

[0017] Despite the recent successes of adoptive cell therapy, there remain some significant hurdles to overcome before the wide spread adoption of this technique. For example, the endogenous $\alpha\beta$ T-cell receptor (TCR) on infused allogeneic (donor derived) engineered T cells may recognize major and minor histocompatibility antigens in the recipient leading to graft-versus-host-disease (GVHD). As a result, the majority of current clinical trials infuse autologous CAR+ T cells (T-cells comprising an expressed CAR encoding sequence) relying on immune tolerance to prevent TCR-mediated deleterious recognition of normal tissues after adoptive transfer. This approach has achieved initial clinical successes targeting CD19+ malignancies, but is limited by the time and expense to manufacture patient-specific T-cell products (Torikai *et al* (2012) *Blood* 119:5697-5705). Another issue that has arisen in the treatment of patients with these engineered T cells is one of relapse, where the patients have been found to initially clear that tumor, only to relapse with a tumor cell that has down regulated the targeted antigen, so-called ‘immune escape’ (Sotillo *et al* (2015) *Cancer Dis* 5(12):1282-1295 and Gardner *et al* (2016) *Blood*; 127(20): 2406–2410).

[0018] Thus, despite the potential demonstrated by the use of adoptive cell therapy, there remains a need for methods and compositions that can be used to increase the efficiency and specificity of armed T cells. Specifically, methods are needed to safely introduce and sustain expression of antigen specific complexes including CARs and engineered TCRs. In addition, methods are needed to develop universal engineered T cells, lacking markers that are recognized by the patient’s immune system (*e.g.*, HLA complexes) that may be used to treat a large number of patients without the need to develop modified cells for each patient to be treated.

SUMMARY

[0019] Disclosed herein are compositions and methods for introduction of a chimeric antigen receptor into an engineered T cell. The methods presented comprise targeted introduction of a gene encoding one or more donor transgenes (*e.g.* CAR(s)) into a cell, including targeted integration of one or more CAR(s) into one or more TCR genes and/or a B2M gene (also referred to as CAR+ cells). The resulting cells of the disclosure thus comprise one or more CAR(s) specific to the antigen of choice (*e.g.*, CD20, CD171, CD19), and/or IL13R α 2), and also lack a functional endogenous TCR and/or HLA complex. In some examples, the cells are further modified at

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) 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. 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.

[0021] In another aspect, the present disclosure refers to 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

B2M and HLA-E- or HLA-G-encoding sequences. In any of the methods described herein, the one or more donors may further comprise one or more of the following: (1) homology arms (to the TCR- α gene) flanking the first and second polynucleotides; (2) one or more promoters that drives expression of the first and/or second
5 polynucleotide; (3) one or more TCR- α enhancer sequences; (4) a 3' untranslated region (3' UTR) and/or a 5' untranslated region (5' UTR); (5) a WPRE sequence (*e.g.*, in the 3' UTR); and/or (5) a sequence from a *Xenopus* beta globin gene (*e.g.*, in the 5' UTR).

[0022] In another example, described herein is an isolated cell (*e.g.*, a
10 eukaryotic cell such as a mammalian cell including a lymphoid cell, a stem cell (*e.g.*, iPSC, embryonic stem cell, MSC or HSC), or a progenitor cell) in which one or more exogenous CAR(s) is expressed following site-specific integration of the CAR gene into the genome of the cell, including for example an endogenous gene. In some examples, the CAR is inserted into a TCR gene (TCRA and/or TRBC), and/or
15 optionally into a checkpoint gene (*e.g.*, CISH, PD1, etc.) and/or B2M gene using nucleases targeted to these genes as described herein or known in the art. In further examples, the CAR is inserted into the TCRA gene, while in others, it is inserted into the TRBC gene. In some examples, it is inserted into a B2M gene while in other examples, it is inserted into a checkpoint gene (*e.g.*, CISH, PD1, etc.) gene in addition
20 to the TCR gene. In the cells produced by this method, introduction of the gene encoding the CAR results in the knock out of an endogenous TCR, checkpoint (*e.g.*, CISH) and/or B2M gene, and results in the knock out of the formation of a functional endogenous TCR or HLA complex and/or the knock out of the check point gene (*e.g.*, CISH). In any of the cells described, if the CAR is integrated into a B2M gene, one
25 or more TCR genes in the cell may be inactivated in the cell (*e.g.*, by nuclease-mediated inactivation) to create a knockout of multiple genes (*e.g.*, a B2M gene and one or more TCR genes and a checkpoint (*e.g.*, CISH) gene). Similarly, if the CAR is integrated into a TCR gene, one or more different TCR genes, a checkpoint (*e.g.*, CISH) and/or a B2M gene may be inactivated in the cell (*e.g.*, by nuclease-mediated
30 inactivation) to create a knockout of multiple genes (*e.g.*, one or more TCR genes, a CISH gene and a B2M gene).

[0023] In further examples, the genetically modified cells (T-cells) described herein further comprise an altered HLA-E or HLA-G gene (transgene) that is inserted into the cells encoding the CAR, for example into a TCR (TCRA) gene

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along with the CAR. In some examples, an HLA-E or HLA-G gene (*e.g.*, exogenous transgene) is co-introduced into the engineered cells with the CAR gene. In some examples, the HLA-E or HLA-G transgene is introduced before or after the CAR gene is introduced. In further examples, the transgene comprises a HLA-E or HLA-G gene sequence that is fused to a sequence encoding a linker which is then fused to a B2M gene sequence (*i.e.*, transgene comprising an HLA-E or HLA-G-coding sequence, a linker and a B2M-coding sequence), thus encoding a modified HLA-E or HLA-G complex where the B2M gene is part of the open reading frame with the HLA-E or HLA-G gene such that upon expression, a stable HLA-E or HLA-G complex may form with the B2M expressed from the transgene. In this example, due to the presence of the linker, the B2M subunit can only associate with the HLA-E or HLA-G protein to form a stable HLA-E or HLA-G complex on the cell surface and cannot associate with any other HLA complexes. In still further examples, the HLA-E or HLA-G-linker-B2M transgene is linked to a sequence encoding a CAR of interest, wherein the construct further comprises a 2A self-cleaving peptide sequence between the CAR gene and the HLA-E or HLA-G-linker-B2M construct. In yet further examples, this CAR-HLA-G or CAR- HLA-E complex also comprises homology arms such that the complex may be integrated into a genome at a specified site. The site of integration may in some examples cause transcription driven by an endogenous promoter at the site of integration, or the CAR- HLA-E or CAR-HLA-G construct may be linked to a promoter that is also integrated such that the expression construct is integrated into the genome but is driven by the heterologous promoter. In some examples, the CAR- HLA-E or CAR-HLA-G complex (transgene) is operably linked to a promoter to allow expression from an episomal (*e.g.* cDNA) nucleic acid. Additional sequences, including, but not limited to, WPRE and/or TCRA enhancer sequences may also be included in the donor construct. In further examples, the promoter is a tissue specific promoter. Further, any of these expression constructs may further comprise AAV ITRs to allow packaging into an AAV particle. In some instances, the AAV is an AAV5, AAV6, AAV9 or any other type of AAV.

