CONTROLLED TRANSGENE EXPRESSION IN REGULATORY T CELLS

SEQUENCE LISTING

[001] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 20, 2020, is named 025297_WO006_SL.txt and is 32,818 bytes in size.

BACKGROUND OF THE INVENTION

[002] A healthy immune system is one that is in balance. Cells involved in adaptive immunity include B and T lymphocytes. There are two general types of T lymphocytes – effector T (Teff) cells and regulatory T (Treg) cells. Teff cells include $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. Teff cells play a central role in cellular-mediated immunity following antigen challenge. A key regulator of the Teff cells and other immune cells is the Treg cells, which prevent excessive immune responses and autoimmunity (*see, e.g.,* Romano et al., *Front Imm.* (2019) 10, art. 43).

[003] Some Tregs are generated in the thymus; they are known as natural Treg (nTreg) or thymic Treg (tTreg). Other Tregs are generated in the periphery following antigen encounter or in cell culture, and are known as induced Tregs (iTreg) or adaptive Tregs. Tregs actively control the proliferation and activation of other immune cells, including inducing tolerance, through cell-to-cell contact involving specific cell surface receptors and the secretion of inhibitory cytokines such as IL-10, TGF-β and IL-35 (Dominguez-Villar and Hafler, *Nat Immunol.* (2018) 19: 665-673). Failure of tolerance can lead to autoimmunity and chronic inflammation. Loss of tolerance can be caused by defects in Treg functions or insufficient Treg numbers, or by unresponsive or over-activated Teff (Sadlon et al., *Clin Transl Imm.* (2018) 7:e1011, doi:10-1002/cti2.1011).

[004] In recent years, there has been much interest in the use of Tregs to treat diseases. A number of approaches, including adoptive cell therapy, have been explored to boost Treg numbers and functions in order to treat autoimmune diseases. Treg transfer

(delivering an activated and expanded population of Tregs) has been tested in patients with autoimmune diseases such as type I diabetes, cutaneous lupus erythematosus, and Crohn's disease (Dominguez-Villar, *supra*). However, these cell populations are polyclonal in nature and thus may not be as effective as hoped. There also is evidence that simply increasing the number of Tregs may not be sufficient to control disease (McGovern et al., *Front Imm.* (2017) 8, art. 1517).

[005] Thus, there remains a need for effective cell therapy that can treat diseases associated with unwanted proliferation and activation of Teff cells.

SUMMARY OF THE INVENTION

[006] The present disclosure provides a genetically engineered mammalian cell (e.g., a human cell) comprising a heterologous sequence in a FOXP3 locus in the genome, wherein the heterologous sequence comprises a transgene, wherein the transgene is under the transcriptional control of a FOXP3 promoter in the FOXP3 locus; and when the promoter is activated, the cell expresses both FOXP3 and a product of the transgene from the locus.

[007] In another aspect, the present disclosure provides a method of making a genetically engineered mammalian cell (e.g., a human cell), comprising contacting a mammalian cell with a nucleic acid construct comprising (i) a heterologous sequence and (ii) a first homologous region (HR) and a second HR flanking the heterologous sequence, wherein the heterologous sequence comprises a transgene, the first and second HRs are homologous to a first genomic region (GR) and a second GR, respectively, in a FOXP3 locus in the mammalian cell; and culturing the cell under conditions that allow integration of the heterologous sequence between the first and second GRs in the FOXP3 locus. In some embodiments, the integration is facilitated by a zinc finger nuclease or nickase (ZFN), a transcription activator-like effector domain nuclease or nickase (TALEN), a meganuclease, an integrase, a recombinase, a transposase, or a CRISPR/Cas system. In some embodiments, the nucleic acid construct is a lentiviral construct, an adenoviral construct, an adeno-associated viral construct, a plasmid, a DNA construct, or an RNA construct.

[008] In some embodiments of the present disclosure, the heterologous sequence comprises (i) an internal ribosome entry site (IRES) immediately upstream of the transgene, or (ii) a coding sequence for a self-cleaving peptide immediately upstream of and in-frame with the transgene. The self-cleaving peptide may be, for example, a 2A peptide, where the 2A peptide is optionally selected from the group consisting of a P2A peptide, an E2A peptide, a F2A peptide, and a T2A peptide.

[009] In some embodiments, the heterologous sequence is inserted into a FOXP3 intron (e.g., intron 4, 9, or 10) upstream of one or more FOXP3 exons, wherein the heterologous sequence comprises (i) a nucleotide sequence coding for the one or more FOXP3 exons, and (ii) a splice acceptor upstream of the nucleotide sequence to allow expression of a full-length FOXP3 mRNA transcript from the locus, and the nucleotide sequence is (a) immediately upstream of the IRES, or (b) immediately upstream of and in-frame with the coding sequence for the self-cleaving peptide.

[010] In some embodiments, the transgene product is a chimeric antigen receptor (CAR) or a T-cell receptor (TCR). The CAR or TCR may be specific for, e.g., (i) an autoantigen, (ii) a B cell antigen optionally selected from CD19 and CD20, or (iii) an allogeneic HLA class I molecule, wherein the class I molecule is optionally HLA-A2. In particular embodiments, the transgene product is a CAR specific for allogeneic HLA-A2 and comprises, from N-terminus to C-terminus, (i) a scFV comprising a heavy chain variable domain (VH) and a light chain variable domain (VL), wherein the VH comprises HCDR1-3 comprising SEQ ID NOs: 1-3, respectively, and VL comprises LCDR1-3 comprising SEQ ID NOs: 5-7, or the VH and VL comprise SEQ ID NOs: 4 and 10, respectively; (ii) a transmembrane (TM) domain and a co-stimulation domain derived from CD28, CD4, or 4-1BB; and (iii) an intracellular CD3ζ signaling sequence.

[011] In other embodiments, the transgene product is a cytokine, a chemokine, a growth factor, or a signaling factor; or an AAV capsid protein selected from VP1, VP2, or VP3.

[012] In some embodiments, the engineered cell is a lymphoid cell (e.g., a Treg cell), a lymphoid progenitor cell, a mesenchymal stem cell, a hematopoietic stem cell, an induced pluripotent stem cell, or an embryonic stem cell. In preferred embodiments, the engineered cell is a human cell. In some embodiments, the cell comprises a null mutation

in a gene selected from a T cell receptor alpha or beta chain gene, a Class II major histocompatibility complex transactivator (CIITA) gene, a transporter associated with antigen processing (e.g., TAP-1 or TAP-2), an HLA Class I or II gene, a minor histocompatibility antigen gene, and a β2-microglobulin (B2M) gene.

[013] In some embodiments, the cell comprises a suicide gene optionally selected from a HSV-TK gene, a cytosine deaminase gene, a nitroreductase gene, a cytochrome P450 gene, or a caspase-9 gene.

[014] In other aspects, the present disclosure provides a method of treating a patient in need of immunosuppression, comprising administering to the patient the present engineered cell; use of the cell in the manufacture of a medicament in treating a patient in need of immunosuppression; and the engineered cell for use in treating a patient in need of immunosuppression. In some embodiments, the patient has an autoimmune disease. In some embodiments, the patient has received or will receive tissue transplantation. In preferred embodiments, the patient is a human.

BRIEF DESCRIPTION OF THE DRAWINGS

[015] **FIGs. 1A-D** are fluorescence activating cell sorting (FACS) plots analyzing Treg cells isolated from a healthy human volunteer.

[016] **FIG. 2A** is a schematic diagram depicting a genome editing approach to integrating a chimeric antigen receptor (CAR) coding sequence into intron 9 of the human *FOXP3* gene. A zinc finger nuclease (ZFN) produced from an introduced mRNA makes a double-stranded break at a specific site (lightning bolt) in intron 9. The donor sequence, introduced by an adeno-associated virus (AAV) 6 vector, contains, from 5' to 3', homology region 1, a splice acceptor (SA), the sequences of *FOXP3* exons 10-12, a coding sequence for self-cleaving peptide 2A, a CAR coding sequence, and homology region 2. The homology regions are homologous to the genomic regions flanking the ZFN cleavage site. The *FOXP3* exons, the 2A coding sequence and the CAR coding sequences are in-frame with each other.

[017] **FIG. 2B** is a schematic diagram depicting a similar genome editing approach to integrating a green fluorescent protein (GFP) coding sequence into intron 9 of the human *FOXP3* gene.

[018] **FIG. 2C** is a bar graph showing the level of allelic genome modification as assessed by MiSeq next generation sequencing in the study shown in FIG. 2B. Indels: insertions/deletions. TI: targeted integration. Mock: cells treated with vehicle. ZFNs: cells transfected with mRNA of a ZFN targeting *FOXP3* intron 9. Donor: cells transfected with the donor construct shown in FIG. 2B. ZFNs + Donor: cells transfected with the ZFN mRNA and the donor construct.

[019] **FIG. 2D** is a table showing the level of allelic genome modification as assessed by MiSeq next generation sequencing in T cells modified at different sites in the *FOXP3* locus using the general scheme shown in FIGs. 2A and 2B. For each targeted site A, B, C, D, E, or F, three T cell samples were tested: T cells treated with ZFN and the donor construct (first row), T cells treated with only the donor construct (second row), and T cells treated with GFP mRNA (third row).

[020] **FIG. 2E** is a bar graph summarizing the data shown in FIG. 2D and indicating the locations of the targeted sites A, B, C, D, E, and F.

[021] **FIGs. 3A-C** are flow cytometry graphs analyzing the same Tregs assessed in FIG. 2C. Mock: cells treated with vehicle. ZFNs: cells transfected with mRNA of a ZFN targeting *FOXP3* intron 9. SA-partial FOXP3-2A-GFP: cells transfected with the donor construct shown in FIG. 2B. ZFNs + Donor: cells transfected with the ZFN mRNA and the donor construct.

