cas12a protein that included an NLS at each of the N-terminal and C-terminal regions of the protein (IDT). The first RNP was formed by incubating the Cas12a protein with the crRNA targeting the TRAC locus and having the target sequence of SEQ ID NO:52 way (30 min incubation at 4ºC). The second RNP was formed by incubating the Cas12a protein with a GM-CSF-targeting crRNA (having target sequence SEQ ID NO:80) in the same way.

A single transfection of the T cells for knock-out of the TCR receptor gene with knock-in of the anti-CD38 DAR construct and knock-out of the GM-CSF gene was performed that used

the two assembled Cas12a RNPs along with the donor fragment having HAs for insertion into the TRAC gene. Transfection was by electroporation using the same conditions as provided in Example 1.

The double-stranded chemically modified donor fragment having the sequence of SEQ ID NO:48 with the nucleotide modifications of primers SEQ ID NO:18 and SEQ ID NO:19 described in Example 7 was used to transfect cells along with the RNPs. The double-stranded donor fragment had HAs of 171 and 161 bp.

Flow cytometry was performed essentially as described in Example 10 to evaluate the efficiency of introducing a different construct into the TRAC locus, where intracellular GM-CSF was detected using eBioscience™ Intracellular Fixation & Permeabilization Buffer Set, (ThermoFisher, 88-8824-00) and stained with PE-GM-CSF antibody [BioLegend, 502306]. CD3 (T cell receptor) was detected with anti-CD3-BV421 antibody SK7 (BioLegend), and anti-CD38 DAR expression was detected with PE conjugated anti-CD38-Fc protein (Chimerigen Laboratories, Allston, MA).

Figure 19 A and 19B shows that only approximately 2% of T cells transfected with the TRAC-targeting RNP and anti-CD38 DAR donor construct express GM-CSF when not stimulated whereas stimulation of the transfected cells with these drugs results in approximately 53% of the cells producing GM-CSF. On the other hand, as seen in Figure 19D, only approximately 15% of T cells transfected with the GM-CSF targeting RNP in addition to the TRAC-targeting RNP and anti-CD38 DAR donor construct produce GM-CSF on stimulation, evidence that simultaneous knock-out of a second gene occurs at a frequency estimated as 72% (52.78-14.9/52.78) in this case.

The same cell populations were analyzed for T cell receptor and anti-CD38 DAR expression in **Figure 19H**. Figure 19E shows that when transfected with the TRAC targeting RNP in the absence of the anti-CD38 DAR donor fragment, nearly 80% (78.57%) of the cells did not express the T cell receptor and as expected, no expression of the anti-CD38 DAR construct was detected. In contrast, when cells were transfected with the TRAC targeting RNP along with the anti-CD38 DAR donor fragment, approximately 70% of the cell population demonstrated expression of the anti-CD38 DAR in the absence of expression of the native T cell receptor (Figure 19F). Finally, addition of a second gene targeting RNP, the GM-CSF targeting RNP, did not drastically lower the TCR knockout/knockin rate, where approximately 60% of the cells transfected with both RNPs (anti-TRAC and anti-GM-CSF) plus the anti-CD38 DAR construct expressed the anti-CD38 DAR construct while failing to express the endogenous T cell

receptor (Figure 19H). In these cultures a high proportion (~49%) of the population are calculated to be GM-CSF knockouts resulting from the inclusion of the anti-GM-CSF RNP to the transfection.

Example 12. Targeted insertion of an anti-CD20 dimeric antibody receptor (DAR) construct into the TRAC gene using Cas12a.