[0024] In some examples, the TCR gene is modulated by modification of exon c2 of the TCRA gene. The B2M gene is modulated by modification of exon 1 of the B2M gene. In certain examples, the modification is within and/or adjacent to a sequence comprising 12-25 (including target sites of 12, 13, 14, 15, 16, 17, 18, 19, 20,

21, 22, 23, 24, 25) or more nucleotides as shown in the target sites of Table 1, within and/or adjacent to a target site as shown in Table 1 (SEQ ID NO:1 and 2; SEQ ID NO:3 and 4, SEQ ID NO:5 and 6; SEQ ID NO:39 and 40; SEQ ID NO:50 and 51); within 1-5, within 1-10 or within 1-20 base pairs on either side (the flanking genomic sequences) of the target sites shown in Table 1 (SEQ ID NO:1-6, 39, 40, 50 or 51); or within TTGAAA for the TCRA ZFN pair, or within GCCTTA for the B2M ZFN pair. Alternatively, or in addition, the modifications may also be made to sequences (*e.g.*, genomic sequences) between paired target sites as described herein (*e.g.*, target sites for the nuclease pairs shown in Table 1, including between the target sites for 55266 and 53853 (between SEQ ID NO:1 and SEQ ID NO:2) or 68813 and 68812 (SEQ ID NO:39 and SEQ ID NO:40) in the TCRA gene; and/or between the target sites for 57071 and 57531 (between SEQ ID NO:3 and SEQ ID NO:4) in the B2M gene; between the target sites for 59488 and 59489 in the CISH gene (SEQ ID NO:50 and SEQ ID NO:51). In additional examples, additional genomic modifications are made between the target sites for 37706 and 48407 in the HPRT gene (SEQ ID NO:5 and 6). The genetic modification may be made by introduction, into a cell, of an exogenous fusion molecule comprising a functional domain (*e.g.*, transcriptional regulatory domain, nuclease domain) and a DNA-binding domain, including but not limited to ZFP DNA-binding domain(s), TALE DNA-binding domains and sg RNA DNA-binding domains of a CRISPR/Cas system. The modified cells may include, but are not limited to: (i) a cell comprising an exogenous transcription factor comprising a DNA-binding domain that binds to a target site as shown in Table 1 and a transcriptional regulatory domain in which the transcription factor modulates B2M and/or TCRA gene expression and/or (ii) a cell comprising an insertion and/or a deletion at or near (within, between or adjacent to) one or more of the target sites shown in Table 1; within 1-5, within 1-10 or within 1-20 base pairs on either side (the flanking genomic sequence) of the target sites shown in Table 1; or within TTGAAA for the TCRA, or GCCTTQ for the B2M; and/or between paired target sites as described herein (*e.g.*, target sites for the nuclease pairs shown in Table 1). The cell may include further modifications, for example an additional inactivated TCR or B2M gene, an inactivated HLA gene, CISH, PD1 and/or CTLA4 gene and/or a transgene encoding an Antibody-coupled T-cell Receptor (ACTR) and/or a transgene encoding a CAR, and/or a transgene encoding an antibody. It may also include an expression construct for a modified HLA-E or HLA-G complex. Pharmaceutical compositions

comprising any cell as described herein are also provided as well as methods of using the cells and pharmaceutical compositions in *ex vivo* therapies for the treatment of a disorder (*e.g.*, a cancer) in a subject.

[0025] Thus, in one example, described herein are genetically modified cells in which, in addition to the integration of a CAR-encoding gene, the expression of a TCR and/or B2M gene is modulated (*e.g.*, activated, repressed or inactivated). In some examples, the cells also comprise a modified HLA-E or HLA-G complex. In preferred examples, exon c2 of a TCRA gene is modulated and/or exon 1 of a B2M gene is modulated. The modulation may be by an exogenous molecule (*e.g.*, engineered transcription factor comprising a DNA-binding domain and a transcriptional activation or repression domain) that binds to the TCR and/or B2M gene and regulates TCR and/or B2M expression and/or via sequence modification of the TCR and/or B2M gene (*e.g.*, using a nuclease that cleaves the TCR or B2M gene and modifies the gene sequence by insertions and/or deletions). In some examples, cells are described that comprise an engineered nuclease (*e.g.*, ZFN, TALEN, CRISPR/Cas nuclease) to cause a knockout of a TCR and/or B2M gene and optionally additional genes such as immunological checkpoint genes (*e.g.*, CISH (nucleases of Table 1), PD1 (U.S. Patent No. 8,563,314), CTLA-4, etc.). In other examples, cells are described that comprise one or more engineered transcription factor (TF) such that the expression of a TCR and/or B2M gene is modulated. In some examples, the cells are T cells. Further described are cells wherein the expression of a TCR and/or B2M gene is modulated and wherein the cells are further engineered to comprise a least one exogenous transgene and/or an additional knock out of at least one endogenous gene (*e.g.*, beta 2 microglobulin (B2M) or TCR gene, and/or immunological checkpoint gene such as PD1 and/or CTLA4) or combinations thereof. The exogenous transgene(s) may be integrated into a TCR or B2M gene (*e.g.*, when the TCR or B2M gene is knocked out) and also may be integrated into one or more genes, including but not limited to a non-TCR or non-B2M gene such as a checkpoint gene (*e.g.*, CISH, PD1) or a safe harbor gene. In certain examples, one or more CAR transgenes (*e.g.*, a CD19-specific CAR) is integrated into a TRAC gene, a CISH gene and/or a B2M gene as well as a safe harbor gene (*e.g.*, AAVS1, Rosa, albumin, HPRT, etc.). The same or different CAR transgenes may be integrated into the same or different loci. In examples in which one or more CARs are integrated into one or more safe harbor genes in addition to a TRAC gene,