[022] **FIGs. 4A-C** depict an AAV-based donor construct containing a chimeric antigen receptor (CAR) specific for human MHC class I molecule HLA-A2. FIG. 4A: a schematic diagram of an HLA-A2 CAR with an anti-HLA-A2 scFv; CD28 hinge, transmembrane (TM) and signaling (co-stimulatory) domains; and a CD3ζ signaling domain (Boardman et al., *Am J Transpl.* (2017) 17:931-43). FIG. 4B: VH and VL amino acid sequences of the HLA-A2 scFv in the CAR (SEQ ID NOs: 95, 4 and 10, respectively, in order of appearance). *Id.* FIG. 4C: a schematic diagram of the AAV donor construct, in which the VH and VL of the HLA-A2 scFv is linked by a $[G_4S]_3$ peptide linker (SEQ ID NO: 90). SA: splice acceptor. Exons 10-12: exons 10-12 of *FOXP3*. 2A: 2A self-cleaving peptide. hGMCSF sig: human GM-CSF signal peptide. Myc: myc tag. TM: transmembrane domain. Co-stim: costimulatory domain. Signal: signaling domain. ITR: Inverted Terminal Repeat of the AAV.

[023] **FIGs. 5A-C** are flow cytometry graphs analyzing Tregs edited with the same ZFN and/or the donor construct shown in FIGs. 4B and 4C.

[024] **FIGs. 6A-C** are flow cytometry graphs analyzing $CD8^+$ effector T cells (Teff) edited with the same ZFN and/or the same donor construct used in FIGs. 4B and 4C.

[025] **FIGs. 7A** and **B** are flow cytometry graphs analyzing the fraction of FOXP3+ cells among the Treg cells of FIGs. 5A-C. FIG. 7A: unedited cells (cells treated with the AAV donor construct alone). FIG. 7B: edited cells (cells treated with the ZFN and the AAV donor construct). "Isotype": control antibody having the same isotype as the anti-FOXP3 antibody (IgG antibody mixture isolated from un-immunized animals).

[026] **FIG. 8** shows an annotated map of the human *FOXP3* gene. The homology arms (HA) flanking each ZFN cleavage site were incorporated into each respective AAV donor to promote homology-directed repair. Regulatory sites within intron 1 (CNS1 & TSDR), intron 2 (CNS2), and the core promoter region are also annotated.

DETAILED DESCRIPTION OF THE INVENTION

[027] The present disclosure provides genetically engineered regulatory T (Treg) cells containing a heterologous sequence (e.g., a transgene) in an endogenous genomic *FOXP3* locus, where the heterologous sequence can be transcribed into RNA under the control of the *FOXP3* transcription regulatory elements such as the *FOXP3* promoter. High FOXP3 expression level is a specific phenotype for Treg cells. As a result, the heterologous sequence is transcribed actively, together with the *FOXP3* gene, in Treg cells and the heterologous sequence is expressed only as long as the cell maintains its Treg, $FOXP3^+$ phenotype. The present disclosure also provides methods for making and using the genetically engineered Treg cells.

[028] Regulatory T cells maintain immune homeostasis and confer immune tolerance. The engineered Treg cells, which may be autologous or allogeneic, can be used in cell-based therapy to treat patients in need of induction of immune tolerance or restoration of immune homeostasis, such as patients receiving organ transplantation or allogeneic cell therapy and patients with an autoimmune disease. The present Treg cells will have enhanced immune-regulatory activities, including improved tissue-specificity (e.g., through expression of an edited-in receptor specific for an antigen in a targeted

tissue) and/or increased immunosuppressive functions (e.g., through secretion of an edited-in immunomodulatory molecule). The Tregs will actively control the proliferation and activation of T effector cells locally and/or systemically through receptor-mediated cell-to-cell contact and secretion of immunosuppressive cytokines (e.g., IL-10, TGF-β and IL-35).

[029] Since the engrafted Tregs can proliferate and self-renew, the present cell therapy can achieve long-term tolerance and protection of the transplant. *See, e.g.*, Dawson et al., *JCI Insight*. (2019) 4(6):e123672, which is incorporated herein by reference in its entirety.

[030] The Treg cells will also have the safety feature that the heterologous sequence will be expressed only as long as the Treg cells maintain their Treg phenotype. Once the cell loses *FOXP3* gene expression, the heterologous sequence will no longer be transcribed.

I. Cells Used for *FOXP3* Genome Editing

[031] The engineered cells of the present disclosure are mammalian cells, such as human cells, cells from a farm animal (e.g., a cow, a pig, or a horse), and cells from a pet (e.g., a cat or a dog). The genome editing described may be performed on Treg cells, or on cells that are not Treg cells but are differentiated into Treg cells after the genome editing. The Treg phenotype is in part dependent on the expression of the master transcription factor forkhead box P3 (FOXP3), which regulates the expression of a network of genes essential for immune suppressive functions.

[032] As used herein, the terms "regulatory T cells," "regulatory T lymphocytes," and Tregs refers to a subpopulation of T cells that modulates the immune system, maintains tolerance to self-antigens, and generally suppresses or downregulates induction and proliferation of T effector cells. Tregs often are marked by the phenotype of $CD4^+CD25^+CD127^{lo}FOXP3^+$. In some embodiments, Tregs are also $CD45RA^+$, $CD62L^{hi}$, and/or $GITR^+$. In particular embodiments, Tregs are marked by $CD4^+CD25^+CD127^{lo}CD62L^+$ or $CD4^+CD45RA^+CD25^{hi}CD127^{lo}$. As used herein, Tregs include (i) "natural" Tregs that develop in the thymus; (ii) induced, adaptive, or peripheral Tregs arising via a differentiation process that takes place outside the thymus

(e.g., in tissues or secondary lymphoid organs, or in laboratory settings under defined culture conditions); and (iii) Tregs that have been created using recombinant DNA technology, including genome editing and gene therapy.

1. Isolation of Treg cells for Genome Editing

[033] The Treg cells on which the genome editing is performed may be isolated from a number of sources, including peripheral blood mononuclear cells (PBMC), bone marrow, lymph node tissue, cord blood, thymus tissue, or spleen tissue. For example, Tregs may be isolated from a unit of blood collected from a subject using well known techniques such as Ficoll™ separation, centrifugation through a PERCOLL™ gradient following red blood cell lysis and monocyte depletion, counterflow centrifugal elutriation, leukapheresis, and subsequent cell surface marker-based magnetic or flow cytometric isolation.

[034] Further enrichment of Treg cells from the isolated white blood cells can be accomplished by positive and/or negative selection with a combination of antibodies directed to unique surface markers using techniques such as flow cytometry cell sorting and/or magnetic immunoadherence involving conjugated beads. For example, to enrich for $CD4^+$ cells by negative selection, a monoclonal antibody cocktail typically may include antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8. To enrich or positively select for Tregs, antibodies to CD4, CD25, CD45RA, CD62L, GITR, and/or CD127 can be used.

[035] In an exemplary and nonlimiting protocol, Treg cells may be obtained as follows (*see* Dawson et al., *JCI Insight*. (2019) 4(6):e123672). $CD4^+$ T cells are isolated from a human donor via RosetteSep™ (STEMCELL Technologies, 15062) and enriched for $CD25^+$ cells (Miltenyi Biotec, 130-092-983) prior to sorting live $CD4^+CD25^{hi}CD127^{lo}$ Tregs or $CD4^+CD127^{lo}CD25^{hi}CD45RA^+$Tregs using a MoFlo® Astrios™ (Beckman Coulter) or FACSAria™ II (BD Biosciences). Sorted Tregs may be stimulated with L cells and anti-CD3 monoclonal antibody (e.g., OKT3, UBC AbLab; 100 ng/ml) in ImmunoCult™-XF T cell expansion media (STEMCELL Technologies,10981) with 1000 U/ml IL-2 (Proleukin) as described in MacDonald et al., *J Clin Invest.* (2016) 126(4):1413-24). One or more days later, the Treg cells may be genome-edited as described below. For phenotypic analysis, cells may be stained with fixable viability dye

(FVD, Thermo Fisher Scientific, 65-0865-14; BioLegend, 423102) and for surface markers before fixation and permeabilization using an eBioscience FOXP3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, 00-5523-00) and staining for intracellular proteins. Samples were read on a CytoFLEX (Beckman Coulter).

[036] Tregs may also be derived from T effector cells *in vitro*, for example, by exposure to IL-10 or TGF-β.

2. Isolation of non-Treg Cells for Genome Editing

[037] The source cells, i.e., cells on which the genome editing is performed, may also be pluripotent stem cells (PSCs). PSCs are cells capable to giving rise to any cell type in the body and include, for example, embryonic stem cells (ESCs), PSCs derived by somatic cell nuclear transfer, and induced PSCs (iPSCs). *See, e.g.,* Iriguchi and Kaneko, *Cancer Sci.* (2019) 110(1):16–22 for differentiating iPSCs to T cells. As used herein, the term "embryonic stem cells" refers to pluripotent stem cells obtained from early embryos; in some embodiments, this term refers to ESCs obtained from a previously established embryonic stem cell line and excludes stem cells obtained by recent destruction of a human embryo.