The T cell receptor alpha constant (TRAC) gene was also targeted with an anti-CD20 DAR construct as the donor DNA. The anti-CD20 DAR construct (SEQ ID NO:81) included a nucleic acid sequence encoding two polypeptides linked by a "self-cleaving" 2A sequence that was used to generate two polypeptides from a single open reading frame. The first encoded polypeptide was a heavy chain polypeptide that included a heavy chain variable (VH) and the first heavy chain constant region (CH1), a hinge region, a transmembrane domain of CD28, and a cytoplasmic domain of 4-1BB and the third ITAM of CD3ζ. This was followed by the *Thosea asigna* virus T2A peptide-encoding sequence (SEQ ID NO:46**)** and then by the sequence encoding the second polypeptide, where the second polypeptide included, proceeding from the N-terminus to the C-terminus, an immunoglobulin light chain variable (VL) plus constant region (kappa). The nucleic acid sequences encoding the heavy chain polypeptide sequence, 2A peptide, and light chain sequence were operably linked to the JeT promoter (SEQ ID NO:3) at the 5' end of the DAR-encoding sequence and an SV40 polyA addition sequence (SEQ ID NO:47) at the 3'end of the DAR-encoding sequence. The entire anti-CD20 DAR construct (JeT promoter, heavy chain-encoding sequences with hinge, transmembrane of CD28, and cytoplasmic domains of 4-1BB and CD3ζ followed by T2A, light chain, and SV40 sequence (SEQ ID NO:48)), was cloned between homology arms of 645 bp (SEQ ID NO:50) and 600 bp (SEQ ID NO:51) in a pAAV vector. The homology arms (HAs) were sequences of the TRAC exon 1 locus on either side of the target sequence (SEQ ID NO:52) in exon 1 of the TRAC gene.

Donor fragment for use in transfection experiments was synthesized by PCR as described in Example 1 using the pAAV anti-CD20 DAR vector construct that included flanking TRAC HAs. The primers used were SEQ ID NO:82 (forward primer) which was 5' phosphorylated and SEQ ID NO:54 (reverse primer) which included 2'-O-methyl modifications on the three 5'-most nucleotides of the primer and phosphorothiate bonds between the first and second, second and third, and third and fourth nucleotides from the 5' end of the primer (**Table 1**). These primers hybridized within the 645 bp and 600 bp homology arms in the vector construct to generate a fragment with homology arms of 192 bp and 159 bp flanking the DAR construct. The resulting PCR product, a double stranded anti-CD20 DAR donor DNA fragment (SEQ ID NO:83) was 2.8 kb in size and including the 2.457 kb anti-CD20 DAR construct (SEQ ID NO:81), a 192 bp

homology arm (SEQ ID NO:55), and a 159 bp homology arm (SEQ ID NO:56) flanking the anti-CD20 DAR-encoding construct (SEQ ID NO:81) and incorporated the 2'-O-methyl and PS modifications of the reverse primer (SEQ ID NO:54) and the 5' terminal phosphate of the forward primer (SEQ ID NO:82) into the donor DNA molecule. The primer modifications of SEQ ID NO:54 incorporated into the first strand but no chemical modifications were introduced into the opposite, or second, strand, which include the 5' terminal phosphate of the primer. The donor molecule was used to transfect activated T cells as a double-stranded molecule together with a Cas12a protein complexed with a crRNA (guide RNA) that included the target sequence (SEQ ID NO:52).

For RNA guide-directed targeting of the TCR alpha (TRAC) gene, the crRNA (ALT-R® CRISPR-Cas12a crRNA) was purchased from IDT (Coralville, IA), where the crRNA was designed to include the target sequence (SEQ ID NO:52) that occurs directly downstream of a Cas12a PAM sequence (TTTA) in first exon of the TRAC gene.

Formation of the Cas12a and guide RNA RNP was performed essentially as described in Example 7. Electroporation of the Cas12a RNP and the double-stranded donor DNA into T cells was also performed essentially according to Example 7. As a control, one T cell population was transfected with the Cas12a RNP but no donor fragment, referred to as the TRAC knockout (KO) control. After transfection, T cells were transferred to complete cell culture medium for expansion.

After ten days, the cultures were analyzed by flow cytometry alongside the TRAC knockout control population as described in Example 1 except CD20 DAR expression was detected by Anti-Rituximab Antibodies (Acro) (**Figure 20A** and **20B**). Only about 16.6% of the cell population that was transfected with a Cas12a RNP targeting the TRAC gene in the absence of a donor fragment ("TRAC KO") expressed the TCR. An even smaller percentage, about 3.5%, of the cell population that was transfected with a Cas12a RNP targeting the TRAC gene along with a CD20 DAR donor fragment ("CD20 DAR-T") expressed the TCR. As expected, none of the cells that did not receive donor DNA were positive for the anti-CD20 constructs (**Figure 20A**). On the other hand, 28.7% of the population transfected with the anti-CD20 DAR construct donor DNA along with a Cas12a RNP (**Figure 20B**) expressed anti-CD20 DAR while not expressing the TCR.