CISH and/or B2M are also preferably inactivated (*e.g.*, via nuclease-mediated inactivation via insertion and/or deletion). In some cases, the exogenous transgene encodes an ACTR. The transgene construct may be inserted by either HDR- or NHEJ- driven processes. In some examples the CAR-comprising cells with modulated TCR and/or B2M expression comprise at least an exogenous ACTR. Some cells comprising a TCR and/or B2M modulator further comprise a knockout of one or more check point inhibitor genes. In some examples, the checkpoint inhibitor gene is CISH (see, Table 1 below and U.S. Provisional Patent application 62/583,724), while in others, the checkpoint inhibitor gene is PD1 (see U.S Patent No. 8,563,314). In other examples, the check point inhibitor is CTLA4. *See, e.g.*, U.S. Patent No. 9,597,357. In further examples, the CAR-comprising TCR and/or B2M modulated cell further comprises a PD1 knockout and/or a CTLA4 knockout and/or a CISH knockout (Table 1 below). In some examples, the TCR gene modulated is a gene encoding TCR β (TCRB). In some examples this is achieved via targeted cleavage of the constant region of this gene (TCR β Constant region, or TRBC). In certain examples, the TCR gene modulated is a gene encoding TCR α (TCRA). In further examples, insertion is achieved via targeted cleavage of the constant region of a TCR α gene, including targeted cleavage of the constant region of a TCR α gene (referred to herein as “TRAC” sequences). In some examples, the TCR gene modified cells are further modified at the B2M gene, the CISH gene (targeting sites disclosed in Table 1), PD1 and/or CTLA4 genes, the HLA-A, -B, -C genes, or the TAP gene, or any combination thereof. In other examples, the regulator for HLA class II, CIITA (*see*, U.S. Patent 8,945,868), is also modified.

[0026] In certain examples, the CAR-comprising cells described herein further comprise a modification (*e.g.*, deletion and/or insertion, binding of an engineered TF to repress TCR expression) to a TCRA gene (*e.g.*, modification of exon c2). In certain examples, the modification is within and/or adjacent to any of the target sites shown in Table 1 (SEQ ID NOs 1 or 2) and/or between paired target sites (*e.g.*, target sites of nuclease pairs shown in Table 1), including modification by binding to, cleaving, inserting and/or deleting one or more nucleotides within any of these sequences and/or within 1-50 base pairs (including any value therebetween such as 1-5, 1-10 or 1-20 base pairs) of the gene (genomic) sequences flanking these sequences in the TCRA gene. In certain examples, the cells comprise a modification (binding to, cleaving, insertions and/or deletions) within one or more of

the following sequences: TTGAAA within a TCRA gene (*e.g.*, exon c2) or GCCTTA within a B2M gene (*e.g.* exon 1). In certain examples, the modification comprises binding of an engineered TF as described herein such that a TCRA gene expression is modulated, for example, repressed or activated. In other examples, the modification is a genetic modification (alteration of nucleotide sequence) at or near nuclease(s) binding (target) and/or cleavage site(s), including but not limited to, modifications to sequences within 1-300 (or any number of base pairs therebetween) base pairs upstream, downstream and/or including 1 or more base pairs of the site(s) of cleavage and/or binding site; modifications within 1-100 base pairs (or any number of base pairs therebetween) of including and/or on either side of the binding and/or cleavage site(s); modifications within 1 to 50 base pairs (or any number of base pairs therebetween) including and/or on either side (*e.g.*, 1 to 5, 1 to 10, 1 to 20 or more base pairs) of the binding and/or cleavage site(s); and/or modifications to one or more base pairs within the nuclease binding site and/or cleavage site. In certain examples, the modification is at or near (*e.g.*, 1-300 base pairs, 1-50, 1-20, 1-10 or 1-5 or any number of base pairs therebetween) and/or between paired target sites (*e.g.*, Table 1) of the TCRA gene sequence within, surrounding or between any of SEQ ID NO:1 and SEQ ID NO:2 and/or SEQ ID NO:39 and 40. In certain examples, the modification includes modifications of a TCRA gene adjacent to one or more of the sequences shown in SEQ ID NO:1 and SEQ ID NO:2, SEQ ID NO:39 and 40 or within TTGAAA of a TCRA gene (*e.g.*, exon c2), for example a modification of 1 or more base pairs to one or more of these sequences. In certain examples, the nuclease-mediated genetic modifications are between paired target sites (when a dimer is used to cleave the target). The nuclease-mediated genetic modifications may include insertions and/or deletions of any number of base pairs, including insertions of non-coding sequences of any length and/or transgenes of any length and/or deletions of 1 base pair to over 1000 kb (or any value therebetween including, but not limited to, 1-100 base pairs, 1-50 base pairs, 1-30 base pairs, 1-20 base pairs, 1-10 base pairs or 1-5 base pairs).

[0027] In certain examples, the modification is within and/or adjacent to any of the target sites shown in Table 1 (SEQ ID NO:3 and SEQ ID NO:4) and/or between paired target sites (*e.g.*, target sites of nuclease pairs shown in Table 1) of a B2M gene (*e.g.*, modification of exon 1), including modification by binding to, cleaving, inserting and/or deleting one or more nucleotides within any of these

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sequences and/or within 1-50 base pairs (including any value therebetween such as 1-5, 1-10 or 1-20 base pairs) of the gene (genomic) sequences flanking these sequences in the B2M gene. In certain examples, the cells comprise a modification (binding to, cleaving, insertions and/or deletions) within GCCTTA within exon 1 of a B2M gene.