[038] In other embodiments, the source cells for genome editing are multipotent cells such as hematopoietic stem cell (HSCs such as those isolated from bone marrow or cord blood), hematopoietic progenitor cells (e.g., lymphoid progenitor cell), or mesenchymal stem cells (MSC). Multipotent cells are capable of developing into more than one cell type, but are more limited than cell type potential than pluripotent cells. The multipotent cells may be derived from established cell lines or isolated from human bone marrow or umbilical cords. By way of example, the HSCs may be isolated from a patient or a healthy donor following G-CSF-induced mobilization, plerixafor-induced mobilization, or a combination thereof. To isolate HSCs from the blood or bone marrow, the cells in the blood or bone marrow may be panned by antibodies that bind unwanted cells, such as antibodies to CD4 and CD8 (T cells), CD45 (B cells), GR-1 (granulocytes), and Iad (differentiated antigen-presenting cells) (*see, e,g.*, Inaba, *et al.* (1992) *J Exp Med.* 176:1693-1702). HSCs can then be positively selected by antibodies to CD34.

[039] In still other embodiments, the source cells for genome editing are non-Treg lymphoid cells that are differentiated into Treg cells after genome editing. *See* above for how to differentiate T effector cells into Treg cells.

[040] The edited non-Treg cells may be differentiated into Treg cells before engrafting into a patient as described above. Alternatively, the edited non-Treg cells may be induced to differentiate into Treg cells after engrafting to a patient.

3. Additional Genome Editing

[041] The present engineered cells may be further genetically engineered, before or after the *FOXP3* genome editing, to make the cells more effective, more useable on a larger patient population, and/or safer.

[042] In some embodiments, the present *FOXP3*-edited cells may be allogeneic cells to the patient. In such instances, the cells may be engineered to reduce host rejection to these cells (graft rejection) and/or these cells' potential attack on the host (graft-versus-host disease). By way of example, the cells may be engineered to have a null genotype for one or more of the following: (i) T cell receptor (TCR alpha chain or beta chain); (ii) a polymorphic major histocompatibility complex (MHC) class I or II molecule (e.g., HLA-A, HLA-B, or HLA-C; HLA-DP, HLA-DM, HLA-DOA, HLA-DOB, HLA-DQ, or HLA-DR; or β2-microglobulin (B2M)); (iii) a transporter associated with antigen processing (e.g., TAP-1 or TAP-2); (iv) Class II MHC transactivator (CIITA); (v) a minor histocompatibility antigen (MiHA; e.g., HA-1/A2, HA-2, HA-3, HA-8, HB-1H, or HB-1Y); and (vi) any combination thereof. The allogeneic engineered cells may also express an invariant HLA or CD47 to protect the engineered Treg cells from host rejection. These further genetic modifications may be performed by the gene editing techniques known in the art and those described herein.

[043] The further-edited allogeneic cells are particularly useful because they can be used in multiple patients without compatibility issues. The allogeneic cells thus can be called "universal" and can be used "off the shelf." The use of "universal" cells greatly improves the efficiency and reduces the costs of adopted cell therapy.

[044] In some embodiments, the present *FOXP3*-edited engineered cells are engineered to include a safety switch such as a suicide gene as further discussed below.

4. Maintenance of Treg Phenotype

[045] Plasticity is a property inherent to nearly all types of immune cells. It appears that Treg cells are able to transition ("drift") to Teff cells under inflammatory and environmental conditions (Sadlon et al., *Clin Transl Imm.* (2018) 7(2):e1011). To maintain the Treg phenotype and/or to increase expression of FOXP3 and the transgene in the engineered Treg cells, the cells may be cultured in tissue culture media containing rapamycin and/or a high concentration of IL-2. *See, e.g.,* MacDonald et al., *Clin Exp Immunol.* (2019) doi: 10.1111/cei.13297.

II. Transgenes for Insertion into the *FOXP3* Locus

[046] The engineered cells of the present disclosure comprise a heterologous sequence integrated in either or both of their endogenous *FOXP3* gene loci. The term "heterologous" here means that the sequence is inserted into a site of the genome where this sequence does not naturally occur. The heterologous sequence may contain a transgene and may contain additional sequences from the genome, such as *FOXP3* sequences (*see, e.g.,* **FIGs. 2A** and **2B**). A transgene integrated into the *FOXP3* locus is co-expressed with *FOXP3* but is no longer expressed when the cell loses its Treg, $FOXP3^+$ phenotype. The transgene may encode a protein that enhances the Treg cells' therapeutic efficacy or provides a separate therapeutic benefit. The following are non-limiting examples of transgenes that may be expressed by the engineered Treg cells of the present disclosure.

1. Soluble Polypeptides

[047] Examples of transgenes useful herein are those encoding cytokines (e.g., IL-10), chemokines (e.g., CCR7), growth factors (e.g., remyelination factors for treatment of multiple sclerosis), and signaling factors (e.g., amphiregulin).

2. Antigen-Binding Receptors

[048] Other examples of transgenes useful herein are those encoding a T cell receptor (TCR) of interest or a chimeric antigen receptor (CAR) such that the engineered Tregs are specific to an antigen of interest.

[049] A CAR is a fusion protein designed to target T cells expressing it to a desired antigen. In its most basic form, a CAR comprises an extracellular antigen-binding

domain and a series of customized intracellular TCR costimulatory/signaling domains. Once the CAR binds to its antigen, it activates the cell expressing it, as a natural TCR would. Antigen-specific engineered Tregs enable enhanced immune suppression by homing to the targeted tissue (e.g., a transplant or a site of autoimmune inflammation). They can interact Teff cells specific for an allo-antigen (in cases of transplantation) or an autoantigen (in cases of autoimmune disease). CARs offer the advantage that, unlike natural TCRs, they bind to antigens without the need to interact with other co-stimulatory molecules or involvement of MHC class I or II molecules, thereby affording them functionality in broader settings.

[050] Tregs can functionally switch to effector-like T cells under certain circumstances (Sadlon, *supra*). Due to the antigen-specific clonality of the engineered CAR or TCR Tregs, functional switching to an effector-like state could induce unintended cytotoxicity, leading to exacerbated pathology. The present engineered Tregs avoid this potential problem because the CAR or TCR is only expressed while the T cells maintain their Treg ($FOXP3^+$) phenotype.

A. Antigen-Binding Domains of CARs

[051] The antigen-binding domain of a CAR may comprise an antibody fragment such as an scFv, a Fv, a Fab, a $(Fab')_2$, a single domain antibody (SDAB), a VH or VL domain, or a camelid VHH domain.

[052] In some embodiments, the CAR is specific for a polymorphic allogeneic MHC molecule, such as one expressed by cells in a solid organ transplant or by cells in a cell-based therapy (e.g., bone marrow transplant, cancer CAR T therapy, or cell-based regenerative therapy). MHC molecules so targeted include, without limitation, HLA-A, HLA-B, or HLA-C; HLA-DP, HLA-DM, HLA-DOA, HLA-DOB, HLA-DQ, or HLA-DR. By way of example, the CAR targets class I molecule HLA-A2. HLA-A2 is a commonly mismatched histocompatibility antigen in transplantation. HLA-A mismatching is associated with poor outcomes after transplantation. Engineered Tregs expressing a CAR specific for an MHC class I molecule are advantageous because MHC class I molecules are broadly expressed on all tissues, so the Tregs can be used for organ transplantation regardless of the tissue type of the transplant. CAR against HLA-A2 offers the additional advantage that HLA-A2 is expressed by a substantial proportion of

the human population and therefore on many donor organs. There has been evidence showing that expression of an HLA-A2 CAR in Treg cells can enhance the potency of the Treg cells in preventing transplant rejection (*see, e.g.,* Boardman, *supra*; MacDonald et al., *J Clin Invest.* (2016) 126(4):1413-24; and Dawson, *supra*).

[053] In some embodiments, the CAR is specific for an autoantigen, i.e., an endogenous antigen expressed prevalently or uniquely at the site of autoimmune inflammation in a specific tissue of the body. Tregs expressing such a CAR can home to the inflamed tissue and exert tissue-specific activity by causing local immunosuppression. Examples of autoantigens are aquaporin water channels (e.g., aquaporin-4 water channel), paraneoplastic antigen Ma2, amphiphysin, voltage-gated potassium channel, N-methyl-d-aspartate receptor (NMDAR), a-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid receptor (AMPAR), thyroid peroxidase, thyroglobulin, anti-N-methyl-D-aspartate receptor (NR1 subunit), Rh blood group antigens, desmoglein 1 or 3 (Dsgl/3), BP180, BP230, acetylcholine nicotinic postsynaptic receptors, thyrotropin receptors, platelet integrin, glycoprotein IIb/IIIa, calpastatin, citrullinated proteins, alpha-beta-crystallin, intrinsic factor of gastric parietal cells, phospholipase A2 receptor 1 (PLA2R1), and thrombospondin type 1 domain-containing 7A (THSD7A). Additional examples of autoantigens are multiple sclerosis-associated antigens (e.g., myelin basic protein (MBP), myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), oligodendrocyte myelin oligoprotein (OMGP), myelin associated oligodendrocyte basic protein (MOBP), oligodendrocyte specific protein (OSP/Claudin 11), oligodendrocyte specific proteins (OSP), myelin-associated neurite outgrowth inhibitor NOGO A, glycoprotein Po, peripheral myelin protein 22 (PMP22), 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), and fragments thereof); joint-associated antigens (e.g., citrulline-substituted cyclic and linear filaggrin peptides, type II collagen peptides, human cartilage glycoprotein 39 peptides, keratin, vimentin, fibrinogen, and type I, III, IV, and V collagen peptides); and eye-associated antigens (e.g., retinal arrestin, S-arrestin, interphotoreceptor retinoid-binding proteins, beta-crystallin Bl, retinal proteins, choroid proteins, and fragments thereof).

