

Engineered T Cells**RELATED APPLICATIONS**

[0001] This application claims the benefit of and priority to U.S. Provisional Application Serial No. 63/612,847, filed on December 20, 2023, and U.S. Provisional Application Serial No. 63/727,648, filed on December 3, 2024, the entire contents of each of which are expressly incorporated herein by reference in their entirety.

SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing file, entitled 12793_0041-00304_SL, was created on December 18, 2024, and is 800,521 bytes in size. The information in electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

BACKGROUND

[0003] Adaptive immunity is a defense mechanism by which the body is able to eliminate foreign pathogens. T cells are immune cells that are capable of mediating this immune response. T cell receptors (TCRs) are protein complexes on the surface of T cells that are capable of recognizing antigens. T cell diversity is derived from rearrangements of TCR alpha and beta loci.

[0004] One feature of adaptive immunity is the ability to distinguish “self” from “non-self” antigens. Autoimmune and autoinflammatory disorders are characterized by pathogenic immune responses against “self” antigens. Some rearrangements of TCR alpha and beta loci generate self-reactive T cells. Owen et al., *Regulatory T Cell Development in the Thymus*, *J Immunol* 203(8) (2019). Many self-reactive T cells are eliminated by clonal deletion in the thymus, but others can escape clonal deletion and elicit deleterious immune responses. *Id.* Specialized T cells called regulatory T cells (Tregs) are used for “self” tolerance. *Id.* Tregs are capable of suppressing excessive immune responses, autoimmune responses, and undesired immune responses, for example in graft versus host disease. *Id.* Dysregulation of Tregs, e.g., if the number of Tregs is insufficient or if Tregs are not functioning properly, may contribute to autoimmune responses. *Id.*

[0005] Current therapies for treating autoimmune disorders aim to suppress the adaptive immune process or the activation of immune cells. While these therapies can suppress deleterious immune responses, e.g., autoimmune responses, they can also suppress beneficial immune responses. Treg therapies have been used to suppress antigen-specific immune responses in different diseases, including graft-versus-host disease (GvHD), in which donor cells mediate an immune attack of host tissues following hematopoietic stem cell transplantation. Pierini et al., *T Cells Expressing Chimeric Antigen Receptor Promoter Immune Tolerance*, *JCI Insight* 2(20) (2017). However, there are still “major challenges to the clinical implementation of Treg-based

therapies.” *Id.* Thus, there remains a need for effective T cell therapies, including Treg therapies, for suppressing immune response(s), including inflammation and autoimmunity.

SUMMARY

[0006] One aspect of the present disclosure relates to an engineered T cell, comprising i) a heterologous nucleic acid sequence encoding a dual mutant transforming growth factor beta 1 (dmTGFB1) under control of a promoter sequence; and ii) a modification of an endogenous a nucleic acid sequence encoding a transforming growth factor beta receptor 2 (TGFB2) wherein the modification knocks down expression of TGFB2.

[0007] In some embodiments, the dmTGFB1 comprises a mutation at two or more amino acids selected from F198, D199, V200, L208, F217, L219, R218, H222, C223, and C225 relative to a wild type human TGFB1. In some embodiments, the dmTGFB1 comprises a mutation at two or more amino acids selected from R218, H222, C223, and C225 relative to a wild type human TGFB1. In some embodiments, the dmTGFB1 comprises two or more amino acid mutations selected from R218C/H, H222D, C223S/R/G, and C225R relative to a wild type human TGFB1. In some embodiments, the dmTGFB1 comprises amino acid mutations R218C/H and C225R relative to a wild type human TGFB1.

[0008] In some embodiments, the engineered T cell is a CD4+ T cell.

[0009] In some embodiments, the engineered T cell further comprises: i) a modification of an endogenous a nucleic acid sequence encoding a tumor necrosis factor alpha (TNFA) wherein the modification knocks down expression of TNFA; ii) a modification of an endogenous nucleic acid sequence encoding an interferon-gamma (IFNG) wherein the modification knocks down expression of the IFNG; or iii) a heterologous nucleic acid sequence encoding a regulatory T cell promoting molecule under control of a promoter sequence.

[00010] In some embodiments, the engineered T cell comprises: i) a modification of an endogenous a nucleic acid sequence encoding a tumor necrosis factor alpha (TNFA) wherein the modification knocks down expression of TNFA; ii) a modification of an endogenous nucleic acid sequence encoding an interferon-gamma (IFNG) wherein the modification knocks down expression of the IFNG; and iii) a heterologous nucleic acid sequence encoding a regulatory T cell promoting molecule under control of a promoter sequence.