5 In certain examples, the modification comprises binding of an engineered TF as described herein such that B2M gene expression is modulated, for example, repressed or activated. In other examples, the modification is a genetic modification (alteration of nucleotide sequence) at or near nuclease(s) binding (target) and/or cleavage site(s), including but not limited to, modifications to sequences within 1-300 (or any number of base pairs therebetween) base pairs upstream, downstream and/or including 1 or more base pairs of the site(s) of cleavage and/or binding site; modifications within 1-100 base pairs (or any number of base pairs therebetween) of including and/or on either side of the binding and/or cleavage site(s); modifications within 1 to 50 base pairs (or any number of base pairs therebetween) including and/or on either side (e.g., 15 1 to 5, 1 to 10, 1 to 20 or more base pairs) of the binding and/or cleavage site(s); and/or modifications to one or more base pairs within the nuclease binding site and/or cleavage site. In certain examples, the modification is at or near (e.g., 1-300 base pairs, 1-50, 1-20, 1-10 or 1-5 or any number of base pairs therebetween) and/or between paired target sites (e.g., Table 1) of the B2M gene sequence within, 20 surrounding or between any of SEQ ID NO:3 and SEQ ID NO:4. In certain examples, the modification includes modifications of a B2M gene within one or more of the sequences shown in SEQ ID NO:3 and SEQ ID NO:4, or within GCCTTA within exon 1 of a B2M gene, for example a modification of 1 or more base pairs to one or more of these sequences. In certain examples, the nuclease-mediated genetic 25 modifications are between paired target sites (when a dimer is used to cleave the target). The nuclease-mediated genetic modifications may include insertions and/or deletions of any number of base pairs, including insertions of non-coding sequences of any length and/or transgenes of any length and/or deletions of 1 base pair to over 1000 kb (or any value therebetween including, but not limited to, 1-100 base pairs, 1-50 base pairs, 1-30 base pairs, 1-20 base pairs, 1-10 base pairs or 1-5 base pairs). 30

[0028] In certain examples, the modification is within and/or adjacent to any of the target sites shown in Table 1 (SEQ ID NO:5 and SEQ ID NO:6) and/or between paired target sites (e.g., target sites of nuclease pairs shown in Table 1) of an

HPRT gene (*e.g.*, modification of intron 1), including modification by binding to, cleaving, inserting and/or deleting one or more nucleotides within any of these sequences and/or within 1-50 base pairs (including any value therebetween such as 1-5, 1-10 or 1-20 base pairs) of the gene (genomic) sequences flanking these sequences in the B2M gene. In certain examples, the cells comprise an insertion into intron 1 such that the HPRT gene is not transcribed. Expression of the transgene inserted into HPRT gene may be driven by the endogenous HPRT promoter or may include an exogenous promoter. In other examples, the modification is a genetic modification (alteration of nucleotide sequence) at or near nuclease(s) binding (target) and/or cleavage site(s), including but not limited to, modifications to sequences within 1-300 (or any number of base pairs therebetween) base pairs upstream, downstream and/or including 1 or more base pairs of the site(s) of cleavage and/or binding site; modifications within 1-100 base pairs (or any number of base pairs therebetween) of including and/or on either side of the binding and/or cleavage site(s); modifications within 1 to 50 base pairs (or any number of base pairs therebetween) including and/or on either side (*e.g.*, 1 to 5, 1 to 10, 1 to 20 or more base pairs) of the binding and/or cleavage site(s); and/or modifications to one or more base pairs within the nuclease binding site and/or cleavage site. In certain examples, the modification is at or near (*e.g.*, 1-300 base pairs, 1-50, 1-20, 1-10 or 1-5 or any number of base pairs therebetween) and/or between paired target sites (*e.g.*, Table 1) of the B2M gene sequence within, surrounding or between any of SEQ ID NO:3 and SEQ ID NO:4. In certain examples, the modification includes modifications of a B2M gene within one or more of the sequences shown in SEQ ID NO:3 and SEQ ID NO:4, or within GCCTTA within exon 1 of a B2M gene, for example a modification of 1 or more base pairs to one or more of these sequences. In certain examples, the nuclease-mediated genetic modifications are between paired target sites (when a dimer is used to cleave the target). The nuclease-mediated genetic modifications may include insertions and/or deletions of any number of base pairs, including insertions of non-coding sequences of any length and/or transgenes of any length and/or deletions of 1 base pair to over 1000 kb (or any value therebetween including, but not limited to, 1-100 base pairs, 1-50 base pairs, 1-30 base pairs, 1-20 base pairs, 1-10 base pairs or 1-5 base pairs).

[0029] In certain examples, the CISH gene is also modified (*e.g.*, inactivation with or without integration), using a nuclease that binds to any of the

CISH target sites shown in Table 1 (SEQ ID NO:50 and SEQ ID NO:51). In certain examples, the modification comprise an insertion and/or deletion such that the CISH gene is not transcribed or such that the mutant transcript is subject to nonsense mediated decay. Expression of one or more transgenes that have been inserted into the CISH gene may be driven by the endogenous CISH promoter or may include an exogenous promoter. In other examples, the modification is a genetic modification (alteration of nucleotide sequence) at or near nuclease(s) binding (target) and/or cleavage site(s), including but not limited to, modifications to sequences within 1-300 (or any number of base pairs therebetween) base pairs upstream, downstream and/or including 1 or more base pairs of the site(s) of cleavage and/or binding site; modifications within 1-100 base pairs (or any number of base pairs therebetween) of including and/or on either side of the binding and/or cleavage site(s); modifications within 1 to 50 base pairs (or any number of base pairs therebetween) including and/or on either side (e.g., 1 to 5, 1 to 10, 1 to 20 or more base pairs) of the binding and/or cleavage site(s); and/or modifications to one or more base pairs within the nuclease binding site and/or cleavage site. In certain examples, the modification is at or near (e.g., 1-300 base pairs, 1-50, 1-20, 1-10 or 1-5 or any number of base pairs therebetween) and/or between paired target sites (e.g., Table 1) of the CISH gene sequence within, surrounding or between any of SEQ ID NO:50 and SEQ ID NO:51. In certain examples, the nuclease-mediated genetic modifications are between paired target sites (when a dimer is used to cleave the target). The nuclease-mediated genetic modifications may include insertions and/or deletions of any number of base pairs, including insertions of non-coding sequences of any length and/or transgenes of any length and/or deletions of 1 base pair to over 1000 kb (or any value therebetween including, but not limited to, 1-100 base pairs, 1-50 base pairs, 1-30 base pairs, 1-20 base pairs, 1-10 base pairs or 1-5 base pairs).