The cells were tested in cytotoxicity assays performed essentially as in Example 1, except that the anti-CD20 DAR cells were incubated with CD20+ Daudi cells as targets for two days. **Figure 21** shows that the level of killing was very high, approaching 90% for effector:target

ratios ranging from 0.625:1 to 5:1, and even at the lowest effector:target ratio of 0.16:1, was approximately 70%. Figure **22A to 22B** demonstrates that the anti-CD20 CAR-T cells secreted a high level of interferon gamma (IFNγ) and GM-CSF when stimulated by CD20+ Daudi cells. Secretion of cytokines by anti-CD19 CAR-T cells (see Example 4) is also shown.

Example 13. Targeted insertion of an anti-CEA CAR construct into the TRAC and CD7 genes using Cas12a.

An anti-CEA CAR construct was also inserted into the TRAC gene using Cas12a. The anti-CEA CAR construct (SEQ ID NO:84) that included the JeT promoter (SEQ ID NO:3) at the 5' end of the CAR-encoding sequence and an SV40 polyA addition sequence (SEQ ID NO:47) at the 3'end of the CAR-encoding sequence was cloned between homology arms of 645 bp (SEQ ID NO:50) and 600 bp (SEQ ID NO:51) in a pAAV vector. The homology arms were sequences of the TRAC exon 1 locus on either side of the target sequence (SEQ ID NO:52) in exon 1 of the TRAC gene.

Donor fragment for use in transfection experiments was synthesized by PCR as described in Example 1. The primers used were SEQ ID NO:82 (forward primer) which was 5' phosphorylated and SEQ ID NO:54 (reverse primer) which included 2'-O-methyl modifications on the three 5'-most nucleotides of the primer and phosphorothiate bonds between the first and second, second and third, and third and fourth nucleotides from the 5' end of the primer (**Table 1**). These primers hybridized within the 645 bp and 600 bp homology arms in the vector construct, to generate a construct with homology arms of 192 bp and 159 bp flanking the DAR construct. The resulting PCR product, a double stranded anti-CEA CAR donor DNA fragment (SEQ ID NO:85) was 2.4 kb in size and including the 2.077 kb anti-CEA CAR construct (SEQ ID NO:84), a 192 bp homology arm (SEQ ID NO:55), and a 159 bp homology arm (SEQ ID NO:56) flanking the anti-CEA CAR construct and incorporated the 2'-O-methyl and PS modifications of the reverse primer (SEQ ID NO:54) and the 5' terminal phosphate of the forward primer (SEQ ID NO:82) into the donor DNA molecule. The primer modifications of SEQ ID NO:54 were incorporated into the first strand of the double-stranded donor DNA but no chemical modifications were introduced into the opposite, or second, strand, which included the 5' terminal phosphate of the primer. The donor molecule was used to transfect activated T cells as a double-stranded molecule together with a Cas12a protein complexed with a crRNA (guide RNA) that included the target sequence (SEQ ID NO:52).

For RNA guide-directed targeting of the TCR alpha (TRAC) gene, the crRNA (ALT-R® CRISPR-Cas12a crRNA (ALT-R® CRISPR-Cas12a crRNA) was purchased from IDT

bp (SEQ ID NO:90) and 170 bp (SEQ ID NO:91) flanking the CAR construct. The resulting PCR product, a double stranded anti-CEA CAR donor DNA fragment (SEQ ID NO:92) was 2.46 kb in size, including the 2.077 kb anti-CEA CAR construct (SEQ ID NO:84), a 212 bp homology arm (SEQ ID NO:90), and a 170 bp homology arm (SEQ ID NO:91) and incorporated the 2'-O-methyl and PS modifications of the forward primer (SEQ ID NO:88) and the 5' terminal phosphate of the reverse primer (SEQ ID NO:89) into the donor DNA molecule. The primer modifications of SEQ ID NO:88 were incorporated into the first strand but no chemical modifications were introduced into the opposite, or second, strand, which include the 5' terminal phosphate of the primer. The donor DNA was used to transfect activated T cells as a double-stranded molecule together with a Cas12a protein complexed with a crRNA (guide RNA) that included the target sequence (SEQ ID NO:87).

For RNA guide-directed targeting of the CD7 gene, the crRNA (ALT-R® CRISPR-Cas12a crRNA) was purchased from IDT (Coralville, IA), where the crRNA was designed to include the target sequence (SEQ ID NO:86) that occurs directly downstream of a Cas12a PAM sequence (TTTA) in first exon of the TRAC gene.

