

another locus (e.g. a check point gene such as CISH or PD1) such that the resulting cells comprise edits at the TCR, B2M and any number of other genes (e.g., CISH) genes, resulting in cells lacking a native TCR, HLA complex and a knock out in additional genes such as the CISH check point gene and comprise a donor transgene (e.g. a CAR). These cells are useful for elimination of other cells bearing the target antigen. In preferred examples, the engineered CAR+ T cells are prepared for adoptive cell therapy. In some examples, the engineered CAR+ T cells express a modified HLA-E and/or HLA-G complex, allowing them to escape NK-mediated killing. Additionally, provided herein are methods and compositions for expressing an antigen on an irrelevant cell type for the purpose of analyzing activity of engineered T cells *in vitro*.

[0020] In one aspect, the present disclosure refers to an ~~An~~ isolated genetically modified T-cell comprising: a first polynucleotide encoding a chimeric antigen receptor (CAR), the first polynucleotide linked to ~~and~~ a second polynucleotide comprising sequences encoding a fusion protein comprising a beta-2-micro-globulin (B2M) protein and HLA-E and/or HLA-G protein, wherein the first and second polynucleotides are integrated into a T-cell receptor-alpha (TCRA) gene and the TCRA gene is inactivated. In one example, provided herein is a genetically modified T-cell (and populations of T cells comprising a plurality of these genetically modified T cells), the genetically modified T-cell comprising: a first polynucleotide encoding a chimeric antigen receptor (CAR) and a second polynucleotide comprising sequences encoding a fusion protein comprising a beta-2-micro-globulin (B2M) protein and HLA-E and/or HLA-G protein, in which the first and second polynucleotides are integrated into a T-cell receptor- α (TCRA) gene. In certain examples, one or more additional genes are inactivated in the cell, for example, a B2M gene and/or one or more immunological checkpoint genes (e.g., PD1, CISH, etc.). In any of the T cells described herein, the first and second polynucleotides may be linked by a self-cleaving peptide (e.g., 2A peptide also referred to as "P2A" or "P2A peptide"). Furthermore, in certain examples, the second polynucleotide further comprises a sequence encoding a linker (e.g., 1, 2, 3, 4, 5, 6 or more copies of a G4S linker) between the B2M and HLA-E- or HLA-G-encoding sequences.

[0020a] In another aspect, the present disclosure refers to a method of making the genetically modified T-cell as described herein, the method comprising: cleaving the TCR- α gene in the isolated T-cell using a zinc finger nuclease comprising a DNA-binding domain that binds to a target site in the TCR- α gene; and introducing a vector comprising the first and second polynucleotides into the T-cell such that the first and second polynucleotides are integrated into the cleaved TCR- α gene.

[0021] ~~In another aspect, the present disclosure refers to~~ Also provided are methods of making genetically modified T-cells as described herein, the method comprising: cleaving a TCR- α gene in the isolated T-cell using a nuclease comprising a DNA-binding domain that binds to a target site in the TCR- α gene; and introducing one or more donors comprising the first and second polynucleotides into the T-cell such that the first and second polynucleotides are integrated into the cleaved TCR- α gene. In certain examples, the donor comprises first and second polynucleotides linked by a self-cleaving 2A peptide. Furthermore, in certain examples, the second polynucleotide further comprises a sequence encoding a linker (e.g., 1, 2, 3, 4, 5, 6 or more copies of a G4S linker) between the

CLAIMS

What is claimed is:

1. An isolated genetically modified T-cell comprising: a first polynucleotide
5 encoding a chimeric antigen receptor (CAR), the first polynucleotide linked to~~and~~ a
second polynucleotide comprising sequences encoding a fusion protein comprising a
beta-2-micro-globulin (B2M) protein and HLA-E and/or HLA-G protein, wherein the
first and second polynucleotides are integrated into a T-cell receptor-alpha (TCRA)
gene and the TCRA gene is inactivated.

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2. The genetically modified T-cell of claim 1, wherein an endogenous B2M
gene is inactivated in the T-cell.

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3. The genetically modified T-cell of claim 1 or 2, wherein an immunological
check point gene is inactivated in the T-cell.

4. The genetically modified T-cell of any of claims 1 to 3, wherein the first
and second polynucleotides are linked by a self-cleaving P2A peptide.

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5. The genetically modified T-cell of any of claims 1 to 4, wherein the second
polynucleotide further comprises a sequence encoding a linker between the B2M and
HLA-E- or HLA-G-encoding sequences.

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6. The genetically modified T-cell of claim 4, wherein the linker comprises 1,
2, 3, 4, 5 or 6 copies of G4S.

7. A method of making the genetically modified T-cell of any of claims 1 to
6, the method comprising:

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cleaving the TCR- α gene in the isolated T-cell using a zinc finger nuclease
comprising a DNA-binding domain that binds to a target site in the TCR- α gene; and

introducing a vector~~one or more donors~~ comprising the first and second
polynucleotides into the T-cell such that the first and second polynucleotides are
integrated into the cleaved TCR- α gene.

8. The method of claim 7, wherein the vector~~donor~~ further comprises homology arms to the TCR- α gene flanking the first and second polynucleotides.

9. The method of claim 7 or claim 8, wherein the vector~~donor~~ further comprises a promoter that drives expression of the first and/or second polynucleotide.

10. The method of any of claims 7 to 9, wherein the vector~~donor~~ further comprises a TCR- α enhancer sequence.

11. The method of any of claims 7 to 10, wherein the vector~~donor~~ further comprises a 3' untranslated region (3' UTR) and a 5' untranslated region (5' UTR).

12. The method of claim 11, wherein the 3' UTR further comprises a WPRE sequence.

13. The method of claim 11 or claim 12, wherein the 5' UTR further comprises a sequence from a *Xenopus* beta globin gene.

14. A pharmaceutical composition comprising the cell of any one of claims 1-6.

15. Use of the cell of any one of claims 1-6 or the pharmaceutical composition of claim 14 in the manufacture of a medicament for treating cancer or graft versus host disease in a subject.