[054] In some embodiments, the autoantigen targeted by the Treg cells is IL23-R (for treatment of, e.g., Crohn's disease, inflammatory bowel disease, or rheumatoid arthritis), MOG (for treatment of multiple sclerosis), or MBP (for treatment of multiple sclerosis).

[055] In some embodiments, the TCR or CAR edited into the *FOXP3* locus may target other antigens of interest (e.g., B cell markers CD19 and CD20). Additionally, rather than using two separate CAR constructs, the CAR transgene itself may encode a bi-specific CAR capable of recognizing, e.g., both CD19 and CD20 (Zah et al., *Cancer Immunol Res.* (2016) 4(6):498-508).

B. CAR Co-Stimulatory and Stimulatory/Activating Domains

[056] The CAR may comprise one or more transmembrane and intracellular costimulatory and activating domains from one or more immune cell surface molecules. A costimulatory signaling domain can be the transmembrane and/or intracellular portion of a costimulatory molecule on a T cell. A costimulatory molecule on a T cell binds to its ligand on an antigen-presenting cell in concert with the TCR's binding to the antigen on the antigen-presenting cell, and allows the activation (e.g., proliferation and secretion of cytokines) of the antigen-bound T cell. A costimulatory molecule can be represented in the following protein families: TNF receptor proteins, immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SLAM proteins), and activating NK cell receptors. Costimulatory domains useful in constructing CARs are well known, including, without limitation, transmembrane and/or intracellular sequences from CD2, CD3 delta, CD3 epsilon, CD3 gamma, CD4, CD7, CD8α, CD8β, CD28, CD137 (4-1BB), TNFR2, and inducible T cell co-stimulator (ICOS). *See also, e.g.,* Chen and Flies, *Nat Rev Immunol.* (2013) 13(4):227-42.

[057] The activating domain of the CAR may be derived from CD3-zeta or CD3-epsilon. The CD3-zeta chain may have the protein sequence provided as GenBank Acc. No. BAG36664.1, or the equivalent residues from a non-human species (e.g., mouse, rodent, monkey, ape and the like). A CD3-zeta activating or stimulatory domain includes the amino acid residues from the cytoplasmic domain of the zeta chain, or functional derivatives thereof, that are sufficient to functionally transmit an initial signal necessary for T cell activation. In one embodiment, the cytoplasmic domain of zeta comprises

residues 52 through 164 of GenBank Acc. No. BAG36664.1 or the equivalent residues from a non-human species.

3. Other Transgenes

[058] The transgene inserted in the *FOXP3* locus may encode other proteins of interest. For example, the transgene may encode a protein that conditions the patient for other therapies such as gene therapy. By way of example, repeated administration of recombinant AAV in gene therapy for genetic diseases such as hemophilia may elicit anti-AAV immune response in the patient, making the therapy less effective in the long run. Thus, it may be desirable to introduce to the patient receiving AAV gene therapy engineered Tregs that express an antigen-specific receptor (e.g., CAR or TCR) toward an AAV capsid protein such as VP1, VP2, and/or VP3, to induce immune tolerance to these proteins in the patient.

[059] Coding sequences for an epitope tag may be included as part of the transgene to allow monitoring of the gene editing. Epitope tags include, for example, one or more copies of FLAG, His tag, myc tag, Tap tag, HA tag, low-affinity nerve growth factor receptor (LNGFR) and/or its antibody-binding domain as a tag, or any other readily detectable amino acid sequence.

4. Signal sequences for surface expression

[060] In some embodiments, the transgene comprises a coding sequence for a signal or leader peptide to facilitate the surface expression of the transgene. For example, the signal sequence may be one derived from the signal sequence of human GM-CSF or CD8.

III. Constructs Carrying the Transgenes

[061] Any gene editing method for targeted integration of a heterologous sequence into a specific genomic site may be used to introduce the heterologous sequence described herein into the *FOXP3* gene locus. In order for the transgene to be co-expressed under the *FOXP3* transcription regulatory elements (e.g., the *FOXP3* promoter), a heterologous sequence carrying the transgene may contain elements that allow the uninterrupted transcription of an intact *FOXP3* transcript despite the integration of the heterologous sequence into the *FOXP3* genomic locus.

[062] In some embodiments, the heterologous sequence is integrated into an *FOXP3* intron, i.e., upstream of at least one *FOXP3* exon. In such embodiments, the heterologous sequence may contain, from 5' to 3', a splice acceptor (SA) sequence, the exon(s) downstream of the heterologous sequence target site, a coding sequence for a self-cleaving peptide, and the transgene. Once integrated, the SA will allow the expression of an RNA transcript encoding an intact (i.e., full-length) FOXP3 polypeptide, the self-cleaving peptide, and the transgene product. Translation of this RNA transcript will yield two separate polypeptide products – the intact FOXP3 polypeptide and the transgene product, due to the self-cleaving nature of the intermediary peptide sequence. Examples of SA sequences are those of the *FOXP3* exons and other SA sequences known in the art. Examples of self-cleaving peptides are 2A peptides, which are viral derived peptides with a typical length of 18-22 amino acids. 2A peptides include T2A, P2A, E2A, and F2A. By way of example, P2A is a peptide of 19 amino acids; after the cleavage, a few amino acid residues from the P2A are left on the upstream gene and a proline is left at the beginning of the second gene.

[063] In other embodiments, instead of the coding sequence for the self-cleaving peptide, the heterologous sequence may carry an internal ribosome entry site (IRES) sequence between the coding sequence for the *FOXP3* exon(s) and the transgene. The IRES sequence will similarly allow the expression of two separate polypeptide products – the intact FOXP3 polypeptide and the transgene product.

[064] In still other embodiments, the heterologous sequence is integrated further downstream just prior to the stop codon in exon 12 in the *FOXP3* locus. In those embodiments, the heterologous sequence may carry a coding sequence for a self-cleaving peptide (as described above) 5' to the transgene, without the need to carry a sequence for any *FOXP3* exon. The self-cleaving peptide will allow the expression of a FOXP3 polypeptide in parallel with the transgene product.

[065] In still other embodiments, the heterologous sequence is integrated into a *FOXP3* exon. In those embodiments, the heterologous sequence will carry the coding sequence(s) for the *FOXP3* exonic sequence(s) downstream of the integration site, including the remaining downstream sequence of the disrupted exon, such that an intact *FOXP3* transcript can still be generated from the engineered genomic locus. Also in

those embodiments, the heterologous sequence may not need to include an SA, while the *FOXP3* sequence and the transgene sequence may be separated by a coding sequence for a self-cleaving peptide or an IRES as described above.

[066] Additional elements may be included in the heterologous sequence. For example, to allow transcription termination, the transgene may include a polyadenylation (polyA) site such as an SV40 polyA site. The heterologous sequence may also include RNA-stabilizing elements such as a Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE).

[067] In some embodiments, the heterologous sequence may also comprise a coding sequence for an invariant HLA or CD47 to promote the resistance of the engineered Treg cells (especially those with HLA class I knockout or knockdown) to the host's natural killer and other immune cells involved in anti-graft rejection. The HLA or CD47 coding sequence may be linked to the primary transgene in the heterologous sequence through a coding sequence for a self-cleaving peptide, such that the *FOXP3* locus can co-express FOXP3, the transgene product, and the invariant HLA or CD47.

[068] To enhance the precision of site-specific integration of the transgene, a construct carrying the heterologous sequence may contain on either or both of its ends a homology region that is homologous to the targeted genomic site. In some embodiments, the heterologous sequence carries in both of 5' and 3' end regions sequences homologous to the target genomic site, e.g., a site within intron 4, intron 9, or intron 10 of the *FOXP3* gene. *See, e.g.,* **FIGs. 2A**, **2B**, and **8**. The lengths of the homology regions on the heterologous sequence may be, for example, 50-1,000 base pairs in length. The homology region in the heterologous sequence can be, but need not be, identical to the targeted genomic sequence. For example, the homology region in the heterologous sequence may be at 80 or more percent (e.g., 85 or more, 90 or more, 95 or more, 99 or more percent) homologous or identical to the targeted genomic sequence (e.g., the sequence that is to be replaced by the homology region in the heterologous sequence). In further embodiments, the construct, when linearized, comprise on one end homology region 1, and on its other end homology region 2, where homology regions 1 and 2 are respectively homologous to genomic region 1 and genomic region 2 flanking the integration site in the genome.

[069] The genomic structure of the *FOXP3* locus is illustrated in **FIGs. 2A** and **8**. The gene sequence and the exon/intron boundaries of the human *FOXP3* gene can be found in Genbank ID 50943. The targeted site for integration may be in an intron (e.g., intron 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11), in a region downstream of the last exon of the *FOXP3* gene, in an exon (e.g., exon 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12), or in a junction between an intron and its adjacent exon.

[070] The construct carrying the heterologous sequence can be introduced to the target cell by any known techniques such as chemical methods (e.g., calcium phosphate transfection and lipofection), non-chemical methods (e.g., electroporation and cell squeezing), particle-based methods (e.g., magnetofection), and viral transduction (e.g., by using viral vectors such as vaccinia vectors, adenoviral vectors, lentiviral vectors, adeno-associated viral (AAV) vectors, retroviral vectors, and hybrid viral vectors). In some embodiments, the construct is an AAV viral vector and is introduced to the target human cell by a recombinant AAV virion whose genome comprises the construct, including having the AAV Inverted Terminal Repeat (ITR) sequences on both ends to allow the production of the AAV virion in a production system such as an insect cell/baculovirus production system or a mammalian cell production system). *See, e.g.,* **FIG. 4**. The AAV may be of any serotype, for example, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV8.2, AAV9, or AAVrh10, of a pseudotype such as AAV2/8, AAV2/5, or AAV2/6.