[00011] In some embodiments, the regulatory T cell promoting molecule is a selected from interleukin-10 (IL10), cytotoxic T-lymphocyte associated protein 4 (CTLA4), T Cell immunoreceptor with Ig And ITIM domains (TIGIT), indoleamine 2,3-dioxygenase 1 (IDO1), ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), 5'-nucleotidase ecto (NT5E), interleukin-22 (IL-22), amphiregulin (AREG), interleukin-35 (IL35), GARP, CD274 molecule (CD274), forkhead box P3 (FOXP3), IKAROS family zinc finger 2 (IKZF2), eosinophilia familial

(EOS), interferon regulatory factor 4 (IRF4), lymphoid enhancer binding factor 1 (LEF1), BTB domain and CNC homolog 2 (BACH2), and interleukin 2 receptor subunit alpha (IL2RA).

[00012] In some embodiments, the regulatory T cell promoting molecule is IL10 or CTLA4.

[00013] In some embodiments, the regulatory T cell promoting molecule is a first regulatory T cell promoting molecule, and further comprising a heterologous nucleic acid sequence encoding a second regulatory T cell promoting molecule under control of a promoter sequence. In some embodiments, the first and the second regulatory T cell promoting molecules are IL10 and CTLA4.

[00014] In some embodiments, the IL10 comprises a mutation at one or more amino acids selected from D25, E96, N21, and R104 relative to a mature wild type human IL10. In some embodiments, the IL10 comprises one or more amino acid mutations selected from D25A/K, E96A, N21A, and R104A relative to a mature wild type human IL10. In some embodiments, the IL10 comprises a combination of amino acid mutations selected from D25K, D25A, D25A/E96A, D25K/E96A, N21A/R104A, D25A/N21A/R104A, D25K/N21A/R104A relative to a mature wild type human IL10. In some embodiments, wherein the IL10 comprises a mature wild type human IL10.

[00015] In some embodiments, the engineered T cell comprises a modification of an endogenous nucleic acid sequence encoding at least one of a T-cell receptor, a MHC class I molecule, or an MHC class II molecule. In some embodiments, the engineered T cell comprises a modification of an endogenous nucleic acid sequence encoding a protein selected from TRAC, HLA-A, HLA-B, and CIITA, wherein the modification knocks down expression of the protein. In some embodiments, the protein is TRAC, and the modification knocks down expression of TRAC. In some embodiments, the protein is HLA-A, and the modification knocks down expression of HLA-A. In some embodiments, the protein is HLA-B, and the modification knocks down expression of HLA-B. In some embodiments, the protein is CIITA, and the modification knocks down expression of CIITA.

[00016] In some embodiments, the engineered T cell further comprises: a) a modification of an endogenous nucleic acid sequence encoding TRAC, wherein the modification knocks down expression of TRAC; b) a modification of an endogenous nucleic acid sequence encoding HLA-A, wherein the modification knocks down expression of HLA-A; c) a modification of an endogenous nucleic acid sequence encoding HLA-B, wherein the modification knocks down expression of HLA-B; and d) a modification of an endogenous nucleic acid sequence encoding CIITA, wherein the modification knocks down expression of CIITA.

[00017] Another aspect of the present disclosure relates to an engineered T cell comprising: i) a modification of a first endogenous nucleic acid sequence encoding a tumor necrosis factor alpha (TNFA), wherein the modification of the first endogenous nucleic acid knocks down expression of

TNFA; ii) a modification of a second endogenous nucleic acid sequence encoding an interferon-gamma (IFNG), wherein the modification of the second endogenous nucleic acid knocks down expression of the IFNG; iii) a modification of a third endogenous nucleic acid sequence encoding a protein selected from TRAC, HLA-A, HLA-B, and CIITA, wherein the modification of the third endogenous nucleic acid knocks down expression of the protein; and iv) a heterologous nucleic acid sequence encoding a regulatory T cell promoting molecule under control of a promoter sequence.

[00018] In some embodiments, the protein is TRAC, and the third modification knocks down expression of TRAC. In some embodiments, the protein is HLA-A, and the third modification knocks down expression of HLA-A. In some embodiments, the protein is HLA-B, and the third modification knocks down expression of HLA-B. In some embodiments, the protein is CIITA, and the third modification knocks down expression of CIITA.