Formation of the Cas12a and guide RNA RNP and electroporation of the Cas12a RNP and the double-stranded donor DNA into T cells was also performed essentially according to Example 7. As a control, one T cell population was transformed with the Cas12a RNP but no donor fragment, referred to as the TRAC knockout (KO) control.

The transfected cultures were analyzed by flow cytometry alongside the TRAC knockout control population as described above (**Figure 23A to 23D**). **Figure 23A** shows that there was no detected expression of the CEA CAR in cells that were not transfected with donor DNA, although 89% of the population that was electroporated with a TRAC guide-RNP did lose expression of the TRAC gene (knockouts). When the donor was included in the electroporation, however, approximately 28.7% of the population failed to express the TCR while expressing the anti-CEA CAR (**Figure 23B**). Targeting the CD7 locus with an anti-CEA CAR donor and Cas12a RNP was even more efficient: approximately 38% of the population were knockin/knockout cells (expression of the anti-CEA CAR in the absence of CD7 expression) (**Figure 23D**). **Figure 23C** provides a basis for comparison of CD7 expression in cells in which the CD7 locus was not targeted (the cells were transfected with an RNP targeting the TRAC locus). In this population about 65% of the cell population expressed CD7 as compared with only about 8% of the population in which the CD7 locus was targeted with an RNP (**Figure 23D**).

Claims

What is claimed is:

1. A method for genetically modifying a primary human T cell at two different genetic loci, comprising introducing into a primary human T cell:

a first ribonucleoprotein (RNP) comprising a first RNA-guided endonuclease and a first guide RNA;

a second RNP comprising a second RNA-guided endonuclease and a second guide RNA; and

a donor DNA molecule comprising at least two nucleic acid modifications;

wherein the first guide RNA comprises a target sequence designed to hybridize with a first target site at a first genetic locus in the target DNA and the donor DNA is inserted into the target DNA molecule at the first target site;

further wherein the second guide RNA comprises a second target sequence designed to hybridize with a second target site at a second genetic locus in the target DNA resulting in a mutation at the second target site.

2. A method according to claim 1, wherein the at least two nucleic acid modifications are on a single strand of the donor DNA molecule.

3. A method according to claim 1 or 2, wherein one or more nucleic acid modifications are a modification of one or more nucleotides or nucleotide linkages within 10 nucleotides of the 5’ end of the modified strand of the donor DNA molecule.

4. A method according to claim 1, wherein one or more nucleic acid modifications is a backbone modification.

5. A method according to claim 4, wherein one or more nucleic acid modifications is a phosphorothioate modification or a phosphoramidite modification, or a combination thereof.

6. A method according to claim1, wherein one or more nucleic acid modifications is a modification or substitution of a nucleobase.

7. A method according to claim 1, wherein one or more nucleic acid modifications is a modification or substitution of a sugar.

8. A method according to claim 7, wherein one or more nucleic acid modifications is a 2’-O-methyl group modification of deoxyribose.

9. A method according to claim 1 or 2, wherein the donor DNA molecule is a double stranded DNA molecule.

10. A method according to claim 9, wherein the donor DNA molecule has a 5’ terminal phosphate on the strand opposite to the modified strand.

11. A method according to claim 10, wherein the donor molecule has between one and three phosphorothiorate modifications on the backbone within ten nucleotides of the 5’ terminus of the modified strand of the donor molecule and between one and three 2’-O-methyl nucleotide modifications within ten nucleotides of the 5’ terminus of the modified strand of the donor molecule.

12. A method according to claim 11, wherein the donor molecule has between one and three phosphorothiorate modifications on the backbone within five nucleotides of the 5’ terminus of the modified strand of the donor molecule and between one and three 2’-O-methyl nucleotide modifications within five nucleotides of the 5’ terminus of the modified strand of the donor molecule.

13. A method according to claim 1, wherein the donor DNA molecule includes homology arms flanking a sequence for integration into the genome.

14. A method according to claim 13, wherein at least one of the homology arms is from 50 to 2000 nucleotides in length.

15. A method according to claim 13, wherein at least one of the homology arms is from 140 to 660 nucleotides in length.

16. A method according to claim 13, wherein at least one of the homology arms is from 140 to 250 nucleotides in length.

17. The method of claim 13, wherein the donor DNA molecule comprises a modified strand and an opposite strand, wherein the modified strand comprises two or more nucleic acid modifications, and the opposite strand comprises a terminal phosphate.