[071] The heterologous sequence may be integrated to the *FOXP3* genomic locus by any site-specific gene knockin technique. Such techniques include, without limitation, homologous recombination, gene editing techniques based on zinc finger nucleases or nickases (collectively "ZFNs" herein), transcription activator-like effector nucleases or nickases (collectively "TALENs" herein), clustered regularly interspaced short palindromic repeat systems (CRISPR, such as those using Cas9 or cpf1), meganucleases, integrases, recombinases, and transposes. As illustrated below in the Working Examples, for site-specific gene editing, the editing nuclease typically generates a DNA break (e.g., a single- or double-stranded DNA break) in the targeted genomic sequence such that a donor polynucleotide having homology to the targeted genomic sequence (e.g., the

construct described herein) is used as a template for repair of the DNA break, resulting in the introduction of the donor polynucleotide to the genomic site.

[072] Gene editing techniques are well known in the art. *See, e.g.,* U.S. Pats. 8,697,359, 8,771,945, 8,795,965, 8,865,406, 8,871,445, 8,889,356, 8,895,308, 8,906,616, 8,932,814, 8,945,839, 8,993,233, 8,999,641, 9,790,490, 10,000,772, 10,113,167, and 10,113,167 for CRISPR gene editing techniques. *See, e.g.,* U.S. Pats. 8,735,153, 8,771,985, 8,772,008, 8,772,453, 8,921,112, 8,936,936, 8,945,868, 8,956,828, 9,234,187, 9,234,188, 9,238,803, 9,394,545, 9,428,756, 9,567,609, 9,597,357, 9,616,090, 9,717,759, 9,757,420, 9,765,360, 9,834,787, 9,957,526, 10,072,062, 10,081,661, 10,117,899, 10,155,011, and 10,260,062 for ZFN techniques and its applications in editing T cells and stem cells. The disclosures of the aforementioned patents are incorporated by reference herein in their entirety.

[073] In gene editing techniques, the gene editing complex can be tailored to target specific genomic sites by altering the complex's DNA binding specificity. For example, in CRISPR technology, the guide RNA sequence can be designed to bind a specific genomic region; and in the ZFN technology, the zinc finger protein domain of the ZFN can be designed to have zinc fingers specific for a specific genomic region, such that the nuclease or nickase domains of the ZFN can cleave the genomic DNA at a site-specific manner. *See* further description in the Working Examples. Depending on the desired genomic target site, the gene editing complex can be designed accordingly. *See, e.g.,* GenBank ID 50943 for the sequence and structure of the human *FOXP3* gene.

[074] Components of the gene editing complexes may be delivered into the target cells, concurrent with or sequential to the transgene construct, by well known methods such as electroporation, lipofection, microinjection, biolistics, virosomes, liposomes, lipid nanoparticles, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA or mRNA, and artificial virions. Sonoporation using, *e.g.*, the Sonitron 2000 system (Rich-Mar) can also be used for delivery of nucleic acids. In particular embodiments, one or more components of the gene editing complex, including the nuclease or nickase, are delivered as mRNA into the cells to be edited.

IV. Safety Switch in Engineered Cells

[075] In cell therapy, it may be desirable for the transplanted cells to contain a "safety switch" in their genomes, such that proliferation of the cells can be stopped when their presence in the patient is no longer desired. A safety switch may, for example, be a suicide gene, which upon administration of a pharmaceutical compound to the patient, will be activated or inactivated such that the cells enter apoptosis. A suicide gene may encode an enzyme not found in humans (e.g., a bacterial or viral enzyme) that converts a harmless substance into a toxic metabolite in the human cell. Examples of suicide genes include, without limitation, genes for thymidine kinases, cytosine deaminases, nitroreductases, intracellular antibodies, telomerases, caspases, and DNases. *See, e.g.,* Zarogoulidis et al., *J Genet Syndr Gene Ther*. (2013) doi:10.4172/2157-7412.1000139. In some embodiments, the suicide gene may be a thymidine kinase (TK) gene from Herpes Simplex Virus (HSV). A HSV-TK gene can be turned on so as to kill the cell by administration of ganciclovir, valganciclovir, famciclovir, or the like to the patient.

[076] A safety switch may also be an "on" or "accelerator" switch, a gene encoding a small interfering RNA, an shRNA, or an antisense that interferences the expression of a cellular protein critical for cell survival.

[077] The safety switch may utilize any suitable mammalian and other necessary transcription regulatory sequences. The safety switch can be introduced into the cell through random integration or site-specific integration using gene editing techniques described herein or other techniques known in the art. It may be desirable to integrate the safety switch in a genomic safe harbor such that the genetic stability and the clinical safety of the engineered cell are maintained. Examples of safe harbors are the AAVS1 locus; the ROSA26 locus; the CLYBL locus; the gene loci for albumin, CCR5, and CXCR4; and the locus where the endogenous gene is knocked out in the engineered cells (e.g., the T cell receptor alpha or beta chain gene locus, the HLA gene locus, the CIITA locus, or the β2-microglobulin gene locus).

V. Use of the Engineered Treg Cells

[078] The genetically engineered Treg cells of the present disclosure can be used in cell therapy to treat a patient (e.g., a human patient) in need of induction of immune

tolerance or restoration of immune homeostasis. The terms "treating" and "treatment" refer to alleviation or elimination of one or more symptoms of the treated condition, prevention of the occurrence or reoccurrence of the symptoms, reversal or remediation of tissue damage, and/or slowing of disease progression.

[079] A patient herein may be one having or at risk of having an undesired inflammatory condition such as an autoimmune disease. Examples of autoimmune diseases are Addison's disease, AIDS, ankylosing spondylitis, anti-glomerular basement membrane disease autoimmune hepatitis, dermatitis, Goodpasture's syndrome, granulomatosis with polyangiitis, Graves' disease, Guillain-Barre syndrome, Hashimoto's thyroiditis, hemolytic anemia, Henoch-Schonlein purpura (HSP), juvenile arthritis, juvenile myositis, Kawasaki disease, inflammatory bowel diseases (such as Crohn's disease and ulcerative colitis), polymyositis, pulmonary alveolar proteinosis, multiple sclerosis, myasthenia gravis, neuromyelitis optica, PANDAS, psoriasis, psoriatic arthritis, rheumatoid arthritis, Sjögren's syndrome, systemic scleroderma, systemic sclerosis, systemic lupus erythematosus, thrombocytopenic purpura (TTP), Type I diabetes mellitus, uveitis, vasculitis, vitiligo, and Vogt-Koyanagi-Harada Disease.

[080] In some embodiments, the Tregs are engineered to express from the *FOXP3* locus a CAR targeting an autoantigen associated with an autoimmune disease, such as myelin oligodendrocyte glycoprotein (multiple sclerosis), myelin protein zero (autoimmune peripheral neuropathy), HIV env or gag protein (AIDS), myelin basic protein (multiple sclerosis), CD37 (systemic lupus erythematosus), CD20 (B-cell mediated autoimmune diseases), and IL-23R (inflammatory bowel diseases such as Crohn's disease or ulcerative colitis).

[081] A patient herein may be one in need an allogeneic transplant, such as an allogeneic tissue or solid organ transplant or an allogeneic cell therapy. The Tregs of the present disclosure, such as those expressing CARs targeting one or more allogeneic MHC class I or II molecules, may be introduced to the patient, where the Tregs will home to the transplant and suppress allograft rejection elicited by the host immune system and/or graft-versus-host rejection. Patient in need of a tissue or organ transplant or an allogeneic cell therapy include those in need of, for example, kidney transplant, heart transplant, liver transplant, pancreas transplant, intestine transplant, vein transplant, bone marrow

transplant, and skin graft; those in need of regenerative cell therapy; those in need of gene therapy (AAV-based gene therapy); and those in need in need of cancer CAR T therapy.

[082] If desired, the patient receiving the engineered Tregs herein (which includes patients receiving engineered pluripotent or multipotent cells that will differentiate into Tregs *in vivo*) is treated with a mild myeloablative procedure prior to introduction of the cell graft or with a vigorous myeloablative conditioning regimen.

[083] The *FOXP3* engineered cells of the present disclosure may be provided in a pharmaceutical composition containing the cells and a pharmaceutically acceptable carrier. For example, the pharmaceutical composition comprises sterilized water, physiological saline or neutral buffered saline (e.g., phosphate-buffered saline), salts, antibiotics, isotonic agents, and other excipients (e.g., glucose, mannose, sucrose, dextrans, mannitol; proteins (e.g., human serum albumin); amino acids (e.g., glycine and arginine); antioxidants (e.g., glutathione); chelating agents (e.g., EDTA); and preservatives). The pharmaceutical composition may additionally comprise factors that are supportive of the Treg phenotype and growth (e.g., IL-2 and rapamycin or derivatives thereof), anti-inflammatory cytokines (e.g., IL-10, TGF-β, and IL-35), and other cells for cell therapy (e.g., CAR T effector cells for cancer therapy or cells for regenerative therapy). For storage and transportation, the cells optionally may be cryopreserved. Prior to use, the cells may be thawed and diluted in a pharmaceutically acceptable carrier.

[084] The pharmaceutical composition of the present disclosure is administered to a patient in a therapeutically effective amount through systemic administration (e.g., through intravenous injection or infusion) or local injection or infusion to the tissue of interest (e.g., infusion through the hepatic artery, and injection to the brain, heart, or muscle). The term "therapeutically effective amount" refers to the amount of a pharmaceutical composition, or the number of cells, that when administered to the patient, is sufficient to effect the treatment.

