

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 isolated genetically modified T-cell comprising: a first polynucleotide encoding a chimeric antigen receptor (CAR), the first polynucleotide linked to 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] 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 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.
3. The genetically modified T-cell of claim 1 or 2, wherein an immunological
15 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.
- 20 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.
6. The genetically modified T-cell of claim 4, wherein the linker comprises 1,
25 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:
cleaving the TCR- α gene in the isolated T-cell using a zinc finger nuclease
30 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.

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

5 9. The method of claim 7 or claim 8, wherein the vector 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 further comprises a TCR- α enhancer sequence.

10 11. The method of any of claims 7 to 10, wherein the vector 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.
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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.
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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.