[00019] Another aspect of the present disclosure relates to engineered T cell comprising: i) a modification of a first endogenous nucleic acid sequence encoding a tumor necrosis factor alpha (TNFA) wherein the modification knocks down expression of TNFA; ii) a modification of a second endogenous nucleic acid sequence encoding an interferon-gamma (IFNG) wherein the modification knocks down expression of the IFNG; iii) a modification of a third endogenous nucleic acid sequence encoding TRAC, wherein the modification of the third endogenous nucleic acid sequence knocks down expression of TRAC; iv) a modification of a fourth endogenous nucleic acid sequence encoding HLA-A, wherein the modification of the fourth endogenous nucleic acid sequence knocks down expression of HLA-A; v) a modification of a fifth endogenous nucleic acid sequence encoding HLA-B, wherein the modification of the fifth endogenous nucleic acid sequence knocks down expression of HLA-B; vi) a modification of a sixth endogenous nucleic acid sequence encoding CIITA, wherein the modification of the sixth endogenous nucleic acid sequence knocks down expression of CIITA; and vii) a heterologous nucleic acid sequence encoding a regulatory T cell promoting molecule under control of a promoter sequence.

[00020] In some embodiments, the regulatory T cell promoting molecule is a selected from interleukin-10 (IL10), cytotoxic T-lymphocyte associated protein 4 (CTLA4), T Cell immunoreceptor with Ig And ITIM domains (TIGIT), indoleamine 2,3-dioxygenase 1 (IDO1), ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), 5'-nucleotidase ecto (NT5E), interleukin-22 (IL-22), amphiregulin (AREG), interleukin-35 (IL35), GARP, CD274 molecule (CD274), forkhead box P3 (FOXP3), IKAROS family zinc finger 2 (IKZF2), eosinophilia familial (EOS), interferon regulatory factor 4 (IRF4), lymphoid enhancer binding factor 1 (LEF1), BTB domain and CNC homolog 2 (BACH2), and interleukin 2 receptor subunit alpha (IL2RA).

[00021] In some embodiments, the regulatory T cell promoting molecule is IL10 or CTLA4.

[00022] In some embodiments, the regulatory T cell promoting molecule is a first regulatory T cell promoting molecule, and further comprising a heterologous nucleic acid sequence encoding a second regulatory T cell promoting molecule under control of a promoter sequence.

[00023] In some embodiments, the first and the second regulatory T cell promoting molecules are IL10 and CTLA4. In some embodiments, the IL10 comprises a mutation at one or more amino acids selected from D25, E96, N21, and R104 relative to a mature wild type human IL10. In some embodiments, the IL10 comprises one or more amino acid mutations selected from D25A/K, E96A, N21A, and R104A relative to a mature wild type human IL10. In some embodiments, the IL10 comprises a combination of amino acid mutations selected from D25K, D25A, D25A/E96A, D25K/E96A, N21A/R104A, D25A/N21A/R104A, D25K/N21A/R104A relative to a mature wild type human IL10. In some embodiments, the IL10 comprises a mature wild type human IL10.

[00024] In some embodiments, the engineered T cell further comprises a modification of an endogenous nucleic acid sequence encoding an interleukin 17A (IL17A), an interleukin-2 (IL2), an interleukin 6 (IL6), a perforin 1 (PRF1), a granzyme A (GZMA), a granzyme B (GZMB), a natural killer cell granule protein 7 (NKG7), a Fas ligand (FASL, NF superfamily, member 6), a ryanodine receptor 2 (RZR2), and colony stimulating factor 2 (CSF2) wherein the modification knocks down expression of the IL17A, the IL2, the IL6, the PRF1, the GZMA, the GZMB, the FASL, the RZR2, or the CSF2 respectively.

[00025] In some embodiments, the engineered T cell further comprises a modification of an endogenous nucleic acid sequence encoding an endogenous T cell receptor (TCR), wherein the modification knocks down expression of the endogenous TCR.

[00026] In some embodiments, the engineered T cell further comprises a heterologous coding sequence for a targeting receptor under control of a promoter sequence. In some embodiments, the targeting receptor is targeted to a ligand selected from mucosal vascular addressin cell adhesion molecule 1 (MADCAM1), tumor necrosis factor alpha (TNFA), CD70 molecule (CD70), CEA cell adhesion molecule 6 (CEACAM6), vascular cell adhesion molecule 1 (VCAM1), citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNF (ligand) super family member 7 (TNFSF7), TNF receptor superfamily member 17 (TNFRSF17), dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), desmoglein 3 (DSG3), and MHC class I HLA-A (HLA-A*02).