[085] In some embodiments, a single dosing unit of the pharmaceutical composition comprises more than 10^4 cells (e.g., from about 10^5 to about 10^6 cells, from about 10^6 to about 10^{10}, from about 10^6 to 10^7, from about 10^6 to 10^8, from about 10^7 to 10^8, from about 10^7 to 10^9, or from about 10^8 to 10^9 cells). In certain embodiments, a single dosing unit of the composition comprises about 10^6, about 10^7, about 10^8, about 10^9, or about

10^{10} or more cells. The patient may be administered with the pharmaceutical composition once every two days, once every three days, once every four days, once a week, once every two weeks, once every three weeks, once a month, or at another frequency as necessary to establish a sufficient population of engineered Treg cells in the patient.

[086] Pharmaceutical compositions comprising any of the zinc finger nucleases or other nucleases and polynucleotides as described herein are also provided.

[087] Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure. In case of conflict, the present specification, including definitions, will control. Generally, nomenclature used in connection with, and techniques of, cardiology, medicine, medicinal and pharmaceutical chemistry, and cell biology described herein are those well-known and commonly used in the art. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Throughout this specification and embodiments, the words "have" and "comprise," or variations such as "has," "having," "comprises," or "comprising," will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. All publications and other references mentioned herein are incorporated by reference in their entirety. Although a number of documents are cited herein, this citation does not constitute an admission that any of these documents forms part of the common general knowledge in the art. As used herein, the term "approximately" or "about" as applied to one or more values of interest refers to a value that is similar to a stated reference value. In certain embodiments, the term refers to a range of values that fall within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context.

[088] In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only and are not to be construed as limiting the scope of the invention in any manner.

EXAMPLES

Example 1: Design of *FOXP3*-Specific Nucleases

[089] *FOXP3*-specific ZFNs were constructed to enable site-specific introduction of double-stranded breaks at the human *FOXP3* gene. ZFNs were designed essentially as described in Urnov et al., *Nature* (2005) 435(7042):646-651; Lombardo et al., *Nat Biotechnol.* (2007) 25(11):1298-306; U.S. Pat. Pub. Nos. 2008/0131962, 2015/016495, 2014/0120622, and 2014/0301990; and U.S. Pat. No. 8,956,828. The ZFN pair targeted intron 4, 9, or 10 in the *FOXP3* gene (**FIGs. 2B** and **2D**). The amino acid sequences of the recognition helices for the exemplary ZFN pair are shown below in Table 1. The target genomic sequence for each ZFN DNA binding domain is shown below in Table 2. Nucleotides in the genome that are targeted by the zinc finger protein (ZFP) recognition helices are indicated in uppercase letters; non-targeted nucleotides indicated in lowercase. Linkers used to join the FokI nuclease domain and the ZFP DNA binding domain are also shown (*see, e.g.*, U.S. Pat. Pub. No. 2015/0132269). For example, the amino acid sequence of the domain linker L0 is DNA binding domain-QLVKS-FokI nuclease domain (SEQ ID NO: 7). Similarly, the amino acid sequences for the domain linker N7a is FokI nuclease domain-SGTPHEVGVYTL-DNA binding domain (SEQ ID NO:8), and N6a is FokI nuclease domain- SGAQGSTLDF-DNA binding domain (SEQ ID NO:9). Further, the expression constructs may comprise a WPRE sequence and a bovine growth hormone polyA sequence.

Table 1 Human *FOXP3* Zinc-Finger Designs

ZFN Name (target intron)	Zinc Finger Amino Acid Sequences (SEQ ID NO)						Domain linker
	F1	F2	F3	F4	F5	F6	
SBS75606 (int. 9)	HAQGLRH (11)	QSGHLSR (12)	IRSTLRD (13)	HRSSLRR (14)	TSGHLSR (15)	QSGHLSR (12)	L0
SBS75609 (int. 9)	TSSNRKT (16)	QSGHLSR (12)	RSDTLSV (17)	DNSTRIK (18)	RSDDLTR (19)	DRSTRRQ (20)	L0
SBS75893 (int.10)	RSDHLSQ (21)	RSAVRKN (22)	QSSNLAR (23)	QSSDLRR (24)	RSDHLSQ (21)	ASSTRTK (25)	L0
SBS75895 (int.10)	SLTYLPT (26)	DRSALAR (27)	RSDHLSE (28)	AKHHRKT (29)	RSAHLSR (30)	TSGSLTR (31)	N6a

SBS75591 (int. 9)	RSAHLSR (30)	QSGHLSR (12)	RSDNLSV (92)	ASWTLTQ (32)	SNQNLTT (33)	DRSHLAR (34)	N7a
SBS75592 (int. 9)	DQSNLRA (35)	RSANLTR (36)	RSDNLST (37)	DNSYLPR (38)	QSGALAR (39)	QSANRTK (40)	L0
SBS75714 (int.10)	QSSDLSR (41)	RTDALRG (42)	DRSNLSR (43)	TSGNLTR (44)	RSDDLSK (45)	DSSHRTR (46)	L0
SBS75715 (int.10)	RSDNLST (37)	DRSALAR (27)	RSDHLST (47)	RSDALAR (48)	RSDNLSE (49)	ARSTRTN (50)	N7a
SBS76695 (int. 4)	RSDVLSE (51)	RLYTLHK (52)	RSDNLSA (53)	RNNDRKT (54)	SNQNLTT (33)	DRSHLAR (34)	L0
SBS76698 (int. 4)	QSSDLSR (41)	QSGNLAR (55)	RSDNLST (37)	FHSCLSA (56)	PYYGRHG (57)	DRSHLAR (34)	N7a
SBS75683 (int. 10)	LRHHLTR (58)	VAEYRYK (59)	QSGHLAR (60)	QRTNLVE (61)	RSDHLSN (62)	QSHDRTK (63)	L0
SBS75684 (int. 10)	RQDCLSL (64)	RNDNRKT (65)	TSGSLSR (66)	TRQNRDT (67)	QSSHLTR (68)	RLDNRTA (69)	N7a
SB75806 (int. 10)	QRNHRTT (70)	LRHHLTR (58)	RSDHLSA (71)	QSGHLSR (12)	TNHLLRT (72)	QSGHLSR (12)	L0
SB75808 (int. 10)	RSDALSR (73)	QSGNLAR (55)	QSADRTK (74)	RSANLTR (36)	QRSNLVR (75)	TSGHLSR (15)	N7a

int: intron.

Table 2 Target Sequences for Human *FOXP3* Zinc Fingers

SBS*	Target Sequence (5′ -> 3′)	SEQ ID NO
SBS75606 (int. 9)	aaGGAGGTGCTCCTGGAATTacttagca	76
SBS75609 (int. 9)	ctCCCCTGACCAAGGAAAATcggggtgg	77
SBS75893 (int.10)	ggGCTAGGGCTGAAGTGAGGtgaaaggt	78
SBS75895 (int.10)	agGTTGGGAGTGGGGTCTTGttcagggc	79
SBS75591 (int. 9)	tcGGCCATCAGAAGGGAGGGaccctgct	80
SBS75592 (int. 9)	gcTAAGTAaTTCCAGGAGCACctcctttt	81
SBS75714 (int.10)	agGGATGGGATGACTTGGCTttaggtca	82
SBS75715 (int.10)	ggACTCAGGTGGGGgGTCTAGgggtgag	83
SBS76695 (int. 4)	acGGCCATTCGCAGGTGCTGacattttg	84
SBS76698 (int. 4)	caGGCTTCTGGCAGaGAAGCTtaaagac	85
SBS75683 (int. 10)	gcTCAGGGCAAGGATGAGGTtagttgtg	86
SBS75684 (int. 10)	gtCAGGGACATGGTTAGGTGgttaggct	87
SB75806 (int. 10)	aaGGATTTGGAAGGGGTAAAgggccagg	88
SB75808 (int. 10)	aaGGTGAAGGGTCAGAAGTGgggtcaag	89

*SBS: Sangamo Biosciences Sequence.

[090] All ZFNs were tested and found to bind to their target sites and found to be active as nucleases. Indels and targeted integration (TI) efficiency were quantified by MiSeq next generation sequencing. ZFNs targeted to each site within introns 4, 9, and 10 yielded high total levels of genome modifications, while intron 9 (site A) yielded the highest TI efficiency (the intended outcome) (**FIGs. 2D** and **2E**). Thus, these ZFNs can be used to make genetic modifications (e.g., insertions and/or deletions) within a human *FOXP3* gene, for example, at a target site shown in any one of SEQ ID NOs: 76-89, at a target site having 12-25 nucleotides of one of these sequences, at a site within 1-50 (*e.g.*, 1 to 10) base pairs of these gene sequences, and/or between paired target sites.

[091] The ZFNs used herein may also include one or more mutations to the phosphate contact residues of the ZFP and/or the FokI domain, for example, the nR-5Qabc mutant (to ZFP backbone) and/or R416S and/or K525S mutants (to FokI), as described in U.S. Pat. Pub. No. 2018/0087072. TALE nucleases and CRISPR/Cas systems targeted the aforementioned target sites can also be used. ZFP, TALE, and sRNA DNA-binding domains that recognize these target sites are also formulated into active engineered transcription factors when associated with one or more regulatory domains. Regulatory domains can include transcriptional activators or repressors, recombinases, integrases, nucleases, and nickases.

Example 2: ZFN Nuclease Activity *in vitro*

[092] The ZFNs described in Table 1 were tested for their nuclease activity in K562 cells. K562 cells were obtained from the American Type Culture Collection and grown as recommended in RPMI medium (Invitrogen) supplemented with 10% qualified fetal bovine serum (FBS, Cyclone).