[0030] The CAR+ modified cells of the disclosure may be a eukaryotic cell, including a non-human mammalian and a human cell such as lymphoid cell (e.g., a T-cell), a stem/progenitor cell (e.g., an induced pluripotent stem cell (iPSC), an embryonic stem cell (e.g., human ES), a mesenchymal stem cell (MSC), or a hematopoietic stem cell (HSC). The CAR+ modified cells are also optionally modified to express a modified HLA-E or HLA-G complex. The stem cells may be totipotent or pluripotent (e.g., partially differentiated such as an HSC that is a pluripotent myeloid or lymphoid stem cell). In other examples, the disclosure

provides methods for producing CAR+ cells that have a null genotype for TCR and/or B2M expression. Any of the CAR+ modified stem cells described herein (modified at the TCRA and/or B2M locus, optionally expressing a modified HLA-E or HLA-G) may then be differentiated to generate a differentiated (*in vivo* or *in vitro*) cell descended from a stem cell as described herein with modified TCRA and/or B2M gene expression. In certain examples, described herein is a population of CAR+ modified cells as described herein in which at least 20% (or any percentage between 20% and 100%), preferably at least 50% (or any percentage between 50% and 100%), even more preferably at least 60% (or any percentage between 60% and 100%), and even more preferably at least 70% to 100% (or any percentage therebetween) of the cells are CAR+ modified.

[0031] In another example, the compositions (CAR+ modified cells, optionally expressing a modified HLA-E or HLA-G complex) and methods described herein can be used, for example, in the treatment or prevention or amelioration of a disorder. The methods typically comprise (a) integration of a CAR-encoding transgene into a cell by cleaving or an endogenous TCR and/or B2M gene in an isolated cell (*e.g.*, T-cell or lymphocyte) using a nuclease (*e.g.*, ZFN or TALEN) or nuclease system such as CRISPR/Cas with an engineered crRNA/tracr RNA, such that the TCR gene is inactivated or down modulated; and (b) introducing the cell into the subject, thereby treating or preventing the disorder. In some examples, the gene encoding the CAR or CAR- HLA-E or CAR-HLA-G expression construct is specifically integrated into TCR β (TCRB) such that expression of TCRB is inactivated. In some examples, inactivation is achieved via targeted cleavage of the constant region of this gene (TCR β Constant region, or TRBC) and integration of a CAR. In preferred examples, the gene encoding the CAR or CAR- HLA-E or CAR-HLA-G expression construct is integrated into TCR α (TCRA) such that TCRA is inactivated. In further preferred examples inactivation is achieved via targeted cleavage of the constant region of this gene (TCR α Constant region, or abbreviated as TRAC). In some examples, the gene encoding the CAR is specifically integrated into B2M such that expression of B2M is inactivated. In some examples, the CAR+ cell further comprises an engineered transcription factor that down modulates a gene. In further examples, the transcription factor is a ZFP-TF, a TALE-TF, or a CRISPR/dCas-TF. In some examples, further genes are modulated including but not limited to a B2M, CISH, PD1 and/or CTLA4 gene.

[0032] In some examples, the additional genes are modulated (knocked-out), for example, B2M, PD1, CISH and/or CTLA4 and/or one or more therapeutic transgenes are present in the cell (episomal, randomly integrated or integrated via targeted integration such as nuclease-mediated integration). The nature of the therapeutic transgene can be to promote the killing activity of the CAR+ T cell, or to act as a killing switch to the modified T cells themselves (making a so-call “smart” CAR T cell, see Hartmann *et al* (2017) *EMBO Mol Medicine*, DOI 10.15252/emmm.201607485). In some examples, the transgene is a suicide gene such that the activation of the gene causes death of the CAR T cell. In some examples, the suicide gene is an HSV-TK gene that encodes the thymidine kinase enzyme which is able to metabolize ganciclovir, an antiviral drug that is normally devoid of antitumor effect, into a toxic compound that interferes with DNA replication and results in cell apoptosis; a cytosine deaminase gene (also coupled to the uracil phosphoribosyltransferase gene) that transforms 5-fluorocytosine (with antifungal properties) into 5-fluorouracil; the nitroreductase gene transforms CB1954 (for [5-(aziridin-1-yl)-2,4-dinitrobenzamide]) into a toxic compound, the 4-hydroxylamine; and the cytochrome P450 gene that transforms ifosfamide to acrolein (nitrogen mustard) (Rouanet *et al* (2017), *Int J Mol Sci*, 18(6): 1231) or inducible caspase-9 (Jones *et al* (2014) *Front Pharmacol*. 5:254). In further examples, the transgene encodes a factor to calm severe side effects and/or toxicities such as cytokine release syndrome (CRS) and/or neurotoxicities (*e.g.* a scFv construct targeted to IL6 or a secretable IL-12 construct- *e.g.* fourth generation “TRUCK” (Chmielewski *et al*, (2014) *Immunol Rev*. 257(1):83-90).

[0033] The transcription factor(s) and/or nuclease(s) can be introduced into a cell or the surrounding culture media as mRNA, in protein form and/or as a nucleic acid sequence encoding the nuclease(s). In certain examples, the isolated CAR+ cell introduced into the subject further comprises additional genomic modification, for example, an integrated exogenous sequence (into the cleaved TCR and/or B2M gene or a different gene, for example a safe harbor gene or locus) and/or inactivation (*e.g.*, nuclease-mediated) of additional genes, for example one or more HLA genes. In some examples, one CAR gene is inserted into a TCR gene and a second (identical or non-identical) CAR gene is inserted into a B2M gene. In both these examples, the CAR gene may be part of a CAR- HLA-E or CAR-HLA-G expression construct. The exogenous sequence or protein may be introduced via a

vector (e.g. Ad, AAV, LV), or by using a technique such as electroporation. In some examples, the proteins are introduced into the cell by cell squeezing (see Kollmannsperger *et al* (2016) *Nat Comm* 7, 10372 doi:10.1038/ncomms10372). In some examples, the composition may comprise isolated cell fragments and/or differentiated (partially or fully) cells.