[00027] In some embodiments, the targeting receptor comprises a chimeric antigen receptor (CAR) or a T cell receptor (TCR).

[00028] In some embodiments, the heterologous nucleic acid sequence encoding the targeting receptor is incorporated into an expression construct. In some embodiments, the heterologous

nucleic acid sequence encoding a targeting receptor is in an expression construct that does not comprise a nucleic acid sequence encoding a regulatory T cell promoting molecule. In some embodiments, the heterologous nucleic acid sequence encoding a targeting receptor is in an expression construct that comprises a nucleic acid sequence encoding a regulatory T cell promoting molecule.

[00029] In some embodiments, the heterologous nucleic acid sequence encoding the first regulatory T cell promoting molecule is incorporated into an expression construct and the heterologous nucleic acid sequence encoding the second regulatory T cell promoting molecule is incorporated in an expression construct. In some embodiments, the heterologous nucleic acid sequence encoding the first regulatory T cell promoting molecule and the heterologous nucleic acid sequence encoding the second regulatory T cell promoting molecule are incorporated into separate expression constructs. In some embodiments, the heterologous nucleic acid sequence encoding the first regulatory T cell promoting molecule and the heterologous nucleic acid sequence encoding the second regulatory T cell promoting molecule are incorporated into a single expression construct.

[00030] In some embodiments, at least one heterologous coding sequence is in an episomal expression construct. In some embodiments, at least one heterologous nucleic acid sequence is inserted into the genome. In some embodiments, the insertion into the genome is an untargeted insertion. In some embodiments, the insertion is a targeted insertion. In some embodiments, the targeted insertion is into a site selected from a TCR gene locus, a TNF gene locus, an IFNG gene locus, IL17A gene locus, IL6 gene locus, IL2 gene locus, an adeno-associated virus integration site 1 (AAVS1) locus. In some embodiments, the TCR gene locus is a T cell receptor alpha constant (TRAC) locus.

[00031] In some embodiments, the modification that knocks down expression of a gene comprises one or more of an insertion, a deletion, or a substitution. In some embodiments, the engineered T cell is a CD4⁺ T cell.

[00032] A further aspect of the present disclosure relates to a population of engineered T cells comprising the engineered T cell as described herein. In some embodiments, the population of engineered T cells comprises the engineered T cell as described herein, wherein at least 30% or at least 40%, of cells of the population comprise a heterologous nucleic acid sequence encoding a dmTGFB1 under control of a promoter sequence; and at least 50% or at least 70%, of cells of the population comprise a modification of an endogenous nucleic acid sequence encoding a TGFBR2. In some embodiments, the population of engineered T cells comprises the engineered T cell as described herein, wherein at least 50%, or at least 70%, of cells of the population comprise a modification of an endogenous nucleic acid sequence encoding a TNFA; at least 50% or at least 70%, of cells of the population comprise a modification of an endogenous nucleic acid sequence

encoding an IFNG; or at least 30% or at least 40%, of cells of the population comprise a heterologous nucleic acid sequence encoding a regulatory T cell promoting molecule under control of a promoter sequence. In some embodiments, the population of engineered T cells comprises the engineered T cell as described herein the percent of cells comprising an insertion or a modification is determined by the percent of reads by next generation sequencing (NGS).

[00033] An aspect of the present disclosure relates to a pharmaceutical composition comprising the engineered T cells or population of engineered T cells as described herein.

[00034] A further aspect of the present disclosure relates to a method or use of administering the engineered T cell, a population of engineered T cells, or a pharmaceutical composition as described herein to a subject. In some embodiments, the subject is in need of immunosuppression. In some embodiments, the method or use described herein is for treatment of an immune disorder. In some embodiments, the method or use described herein is for treatment of an autoimmune disease.

[00035] In some embodiments, the autoimmune disease is selected from ulcerative colitis, Crohn's disease, rheumatoid arthritis, psoriasis, multiple sclerosis, systemic lupus erythematosus, and type 1 diabetes. In some embodiments, the method or use described herein is for treatment of graft versus host disease (GvHD).