[093] To test DNA cleavage activity, mRNAs encoding pairs of human *FOXP3*-specific ZFNs were transfected into the K562 cells (SBS75606/SBS75609 (targeting intron 9), SBS75893/SBS75895 (targeting intron 10), SBS75591/SBS75592 (targeting intron 9), and SBS75714/SBS75715 (targeting intron 10)). For mRNA generation, open reading frames for the ZFNs were cloned into an expression vector optimized for

production of mRNA bearing 5' and 3' UTRs and a synthetic polyA signal. The expression vector was either a pVAX-based vector containing a T7 promoter, the ZFN coding sequence, and a polyA motif for enzymatic addition of a polyA tail following the *in vitro* transcription reaction; or a pGEM-based vector containing a T7 promoter, a 5'UTR, the ZFN coding sequence, a 3'UTR and a 64 bp polyA stretch (SEQ ID NO: 93); or a PCR amplicon containing a T7 promoter, a 5'UTR, the ZFN coding sequence, a 3'UTR and a 60 bp polyA stretch (SEQ ID NO: 94). The mRNAs were generated from the expression vector using the mMessage mMachine™ T7 Ultra kit (Ambion) following manufacturer's instructions.

[094] For transfection, one million K562 cells were mixed with 15µg/mL or 3.75 µg/mL of each ZFN-encoding mRNA mix. Transfection was done in an Amaxa Nucleofector™ IITM using program T-16. Transfected cells were recovered into 1.4 mL warm RPMI medium + 10% FBS. Three days following transfection, nuclease activity was assessed by deep sequencing (MiSeq, Illumina) as per standard protocols. The results are presented below in Table 3.

Table 3 Zinc Finger Nuclease Activity

Pair #	ZFN pair	Target Intron	Indels (15 µg/mL)	Indels (3.75 µg/mL)
1	SBS75606/SBS75609	9	91.4	70.1
2	SBS75893/SBS75895	10	91.3	76.6
3	SBS75591/SBS75592	9	91.2	80.0
4	SBS75714/SBS75715	10	90.7	84.0

*Indels: insertions and/or deletions.

[095] The above data show that the ZFN nucleases described herein were active and induced cleavage and genomic modifications at the target sites.

Example 3: Purification of Regulatory and Effector T Cells

[096] Tregs were isolated from fresh leukapheresis products (Leukopak) (Stem Cell Technologies, Canada) obtained from non-smoking, male donors under the age of 40. On the same day of delivery, the Leukopak was washed one time in CliniMACS® buffer (Miltenyi, Germany) supplemented with 2% human serum albumin (Octapharma, USA). The cells were then processed for Treg isolation using the EasySep™ Human

CD4+CD127lowCD25+ Regulatory T cell Isolation Kit (Stem Cell Technologies, Canada) following manufacturer's instructions.

[097] CD4+CD25- responder T (Tresponder) cells were isolated from the non-Treg fraction of the Leukopak using the same isolation kit. Donor-matching CD8+ Teffs were isolated from a portion of the Leukopak using the EasySep™ Human CD8 Positive Selection Kit II (StemCell Technologies, Canada) following manufacturer's instructions. Purity of the Tregs, Tresponders, and Teffs was assessed by flow cytometry. Cells were then frozen in CS10 (BioLife Solutions, USA) and stored in liquid nitrogen.

[098] Treg, Teff, Tresponder phenotypes were assessed following purification and modification using antibodies specific for the following antigens: CD4, CD25, CD127, CD69, and FOXP3. A fixable viability dye was included to exclude dead or dying cells. FOXP3 intracellular staining was performed using the eBioscience Foxp3/Transcription Factor Staining Buffer set (Thermofisher, USA) after cell surface staining. CD8+ Teff purity was assessed by staining for CD8. Tresponders were CD4+CD25-.

[099] **FIG. 1A** shows that the cell population was 87.8% lymphocytes. **FIG. 1B** shows that 93.6% of the lymphocytes were CD4+CD25+. **FIG. 1C** shows that among the CD4+CD25+ cells, only 0.25% was CD127+, while **FIG. 1D** shows that among the CD4+CD25+CD127- cells, 90.4% was FOXP3+. This phenotype, CD4+CD25+CD127-FOXP3+ is characteristic of Treg cells. The high levels of CD4+CD25+CD127-FOXP3+ cells indicate a highly purified population of Treg cells.

Example 4: Targeted Integration of a GFP Transgene and an HLA-A2 CAR in Isolated Tregs and CD8+ Teffs

[0100] ZFN mRNAs (SBS75591/SBS75592 pair) targeting an intronic region between Exons 9 and 10 of the human *FOXP3* locus were engineered as described in Example 1. An AAV6 vector encoding a 5' splice acceptor, partial *FOXP3* cDNA encompassing Exons 10-12, a coding sequence for a 2A self-cleaving peptide, and a coding sequence for either a green fluorescent protein (GFP; **FIG. 2B**) or an anti-HLA-A2 CAR (Boardman et al., *supra*; **FIG. 5**) were generated by standard cloning technology.

[0101] The HLA-A2 CAR construct is illustrated in **FIGs. 2A** and **4A-C**. The CAR's antigen recognition domain is a scFv comprising a heavy chain variable domain (VH) from 3PB2 and a light chain variable domain (VL) from DPK1, wherein the VH and the VL are linked by a peptide linker sequence $[G_4S]_3$ (SEQ ID NO: 90). The HLA-A2 CAR construct comprises sequences encoding a hGMCSF surface expression signal sequence, the scFv, a Myc tag, the CD28 transmembrane (TM) domain, the CD28 co-stimulation domain and the CD3ζ signaling sequence (SEQ ID NO: 91).

[0102] Before use, Tregs were thawed and cultured for two days in RPMI supplemented with 10% human AB serum (Valley Biomedical) and 1000 U/mL of recombinant human IL-2 (CTS Thermofisher, USA). The Tregs were activated in the presence of CD3/CD28 Dynabeads™ (CTS Thermofisher, USA) prior to manipulation. Teffs were thawed and cultured for two days in the same media with 100 U/mL of recombinant human IL-2 and activated in the presence of CD3/CD28 Dynabeads™.

[0103] The ZFN mRNAs were introduced to cells by electroporation via a MaxCyte device following manufacturer's instructions or by BTX ECM® 830 Square Wave Electroporator (Harvard Bioscience, USA). The AAV transgene donor constructs were introduced into the cells at $1x10^5$ viral genomes (vg) per cell.

[0104] All cell samples were analyzed on an Attune™ NxT Flow Cytometer (Thermofisher, USA) and data was analyzed using FlowJo software. Detection of HLA-A2 CAR in modified Tregs and Teffs was performed by first permeabilizing cells with the eBioscience Foxp3/Transcription Factor Staining Buffer set. Cells were then stained with an HLA-A2 dextramer conjugated to a fluorophore (Immudex, Denmark) and analyzed within two hours of staining as recommended by the manufacturer. GFP was detected through flow cytometry within the appropriate fluorescent channel.

[0105] As shown in **FIG. 2C**, the ZFNs alone induced >90% of total genome modification (indel: insertions/deletions). When ZFNs were co-delivered with the AAV GFP transgene construct, about 70% of the genomic modifications were targeted integration (TI) of the GFP transgene at the intended target site. The data in **FIGs. 3A-C** show that the fraction of cells expressing GFP and the level of GFP expression were similar between as Mock untreated cells in Tregs subjected to ZFNs (**FIG. 3A**) or AAV

donor alone (**FIG. 3B**), whereas Tregs subjected to ZFNs as well as donor (**FIG. 3C**) yielded >60% of cells expressing GFP driven from the endogenous FOXP3 promoter.

[0106] For HLA-A2 CAR-engineered T cells, the level of CAR+ cells that bound HLA-A2 dextramer was assessed by flow cytometry. In the Treg study, the fraction and level of HLA-A2-targeted scFv binding was shown to be the same as Mock untreated cells in Tregs subjected to ZFNs (**FIG. 5A**) or subjected to AAV donor alone (**FIG. 5B**). By contrast, Tregs subjected to ZFNs and the AAV donor (**FIG. 5C**) yielded >70% of Treg cells expressing HLA-A2 CAR under the control of the endogenous *FOXP3* promoter. In the Teff study, the fraction and level of HLA-A2-targeted scFv binding was shown to be the same as Mock untreated cells in Teffs subjected to ZFNs (FIG. 6A) or subjected AAV donor alone (**FIG. 6B**). Unlike the Tregs, Teffs subjected to ZFNs and the AAV donor (**FIG. 6C**) also showed no CAR expression. These results indicate that only cells expressing FOXP3 (Tregs) expressed the HLA-A1 CAR and that the expression was regulated by the endogenous *FOXP3* promoter, which was active on Treg cells but not in Teff cells.

[0107] The data in **FIG. 7A** show that among the Treg cells transfected with the HLA-A2 CAR donor, 90.1% of the cells retained the FOXP3+ Treg phenotype. The data in **FIG. 7B** show that among the Tregs subjected to both the ZFNs and the AAV donor, a similarly high percentage of the cells (85.8%) retained the FOXP3+ Treg phenotype.

LIST OF SEQUENCES

[0108] The table below lists the amino acid and nucleotide sequences in the present disclosure and their respective SEQ ID NOs (SEQ).