18. The method of claim 1, wherein the donor DNA is from about 500 to about 5000 bp in length.

19. The method of claim 18, wherein the donor DNA is from about 500 to about 3500 bp in length.

20. The method of claim 1, wherein the donor DNA comprises a chimeric antigen receptor (CAR) or dimeric antigen receptor (DAR) construct.

21. A method according to claim 1, wherein the first and/or the second RNA-guided endonuclease is Cas9.

22. A method according to claim 21, wherein the first and/or the second RNA-guided endonuclease is Cas12a.

23. A method according to claim 22, wherein the first and the second RNA-guided endonuclease are Cas12a.

24. A method according to claim 21, wherein the first RNA-guided endonuclease is Cas12a and the second RNA-guided endonuclease is Cas9 or wherein the first RNA-guided endonuclease is Cas9 and the second RNA-guided endonuclease is Cas12a.

25. A method according to claim 1, wherein the first RNP and the donor DNA are introduced at the same time.

26. A method according to claim 25, wherein the first RNP, the donor DNA, and the second RNP are introduced into the cell at the same time.

27. A method according to claim 1, wherein the first RNP and the donor DNA are introduced into the cell at the same time, and the second RNP is introduced into the cell at a different time.

28. The method of claim 27, wherein the RNP is introduced into the cell by electroporation or liposome transfer.

29. An engineered primary T cell comprising:
a non-native genetic construct integrated into the genome at a first genetic locus comprising a first target site of an RNA-guided nuclease and a mutation at a second genetic locus comprising a second target site of an RNA-guided nuclease, wherein the engineered primary T cell is produced by the method of any of claims 1-28.

30. A population of primary human T cells transfected with a genetic construct, wherein the cell population comprises cells having a non-native genetic construct integrated into the genome at a first genetic locus, and further have a mutation in a gene at a second genetic locus, wherein at least 25% of the cells of the population express the genetic construct and exhibit reduced expression of the gene at the second genetic locus.

31. A population of primary human T cells according to claim 30, wherein the first RNA-guided endonuclease target site and the second RNA-guided endonuclease site are Cas12a target sites.

32. A population of human T cells according to claim 30, wherein the first RNA-guided endonuclease target site is a Cas12a target site and the second RNA-guided endonuclease site is a Cas9 target site.

33. A population of human T cells according to claim 30, wherein the first RNA-guided endonuclease target site is a Cas9 target site and the second RNA-guided endonuclease site is a Cas12a target site.

34. A method for site-specific integration of a donor DNA into a target DNA molecule, comprising:

introducing into a cell:

an RNP comprising a Cas12a endonuclease and an engineered guide RNA; and

a donor DNA molecule comprising at least two nucleic acid modifications;

wherein the guide RNA comprises a target sequence designed to hybridize with a target site in the target DNA and the donor DNA is inserted into the target DNA molecule at the target site.

35. A method according to claim 34, wherein the at least two nucleic acid modifications are on a single strand of the donor DNA molecule.

36. A method according to claim 34 or 35, wherein one or more nucleic acid modifications are a modification of one or more nucleotides or nucleotide linkages within 10 nucleotides of the 5' end of the modified strand of the donor DNA molecule.

37. A method according to claim 34, wherein one or more nucleic acid modifications is a backbone modification.

38. A method according to claim 37, wherein one or more nucleic acid modifications is a phosphorothioate modification or a phosphoramidite modification, or a combination thereof.

39. A method according to claim 33, wherein one or more nucleic acid modifications is a modification or substitution of a nucleobase.

40. A method according to claim 33, wherein one or more nucleic acid modifications is a modification or substitution of a sugar.

41. A method according to claim 40, wherein one or more nucleic acid modifications is a 2'-O-methyl group modification of deoxyribose.

42. A method according to claim 34, wherein the donor DNA molecule is a double stranded DNA molecule.

43. A method according to claim 42, wherein the donor DNA molecule has a 5' terminal phosphate on the strand opposite to the modified strand.

44. A method according to claim 43, wherein the donor molecule has between one and three phosphorothiorate modifications on the backbone within ten nucleotides of the 5' terminus of the modified strand of the donor molecule and between one and three 2'-O-methyl nucleotide modifications within ten nucleotides of the 5' terminus of the modified strand of the donor molecule.