[0034] In some examples, the CAR+ cells may be used for cell therapy, for example, for adoptive cell transfer. In other examples, the CAR+ cells may be used in a T cell transplant and may contain other gene modification(s) of interest. In one example, the CAR+ T cells contain a CAR specific for a cancer marker. In a further example, the inserted CAR is specific for the CD19 marker characteristic of B cells, including B cell malignancies. Targeted integration of the CAR into the B2M gene would generate ‘universal’ cells useful in a therapeutic composition for treating patients without having to match HLA, and so would be able to be used as an “off-the-shelf” therapeutic for any patient in need thereof. Targeted integration of the CAR- HLA-E or CAR-HLA-G expression construct may create an “off-the-shelf” universal cell that will not be subject to NK-mediated killing within the treated patient, and thus may survive for a longer period in the body.

[0035] In some examples, the CAR encoding sequence is inserted via targeted integration where the CAR donor sequence comprises flanking homology arms that have homology to the sequence flanking the cleavage site of the engineered nuclease. In some examples the CAR donor sequence or CAR- HLA-E or CAR-HLA-G donor sequence further comprises a promoter and/or other transcriptional regulatory sequences. In other examples, the CAR or CAR- HLA-E or CAR-HLA-G donor sequence lacks a promoter. In further examples, the CAR or CAR- HLA-E or CAR-HLA-G donor sequence comprises a switch whereby separate extracellular antigen-binding domain and intracellular signaling components can be assembled, for example, through an FKBP-FRB module only in the presence of heterodimerizing small molecules (rapamycin analog AP21967) (see Wu *et al* (2015) *Science*. 350(6258):aab4077). Any of the donor constructs described herein may further include additional sequences, including but not limited to homology arms (of any length); WPRE sequences and/or TCRA enhancer sequences.

[0036] In another example, the CAR+ or CAR+, modified HLA-G+ or modified HLA E+ TCR- or B2M-modulated (modified) T cells further contain an inserted Antibody-coupled T-cell Receptor (ACTR) donor sequence. In some examples,

the ACTR donor sequence is inserted into a TCR and/or gene to disrupt expression of that TCR and/or B2M gene following nuclease induced cleavage. In other examples, the donor sequence is inserted into a “safe harbor” locus, such as the AAVS1, HPRT, albumin and CCR5 genes.

5 **[0037]** In another aspect, the present disclosure refers to a pharmaceutical composition comprising the cell as disclosed herein. Also provided are pharmaceutical compositions comprising the modified cells as described herein (*e.g.*, CAR+, or CAR+, modified HLA-E+ or HLA-G+ T cells or stem cells with inactivated TCR and/or B2M gene), or pharmaceutical compositions comprising one
10 or more of the TCR and/or B2M gene binding molecules (*e.g.*, engineered transcription factors and/or nucleases) as described herein. In certain examples, the pharmaceutical compositions further comprise one or more pharmaceutically acceptable excipients. The modified cells, TCR and/or B2M gene binding molecules (or polynucleotides encoding these molecules) and/or pharmaceutical compositions
15 comprising these cells or molecules are introduced into the subject via methods known in the art, *e.g.* through intravenous infusion, infusion into a specific vessel such as the hepatic artery, or through direct tissue injection (*e.g.* muscle). In some examples, the subject is an adult human with a disease or condition that can be treated or ameliorated with the composition. In other examples, the subject is a pediatric
20 subject where the composition is administered to prevent, treat or ameliorate the disease or condition (*e.g.*, cancer, graft versus host disease, etc.).

[0038] In another aspect, the present disclosure refers to the use of the cell as disclosed herein or the pharmaceutical composition as disclosed herein in the manufacture of a medicament for treating cancer or graft versus host disease in a
25 subject. In some examples, a composition (modulated cells comprising a CAR or a CAR and a modified HLA-E or HLA-G) can comprise a CAR comprising an antibody antigen binding domain. In some examples, the CAR is useful for arming an engineered T cell to prevent or treat a condition. In some examples, the CAR recognizes an antigen associated with a tumor cell or with cancer associated processes
30 such as BCMA, EpCAM, CEA (pancreatic adenocarcinoma, breast cancer, colorectal carcinoma), gpA33, mucins, TAG-72, CAIX (renal cell carcinoma (RCC)), folate-binding antibodies, CD19 (B-cell malignancies), CD20, CD22, EGFR (epithelial carcinoma, glioma), EGFRvIII (glioblastoma), EpCam, EphA2, ERBB2/HER2 (breast, ovarian, colon cancers; osteosarcoma, medullablastoma) ERBB3, FAP
35 (malignant pleural mesothelioma), MET, MSLN (mesothelioma, ovarian cancer, pancreatic adenocarcinoma), Igκ, IL-1RAP, IGF1R, EPHA3, Lewis Y, NKG2D ligands, ROR1, FR-a (ovarian cancer), GD2 (neuroblastoma, melanoma), GPC3 (hepatocellular carcinoma), IL-13Rα2 (glioma), L1-CAM (neuroblastoma, melanoma,

ovarian adenocarcinoma), MUC1 (seminal vesicle cancer), CA125 (epithelial ovarian cancers), PD-L1, PSCA, CTAG1B (melanoma and ovarian cancer), PSMA (prostate cancer), VEGFR2, c-MET, CD133 (glioblastoma, cholangiocarcinoma (CCA)), TRAILR1, TRAILR2, RANKL, FAP, VEGF, VEGFR, $\alpha V\beta 3$ and $\alpha 5\beta 1$ integrins, CD30, CD33, CD52, CD70, CD123, CD138, CD16V, CTLA4, and enascin (Scott *et al* (2012) *Nat Rev Cancer* 12:278; Hartmann *et al* (2017) *EMBO Mol Med* ; DOI 10.15252/emmm.201607485, Yu *et al* (2017) *J Hematol Oncol* 10:78). In other examples, the CAR recognizes an antigen associated with an infectious disease such as HIV, HCV and the like (see *e.g.* Carillo *et al* (2017) *Transl Res.* pii: S1931-5244(17)30233-5. doi: 10.1016/j.trsl.2017.07.002). In some examples, the engineered T cell may comprise 2 or more non-identical CAR sequences integrated into the genome (*e.g.* specific for CD19 and CD20). In certain examples, the CAR comprises a CAR designated FMC63-CD8BBZ, in which FMC63 is an anti-CD19 scFv sequence (see, U.S. Patent No. 9,701,758) and CD8BBZ refers to the portions of the CAR excluding the scFv (CD8 hinge/transmembrane domain (CD8), costimulation domain from 41BB gene (BB), activation domain from CD3z gene (Z)).