[00036] In some embodiments, the subject receives a transplant or transfusion. In some embodiments, the subject will receive a transplant or transfusion. In some embodiments, the subject is receiving a transplant or transfusion. In some embodiments, the subject has received a transplant or infusion. In some embodiments, the transplant or transfusion comprises a solid organ or a population of cells. In some embodiments, the transplant or transfusion comprises cells from a donor that is not the subject.

[00037] In some embodiments, the subject is administered the engineered T cell, the population of engineered T cells, or the pharmaceutical composition as described herein prior to receiving the transplant or transfusion. In some embodiments, the subject is administered the engineered T cell, the population of engineered T cells, or the pharmaceutical composition as described herein concurrently with receiving the transplant or transfusion. In some embodiments, the subject is administered the engineered T cell, the population of engineered T cells, or the pharmaceutical composition described herein after receiving the transplant or transfusion.

BRIEF DESCRIPTION OF THE DRAWINGS

[00038] Fig. 1 shows the percent suppression of cell proliferation by engineered T cells as measured by CTV dilution.

- [00039] Fig. 2 shows the percentage of survival over time after injection of NOG mice with engineered T cells.
- [00040] Fig. 3 shows the persistence profile of engineered T cells.
- [00041] Fig. 4 shows in vivo IL-10 production (pg/ml).
- [00042] Figure 5 shows results of in vivo study for CAR targeting of synTregs in a xenoGvHD mouse model, depicting percent survival of mice in each cohort surviving at each timepoint after injection.
- [00043] Figure 6 shows results of in vivo study for CAR targeting of synTregs in a xenoGvHD mouse model, depicting the mean percentage of synTregs among all human CD45+ cells recovered from mouse spleen at time of sacrifice.
- [00044] Figure 7 shows results of in vivo study for CAR targeting of synTregs in a xenoGvHD mouse model, depicting the mean GvHD score for each experimental condition.
- [00045] Figure 8 shows results of in vivo study for CAR targeting of synTregs in a xenoGvHD mouse model, depicting the mean IL-10 serum levels for mice in each treatment group.
- [00046] Figure 9 shows the suppressive activity of synTregs isolated from spleen as measured by suppressive activity against proliferation of Tresp.
- [00047] Figures 10A-10D show secretion of proinflammatory cytokines (IFN-gamma, Fig. 10A; TNF-alpha, Fig. 10B; IL-2, Fig. 10C; IL-10, Fig. 10D) by Tconv as compared to synTreg.
- [00048] Figures 11A-11C show activation profile of Tconv (Fig. 11A), synTreg (Fig. 11B), and targeting CAR synTreg (Fig. 11C) as measured by CD69 levels upon exposure to murine MAdCAM-1.
- [00049] Fig. 12 shows the mean cytokine secretion upon restimulation in pg/ml for INFg, TNFa, IL-10, and TFG-b. Cells were stimulated with murine MAdCAM-1 (MC-1) or Immunocult (IC).
- [00050] Fig. 13 shows mean percent suppression of cell proliferation in a mixed lymphocyte reaction using synTregs and T cells derived from ulcerative colitis patients. HD, healthy donor. UC, ulcerative colitis.
- [00051] Fig. 14 shows the mean cytokine secretion of IFNg (Fig. 14A) and TNFa (Fig. 14B) in a mixed lymphocyte reaction using synTregs and T cells derived from ulcerative colitis patients. HD, healthy donor. UC, ulcerative colitis.
- [00052] Fig. 15 shows colon weight:length ratio at the time of sacrifice.
- [00053] Fig 16 shows the mean histology score for colon samples.

- [00054] Fig. 17 shows colon weight:length ratio at the time of sacrifice.
- [00055] Fig. 18 shows the mean percentage of synTregs among the CD4+ cell population in mouse spleens.
- [00056] Fig. 19 shows the mean percentage of synTregs among the CD4+ cell population in mouse mesenteric lymph nodes (MLNs).
- [00057] Fig. 20 shows colon weight:length ratio after treatment in a syngeneic colitis model.
- [00058] Fig. 21 shows histology score for colon sections after treatment in a syngeneic colitis model.
- [00059] Figs. 22A-D show cytokine expression on CD4+ cells from mesenchymal lymph nodes after treatment in a syngeneic colitis model for TNFa (Fig. 22A), IFNg (Fig. 22B), IL-22 (Fig. 22C), and IL-17 (Fig. 22D), respectively.
- [00060] Figs. 23A-B show cytokine levels in harvested media after colon explant culture for IFNg (Fig. 23A) and TNFa (Fig. 23B), respectively.
- [00061] Fig. 24 shows lipocalin levels in stool after treatment in a syngeneic colitis model.