45. A method according to claim 44, wherein the donor molecule has between one and three phosphorothiorate modifications on the backbone within five nucleotides of the 5' terminus of the modified strand of the donor molecule and between one and three 2'-O-methyl nucleotide modifications within five nucleotides of the 5' terminus of the modified strand of the donor molecule.

46. A method according to claim 34, wherein the donor DNA molecule includes homology arms flanking a sequence for integration into the genome.

47. A method according to claim 46, wherein at least one of the homology arms is from 50 to 2000 nucleotides in length.

48. A method according to claim 47, wherein at least one of the homology arms is from 140 to 660 nucleotides in length.

49. A method according to claim 48, wherein at least one of the homology arms is from 140 to 250 nucleotides in length.

50. The method of claim 34, wherein the donor DNA molecule comprises a modified strand and an opposite strand, wherein the modified strand comprises two or more nucleic acid modifications, and the opposite strand comprises a terminal phosphate.

51. The method of claim 34, wherein the donor DNA is from about 500 to about 5000 bp in length.

52. The method of claim 51, wherein the donor DNA is from about 500 to about 3500 bp in length.

53. The method of claim 34, wherein the donor DNA comprises a chimeric antigen receptor (CAR) or dimeric antigen receptor (DAR) construct.

54. A method according to claim 34, wherein the guide RNA is a crRNA.

55. A method according to claim 54, further comprising introducing a tracr RNA into the cell.

56. The method of claim 34, wherein the RNP is introduced into the cell by electroporation or liposome transfer.

57. The method of claim 34, wherein the donor DNA and the RNP are introduced into the cell simultaneously or separately.

58. A system for targeted integration of a donor DNA into a target locus, comprising:
a Cas12a endonuclease;
a guide RNA; and
a double-stranded donor DNA molecule, wherein the donor DNA molecule includes one or more phosphorothioate bonds on a single modified strand of the double stranded DNA molecule within ten nucleotides of the 5’ terminus of the modified strand of the double stranded DNA molecule.

59. The system of claim 58, wherein the donor DNA molecule further comprises at least one modification of a sugar moiety or nucleobase of the modified strand within ten nucleotides of the 5’ terminus of the modified strand of the double stranded DNA molecule.

60. The system of claim 58, wherein the donor DNA has homology arms flanking a sequence of interest for integration into the genome.

61. The system of claim 58, wherein the one or more phosphorothioate bonds on the single modified strand of the double stranded DNA molecule is within five nucleotides of the 5' terminus of the modified strand of the double stranded DNA molecule.

62. The system of claim 61, wherein the at least one modification of a sugar moiety or nucleobase of the modified strand is within five nucleotides of the 5' terminus of the modified strand of the double stranded DNA molecule.

63. The system of claim 61, wherein the at least one modification of a sugar moiety comprises a 2'-O methylation.

64. The system of claim 60, wherein the sequence of interest comprises an expression cassette.

65. The system of claim 64, wherein the expression cassette comprises a construct comprising one or more antibody or receptor domains.

66. A system according to claim 60, wherein at least one of the homology arms is from 50 to 2000 nucleotides in length.

67. A system according to claim 66, wherein at least one of the homology arms is from 140 to 660 nucleotides in length.

68. A system according to claim 66, wherein at least one of the homology arms is from 140 to 250 nucleotides in length.

69. The system of claim 61, wherein the donor DNA molecule comprises a modified strand and an opposite strand, wherein the modified strand comprises two or more nucleic acid modifications, and the opposite strand comprises a terminal phosphate.

70. The system of claim 58, wherein the donor DNA is from about 500 to about 5000 bp in length.

71. The system of claim 58, wherein the donor DNA is from about 500 to about 3500 bp in length.

72. The system of claim 64, wherein the donor DNA comprises a chimeric antigen receptor (CAR) or dimeric antigen receptor (DAR) construct.

73. The system of claim 58, wherein the guide RNA is a crRNA.

74. The system of claim 58, wherein the guide RNA comprises one or more phosphorothioate (PS) oligonucleotides.

75. The system of claim 58, comprising a ribonucleoprotein complex comprising the cas12a endonuclease and the guide RNA.

76. A primary human T cell having a CAR or DAR construct inserted into the CD7 gene.

77. A primary human T cell according to claim 76, wherein the CAR or DAR construct is a CEA CAR or DAR construct.

78. A population of T cells according to claim 76.