[0039] The DNA-binding domain of the transcription factor or nuclease (*e.g.*, ZFP, TALE, sgRNA) may bind to a target site in a TCRA or B2M gene comprising 9, 10, 11, 12 or more (*e.g.*, 13, 14, 15, 16, 17, 18, 19, 20 or more) nucleotides of any of the target sites shown herein (*e.g.*, Table 1 as shown in SEQ ID NOs:1 - 4). The zinc finger proteins may include 1, 2, 3, 4, 5, 6 or more zinc fingers, each zinc finger having a recognition helix that specifically contacts a target subsite in the target gene. In certain examples, the zinc finger proteins comprise 4 or 5 or 6 fingers (designated F1, F2, F3, F4, F5 and F6 and ordered F1 to F4 or F5 or F6 from N-terminus to C-terminus), for example as shown in Table 1. The ZFPs as described herein may also include one or more mutations to phosphate contact residues of the zinc finger protein, for example, the nR-5Qabc mutant described in U.S. Provisional Nos. 62/378,978 and 62/443,981. In other examples, the single guide RNAs or TAL-effector DNA-binding domains may bind to a target site as described herein (*e.g.*, target sites of Table 1 shown in any of SEQ ID NOs: 1-4) or 12 or more base pairs within any of these target sites or between paired target sites. The nucleases described herein (comprising a ZFP, a TALE or a sgRNA DNA-binding domain) are capable of making genetic modifications within a TCRA gene or B2M gene comprising any of SEQ ID NO:1-4, including modifications (insertions and/or

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deletions) within any of these sequences (SEQ ID NO:1-4) and/or modifications to TCRA and/or B2M gene sequences flanking the target site sequences shown in SEQ ID NO:1-4, for instance modifications within exon c2 of a TCRA gene within TTGAAA.

5 **[0040]** Any of the proteins described herein may further comprise a cleavage domain and/or a cleavage half-domain (*e.g.*, a wild-type or engineered *FokI* cleavage half-domain). Thus, in any of the nucleases (*e.g.*, ZFNs, TALENs, CRISPR/Cas systems) described herein, the nuclease domain may comprise a wild-type nuclease domain or nuclease half-domain (*e.g.*, a *FokI* cleavage half domain). In other
10 examples, the nucleases (*e.g.*, ZFNs, TALENs, CRISPR/Cas nucleases) comprise engineered nuclease domains or half-domains, for example engineered *FokI* cleavage half domains that form obligate heterodimers. *See, e.g.*, U.S. Patent No. 7,914,796 and 8,034,598. In certain examples, one or more *FokI* endonuclease domains of the nucleases described herein may also comprise phosphate contact mutants (*e.g.*, R416S
15 and/or K525S) as described in U.S. Publication No. 20180087072.

[0041] In another example, the disclosure provides a polynucleotide encoding any of the proteins, fusion molecules and/or components thereof (*e.g.*, sgRNA or other DNA-binding domain) described herein. The polynucleotide may be part of a viral vector, a non-viral vector (*e.g.*, plasmid) or be in mRNA form. Any of the
20 polynucleotides described herein may also comprise sequences (donor, homology arms or patch sequences) for targeted insertion into the target gene (*e.g.*, TCRA, TCRB, checkpoint and/or B2M gene). In yet another example, a gene delivery vector comprising any of the polynucleotides described herein is provided. In certain examples, the vector is an adenoviral vector (*e.g.*, an Ad5/F35 vector) or a lentiviral
25 vector (LV) including integration competent or integration-defective lentiviral vectors or an adeno-associated vector (AAV). Thus, also provided herein are viral vectors comprising a sequence encoding a nuclease (*e.g.* ZFN or TALEN) and/or a nuclease system (CRISPR/Cas or Ttgo) and/or a donor sequence for targeted integration into a target gene. In some examples, the donor sequence and the sequences encoding the
30 nuclease are on different vectors. In other examples, the nucleases are supplied as polypeptides. In preferred examples, the polynucleotides are mRNAs. In some examples, the mRNA may be chemically modified (*See e.g.* Kormann *et al.*, (2011) *Nature Biotechnology* 29(2):154-157). In other examples, the mRNA may comprise an ARCA cap (see U.S. Patents 7,074,596

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and 8,153,773). In some examples, the mRNA may comprise a cap introduced by enzymatic modification. The enzymatically introduced cap may comprise Cap0, Cap1 or Cap2 (see *e.g.* Smietanski *et al.*, (2014) *Nature Communications* 5:3004). In further examples, the mRNA may be capped by chemical modification. In further examples, the mRNA may comprise a mixture of unmodified and modified nucleotides (see U.S. Patent Publication 2012-0195936). In still further examples, the mRNA may comprise a WPRE element (see U.S. Patent Publication No. 20160326548). In some examples, the WPRE element may comprise one or more mutations (see U.S. Patent 7,419,829). In some examples, the mRNA is double stranded (See *e.g.* Kariko *et al.* (2011) *Nucl Acid Res* 39:e142).

[0042] In yet another example, the disclosure provides an isolated cell comprising any of the proteins, polynucleotides and/or vectors described herein. In certain examples, the cell is selected from the group consisting of a stem/progenitor cell, or a T-cell (*e.g.*, CD4⁺ T-cell). In a still further example, the disclosure provides a CAR+ or a CAR+, modified HLA-E+ and/or HLA-G+ cell or cell line which is descended from a cell or line comprising any of the proteins, polynucleotides and/or vectors described herein, namely a cell or cell line descended (*e.g.*, in culture) from a cell in which TCR and/or B2M has been inactivated by one or more ZFNs and/or in which a donor polynucleotide (*e.g.* ACTR and/or CAR) has been stably integrated into the genome of the cell. Thus, descendants of cells as described herein may not themselves comprise the proteins, polynucleotides and/or vectors described herein, but, in these cells, a TCR and/or B2M gene is inactivated and/or a donor polynucleotide is integrated into the genome and/or expressed.