DETAILED DESCRIPTION

- [00062] Reference will now be made in detail to certain embodiments of aspects of the present disclosure, examples of which are illustrated in the accompanying drawings. While the present disclosure is described in conjunction with the illustrated embodiments, it will be understood that they are not intended to limit the present disclosure to those embodiments. On the contrary, the present disclosure is intended to cover all alternatives, modifications, and equivalents, which may be included within the present disclosure as defined by the appended embodiments.
- [00063] The section headings used herein are for organizational purposes only and are not to be construed as limiting the desired subject matter in any way. In the event that any material incorporated by reference contradicts any term defined in this specification or any other express content of this specification, this specification controls.

I. Definitions

- [00064] Before describing the present teachings in detail, it is to be understood that the disclosure is not limited to specific compositions or process steps, as such may vary. It should be noted that, as used in this specification and the appended embodiments, the singular form “a”, “an” and “the” include plural references unless the context clearly dictates otherwise. Thus, for example, reference to “a conjugate” includes a plurality of conjugates and reference to “a cell” includes a plurality or population of cells and the like. As used herein, the term “include” and its grammatical

variants are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or added to the listed items.

[00065] Numeric ranges are inclusive of the numbers defining the range. Measured and measurable values are understood to be approximate, taking into account significant digits and the error associated with the measurement. Also, the use of “comprise”, “comprises”, “comprising”, “contain”, “contains”, “containing”, “include”, “includes”, and “including” are not intended to be limiting. It is to be understood that both the foregoing general description and detailed description are exemplary and explanatory only and are not restrictive of the teachings.

[00066] Unless specifically noted in the specification, embodiments in the specification that recite “comprising” various components are also contemplated as “consisting of” or “consisting essentially of” the recited components; embodiments in the specification that recite “consisting of” various components are also contemplated as “comprising” or “consisting essentially of” the recited components; and embodiments in the specification that recite “consisting essentially of” various components are also contemplated as “consisting of” or “comprising” the recited components (this interchangeability does not apply to the use of these terms in the claims).

[00067] The term “or” is used in an inclusive sense, *i.e.*, equivalent to “and/or,” unless the context clearly indicates otherwise.

[00068] The term “about”, when used before a list or range, modifies each member of the list or each endpoint of the range. The term “about” or “approximately” means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. The term “about” is used herein to mean within the typical ranges of tolerances in the art. For example, “about” can be understood as about 2 standard deviations from the mean. In certain embodiments, about means +/-10%. In certain embodiments, about means +/-5%.

[00069] In some embodiments a population of cells refers to a population of at least 10^3 , 10^4 , 10^5 or 10^6 cells, *e.g.*, at least 10^7 , 2×10^7 , 5×10^7 , or 10^8 cells.

[00070] The term “at least” prior to a number or series of numbers is understood to include the number adjacent to the term “at least”, and all subsequent numbers or integers that could logically be included, as clear from context. For example, the number of nucleotides in a nucleic acid molecule must be an integer. For example, “at least 17 nucleotides of a 20 nucleotide nucleic acid molecule” means that 17, 18, 19, or 20 nucleotides have the indicated property. When at least is present before a series of numbers or a range, it is understood that “at least” can modify each of the numbers in the series or range.

[00071] As used herein, “no more than” or “less than” is understood as the value adjacent to the phrase and logical lower values or integers, as logical from context, to zero. For example, a duplex region of “no more than 2 nucleotide base pairs” has a 2, 1, or 0 nucleotide base pairs. When “no more than” or “less than” is present before a series of numbers or a range, it is understood that each of the numbers in the series or range is modified.

[00072] As used herein, ranges include both the upper and lower limit.

[00073] As used herein, it is understood that when the maximum amount of a value is represented by 100% (e.g., 100% inhibition or 100% encapsulation) that the value is limited by the method of detection. For example, 100% inhibition is understood as inhibition to a level below the level of detection of the assay, and 100% encapsulation is understood as no material intended for encapsulation can be detected outside the vesicles.

[00074] In the event of a conflict between a sequence in the application and an indicated accession number or position in an accession number, the sequence in the application predominates.

[00075] Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings.