SEQ	SEQUENCES
1	DYGMH
2	FIRNDGSDKYYADSVKG
3	NGESGPLDYWYLDL
4	QVQLVQSGGGVVQPGGSLRVSCAASGVTLS**DYGMH**WVRQAPGKGLEWVA**FIRNDGSDKYYADS** **VKG**RFTISRDNSEKTVSLQMSSLRAEDTAVYYCAK**NGESGPLDYWYLDL**WGRGT (3PB2 VH; CDRs are in boxes)
5	LAGLIDADG
6	MVSKIRTFGWVQNPGKFENLKRVVQVFDRNSKVHNEVKNIKIPTLVKESKIQKEL VAIMNQHDLIYTYKELVGTGTSIRSEAPCDAIIQATIADQGNKKGYIDNWSSDGFL RWAHALGFIEYINKSDSFVITDVGLAYSKSADGSAIEKEILIEAISSYPPAIRILT LLEDGQHLTKFDLGKNLGFSGESGFTSLPEGILLDTLANAMPKDKGEIRNNWEGSS DKYARMIGGWLDKLGLVKQGKKEFIIPTLGKPDNKEFISHAFKITGEGLKVLRRAK GSTKFTRVPKRVYWEMLATNLTDKEYVRTRRALILEILIKAGSLKIEQIQDNLKKL GFDEVIETIENDIKGLINTGIFIEIKGRFYQLKDHILQFVIPNRGVTKQLVKSELE EKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSR KPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEENQTRNKHINPNE WWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIK AGTLTLEEVRRKFNNGEINF
7	QLVKS
8	SGTPHEVGVYTL
9	SGAQGSTLDF
10	DIQMTQSPSSLSASVGDRVTITC**QASQDISNYLN**WYQQKPGKAPKLLIY**DASNLET**GVPSRFS GSGSGTDFTFTISSLQPEDIATYYC**QQYDNLP** (DPK1 VL; CDRs are in boxes)
11	HAQGLRH
12	QSGHLSR
13	IRSTLRD
14	HRSSLRR
15	TSGHLSR
16	TSSNRKT
17	RSDTLSV
18	DNSTRIK
19	RSDDLTR
20	DRSTRRQ
21	RSDHLSQ
22	RSAVRKN
23	QSSNLAR
24	QSSDLRR
25	ASSTRTK
26	SLTYLPT
27	DRSALAR
28	RSDHLSE
29	AKHHRKT
30	RSAHLSR
31	TSGSLTR
32	ASWTLTQ
33	SNQNLTT

34	DRSHLAR
35	DQSNLRA
36	RSANLTR
37	RSDNLST
38	DNSYLPR
39	QSGALAR
40	QSANRTK
41	QSSDLSR
42	RTDALRG
43	DRSNLSR
44	TSGNLTR
45	RSDDLSK
46	DSSHRTR
47	RSDHLST
48	RSDALAR
49	RSDNLSE
50	ARSTRTN
51	RSDVLSE
52	RLYTLHK
53	RSDNLSA
54	RNNDRKT
55	QSGNLAR
56	FHSCLSA
57	PYYGRHG
58	LRHHLTR
59	VAEYRYK
60	QSGHLAR
61	QRTNLVE
62	RSDHLSN
63	QSHDRTK
64	RQDCLSL
65	RNDNRKT
66	TSGSLSR
67	TRQNRDT
68	QSSHLTR
69	RLDNRTA
70	QRNHRTT
71	RSDHLSA
72	TNHLLRT
73	RSDALSR
74	QSADRTK
75	QRSNLVR
76	aaGGAGGTGCTCCTGGAATTacttagca
77	ctCCCCTGACCAAGGAAAATcggggtgg
78	ggGCTAGGGCTGAAGTGAGGtgaaaggt
79	agGTTGGGAGTGGGGTCTTGttcagggc
80	tcGGCCATCAGAAGGGAGGGaccctgct
81	gcTAAGTAaTTCCAGGAGCACctccttt
82	agGGATGGGATGACTTGGCTttaggtca
83	ggACTCAGGTGGGGgGTCTAGgggtgag
84	acGGCCATTCGCAGGTGCTGacattttg
85	caGGCTTCTGGCAGaGAAGCTtaaagac
86	gcTCAGGGCAAGGATGAGGTtagttgtg
87	gtCAGGGACATGGTTAGGTGgttaggct

88	aaGGATTTGGAAGGGGTAAAgggccagg
89	aaGGTGAAGGGTCAGAAGTGgggtcaag
90	GGGGSGGGGSGGGGS
91	MLLLVTSLLLCELPHPAFLLIPQVQLVQSGGGVVQPGGSLRVSCAASGVTLS**DYGMH**WVRQAP GKGLEWVA**FIRNDGSDKYYADS**VKGRFTISRDNSEKTVSLQMSSLRAEDTAVYYCAK**NGESGP** **LDYWYLDL**WGRGT**GGGGSGGGGSGGGGS**DIQMTQSPSSLSASVGDRVTITC**QASQDISNYLN**W YQQKPGKAPKLLIY**DASNLET**GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC**QQYDNLP**EQK LISEEDLAAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGVLACYS LLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAP AYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (CDRs are in boxes)
92	RSDNLSV
93	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
94	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
95	QVQLVESGGGVVQPGGSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAFIRYDGSNKYYADSV KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK

What is claimed is:

1. A genetically engineered mammalian cell comprising a heterologous sequence in a *FOXP3* locus in the genome, wherein

the heterologous sequence comprises a transgene, wherein the transgene is under the transcriptional control of a *FOXP3* promoter in the *FOXP3* locus; and

when the promoter is activated, the cell expresses both FOXP3 and a product of the transgene from the locus.

2. A method of making a genetically engineered mammalian cell, comprising:

contacting a mammalian cell with a nucleic acid construct comprising (i) a heterologous sequence and (ii) a first homologous region (HR) and a second HR flanking the heterologous sequence flanking the heterologous sequence, wherein

the heterologous sequence comprises a transgene,

the first and second HRs are homologous to a first genomic region (GR) and a second GR, respectively, in a *FOXP3* locus in the mammalian cell; and

culturing the cell under conditions that allow integration of the heterologous sequence between the first and second GRs in the *FOXP3* locus.

3. The method of claim 2, wherein the integration is facilitated by a zinc finger nuclease or nickase (ZFN), a transcription activator-like effector domain nuclease or nickase (TALEN), a meganuclease, an integrase, a recombinase, a transposase, or a CRISPR/Cas system.

4. The method of claim 2 or 3, wherein the nucleic acid construct is a lentiviral construct, an adenoviral construct, an adeno-associated viral construct, a plasmid, a DNA construct, or an RNA construct.

5. The cell or method of any one of the preceding claims, wherein the heterologous sequence comprises (i) an internal ribosome entry site (IRES) immediately upstream of

the transgene, or (ii) a coding sequence for a self-cleaving peptide immediately upstream of and in-frame with the transgene.

6. The cell or method of claim 5, wherein the self-cleaving peptide is a 2A peptide, optionally selected from the group consisting of a P2A peptide, an E2A peptide, an F2A peptide, and a T2A peptide.

7. The cell or method of claim 6, wherein the heterologous sequence is inserted into a *FOXP3* intron upstream of one or more *FOXP3* exons, wherein

the heterologous sequence comprises (i) a nucleotide sequence coding for the one or more *FOXP3* exons, and (ii) a splice acceptor upstream of the nucleotide sequence to allow expression of a full-length *FOXP3* mRNA transcript from the locus, and

the nucleotide sequence is (a) immediately upstream of the IRES, or (b) immediately upstream of and in-frame with the coding sequence for the self-cleaving peptide.

8. The cell or method of claim 7, wherein the *FOXP3* intron is intron 4, 9, or 10.

9. The cell or method of any one of claims 1-8, wherein the transgene product is a chimeric antigen receptor (CAR) or a T-cell receptor (TCR).

10. The cell or method of claim 9, wherein the CAR or TCR is specific for (i) an autoantigen, (ii) a B cell antigen optionally selected from CD19 and CD20, or (iii) an allogeneic HLA class I molecule, wherein the class I molecule is optionally HLA-A2.

11. The cell or method of any one of claims 1-8, wherein the transgene product is a cytokine, a chemokine, a growth factor, or a signaling factor; or an AAV capsid protein selected from VP1, VP2, or VP3.

12. The cell or method of any one of the preceding claims, wherein the cell is a lymphoid cell, a lymphoid progenitor cell, a mesenchymal stem cell, a hematopoietic stem cell, an induced pluripotent stem cell, or an embryonic stem cell.

13. The cell or method of claim 12, wherein the cell is a regulatory T (Treg) cell.

14. The cell or method of any one of the preceding claims, wherein the cell comprises a null mutation in a gene selected from

a T cell receptor alpha or beta chain gene,

a Class II major histocompatibility complex transactivator (CIITA) gene,

an HLA Class I or II gene,

a transporter associated with antigen processing,

a minor histocompatibility antigen gene, and

a β2 microglobulin (B2M) gene.

15. The cell or method of any one of the preceding claims, wherein the cell is a human cell.

16. The cell or method of any one of the preceding claims, wherein the cell comprises a suicide gene optionally selected from a HSV-TK gene, a cytosine deaminase gene, a nitroreductase gene, a cytochrome P450 gene, or a caspase-9 gene.

17. A method of treating a patient in need of immunosuppression, comprising administering to the patient a cell of any one of claims 1 and 5-16.

18. Use of the cell of any one of claims 1 and 5-16 in the manufacture of a medicament in treating a patient in need of immunosuppression.

19. A cell of any one of claims 1 and 5-16 for use in treating a patient in need of immunosuppression.

20. The method, use, or cell for use of any one of claims 17-19, wherein the patient has an autoimmune disease.

21. The method, use, or cell for use of any one of claims 17-19, wherein the patient has received or will receive tissue transplantation.

22. The method, use, or cell for use of any one of claims 17-21, wherein the patient is a human.