[0043] In another example, described herein are methods of inactivating at least one TCR gene in a cell (*e.g.*, T-cell) and providing for the expression of an exogenous CAR in a cell by introducing one or more proteins, polynucleotides and/or vectors into the cell as described herein. In any of the methods described herein the nucleases may induce targeted mutagenesis, deletions of cellular DNA sequences, and/or facilitate targeted recombination at a predetermined chromosomal locus. Thus, in certain examples, the nucleases delete and/or insert one or more nucleotides from or into the target gene. In some examples the TCR and/or HLA gene is inactivated by nuclease cleavage followed by non-homologous end joining. In other examples, a genomic sequence in the target gene is replaced, for example using a nuclease (or vector encoding said nuclease) as described herein and a “donor”

sequence (*e.g.*, CAR) that is inserted into the gene following targeted cleavage with the nuclease. The donor sequence may be present in the nuclease vector, present in a separate vector (*e.g.*, AAV, Ad or LV vector) or, alternatively, may be introduced into the cell using a different nucleic acid delivery mechanism. In any of the methods described herein, the nuclease(s) and donors may be co-administered or administered sequentially in any order. Administration of the nuclease(s) and/or one or more donors may be repeated, for example the one or more donors (*e.g.*, one or more CARs) may be administered before, with and/or after a single nuclease (one, two, three, four or more nucleases targeted to different genes) administration; the one or more donors may be administered repeatedly before, with and/or after a single nuclease administration; or the one or more donors may be administered repeatedly before, with and/or after repeated nuclease administrations. In certain examples, TRAC, CISH and/or B2M-specific nucleases and one or more CAR donors are co-administered to provide a multi-knockout CAR-expressing cell in a single editing step method. In some examples, the methods further comprise inactivating one or more additional genes (*e.g.*, immunological checkpoint such as CISH, PD1 or CTLA4) and/or integrating one or more transgenes into the genome of the cell, including, but not limited to, integration of one or more transgenes into the inactivated TCR (and optionally additional genes B2M gene, etc.) and/or into one or more safe harbor genes. In certain examples, the methods described herein result in a population of cells in which at least 80-100% (or any value therebetween), including least 90-100% (or any value therebetween) of the cells include the knockout(s) and/or the integrated transgene(s).

[0044] Furthermore, any of the methods described herein can be practiced *in vitro*, *in vivo* and/or *ex vivo*. In certain examples, the methods are practiced *ex vivo*, for example to modify T-cells, to make them useful as therapeutics in an autologous or allogenic setting to treat a subject (*e.g.*, a subject with cancer). Non-limiting examples of cancers that can be treated and/or prevented include lung carcinomas, pancreatic cancers, liver cancers, bone cancers, breast cancers, colorectal cancers, leukemias, ovarian cancers, lymphomas, brain cancers and the like.

[0045] In another example, described herein is a method of integrating one or more transgenes into a genome of an isolated cell, the method comprising: introducing, into the cell, (a) one or more donor vectors (*e.g.*, AAVs, plasmids, Ads, mRNAs, etc.) comprising the one or more transgenes and (b) at least one non-

naturally occurring nuclease in mRNA form, wherein the at least one nuclease cleaves the genome of the cell such that the one or more transgenes are integrated into the genome of the cell (*e.g.*, one or more CAR gene(s) or CAR- HLA-E or CAR-HLA-G expression construct into a TCR or HLA receptor), wherein the donor vector is
5 introduced into the electroporation buffer comprising the isolated cell and the mRNA immediately before or immediately after electroporation of the nuclease into the cell. In certain examples, the donor vector is introduced into the electroporation buffer after electroporation and prior to transfer of the cells into a culture medium. *See, e.g.*, U.S. Publication Nos. 20150174169 and 20150110762.
10 The methods may be used to introduce the CAR transgene(s) or CAR- HLA-E or CAR-HLA-G expression construct into any genomic location, including, but not limited to, a TCR gene, a B2M gene and/or a safe harbor gene.

BRIEF DESCRIPTION OF THE DRAWINGS

15 [0046] **Figure 1** is a depiction of the HPRT gene showing the organization of introns and exons on the genomic version of the gene and the design of the CD19 expressing transgene for insertion into Intron 1. The transgene donor construct has homology arms (“homology”) flanking the CD19 transgene and also has a splice acceptor (SA) such that upon maturation of the transcript, exon 1 of HPRT will be
20 linked to the CD19 gene. The construct also comprises a 2A self-cleaving site between exon1 of HPRT and CD19. Also present is a poly A sequence at the end of the CD19 coding sequence. When transcribed and matured, the construct will result in eventual translation of only the CD19 coding sequences. Cells comprising the inserted CD19 transgene cassette will not express the HPRT gene, and thus will be resistant to
25 6-thioguanine (“6-TG”), allowing selection of only those cells with the inserted cassette in the presence of 6-TG.

[0047] **Figures 2A and 2B** are graphs depicting the expression of CD19 in K562 cells transfected with the CD19 expression cassette shown in Figure 1. To assess CD19 expression, cells were stained with a PE-Cy7-conjugated antibody
30 targeting human CD19 extracellular domain. Unmodified (“naïve”) K562 cells were similarly treated and stained (Figure 2A) as a negative control. Figure 2B shows the transfected K562 cells after 6-TG selection and demonstrates that nearly all the cells (98.3%) were positive for CD19 expression.

CLAIMS

What is claimed is:

1. An isolated genetically modified T-cell comprising: a first polynucleotide
5 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, wherein the first and second
polynucleotides are integrated into a T-cell receptor-alpha (TCRA) gene.
- 10 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
check point gene is inactivated in the T-cell.
- 15 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.
5. The genetically modified T-cell of any of claims 1 to 4, wherein the second
20 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,
2, 3, 4, 5 or 6 copies of G4S.
- 25 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 nuclease comprising a
DNA-binding domain that binds to a target site in the TCR- α gene; and
30 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.

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

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

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

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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.