[00076] As used herein, “knockdown” refers to a decrease in expression of a particular gene product (e.g., full-length or wild-type mRNA, protein, or both), e.g., in a cell, population of cells, tissue, or organ, by gene editing. In some embodiments, gene editing can be assessed by sequence, e.g., next generation sequencing (NGS). Expression may be decreased by at least 70%, 75%, 80%, 85%, 90%, 95%, or to below the level of detection of the assay as compared to a suitable control, e.g., wherein the gene sequence has not been modified. Knockdown of a protein can be measured by detecting the amount of the protein from a tissue, cell population, or fluid of interest. Methods for measuring knockdown of mRNA are known and include sequencing of mRNA isolated from a tissue or cell population of interest. Flow cytometry analysis is a known method for measuring knockdown of protein expression. For secreted proteins, knockdown may be assessed in a fluid such as tissue culture media or blood, or serum or plasma derived therefrom. In some embodiments, “knockdown” may refer to some loss of expression of a particular gene product, for example a decrease in the amount of full-length, wild-type mRNA transcribed or translated into full-length protein, or a decrease in the amount of protein expressed by a population of cells. It is well understood what changes in an mRNA sequence would result in decreased expression of a wild-type or full-length protein. In some embodiments, “knockdown” may refer to some loss of expression of a particular gene product, for example, a TGFBR2, IFNG, or TNFA gene product in a body fluid or tissue culture media. A modification of an endogenous nucleic acid sequence, e.g., encoding TGFBR2, IFNG, or TNFA, may result in a knockdown.

[00077] As used herein, “T cell receptor” or “TCR” refers to a receptor in a T cell. In general, a TCR is a heterodimer receptor molecule that contains two TCR polypeptide chains, α and β . α and β chain TCR polypeptides can complex with various CD3 molecules and elicit immune response(s), including inflammation and autoimmunity, after antigen binding. As used herein, a knockdown of TCR refers to a knockdown of any TCR gene in part or in whole, e.g., deletion of part of the TRBC1 gene, alone or in combination with knockdown of other TCR gene(s) in part or in whole.

[00078] “TRAC” is used to refer to the T cell receptor α chain. A human wild-type TRAC sequence is available at NCBI Gene ID: 28755; Ensembl: ENSG00000277734. T-cell receptor Alpha Constant, TCRA, IMD7, TRCA and TRA are gene synonyms for TRAC.

[00079] “TRBC” is used to refer to the T-cell receptor β -chain, e.g., TRBC1 and TRBC2. “TRBC1” and “TRBC2” refer to two homologous genes encoding the T-cell receptor β -chain, which are the gene products of the TRBC1 or TRBC2 genes.

[00080] A human wild-type TRBC1 sequence is available at NCBI Gene ID: 28639; Ensembl: ENSG00000211751. T-cell receptor Beta Constant, V_segment Translation Product, BV05S1J2.2, TCRBC1, and TCRB are gene synonyms for TRBC1.

[00081] A human wild-type TRBC2 sequence is available at NCBI Gene ID: 28638; Ensembl: ENSG00000211772. T-cell receptor Beta Constant, V_segment Translation Product, and TCRBC2 are gene synonyms for TRBC2.

[00082] As used herein, an “immune response” refers to one or more immune system reaction(s), e.g., increased production or activity of immune system cells, such as, but not limited to T cells, B cells, natural killer cells, monocytes, neutrophils, eosinophils, basophils, mast cells, erythrocytes, dendritic cells, antigen presenting cells, macrophages, or phagocytes as compared to an unstimulated control immune system. Exposure of the immune system to an antigen, e.g., a foreign or self-antigen such as but not limited to a pathogen (microorganism, virus, prion, fungus, etc.), an allergen (dust, pollen, dust mite, etc.), a toxin (chemical, drug, etc.), or physiological changes (hypercholesterolemia, obesity, organ transplant, etc.), may cause an immune response. An immune response can also include a response in which donor cells mediate an immune attack of host tissues following hematopoietic stem cell transplantation in GvHD. The immune response may result in inflammation. Alternatively, or in addition, an immune response can also include a response in which host cells mediate an immune attack of donor tissues following solid organ transplant or the introduction of other non-host cells by way of transplantation or transfusion. The immune response may target, attack, remove, or neutralize the antigen, e.g., foreign or self. The immune response may or may not be desirable. The immune response may be acute or chronic. The

immune response may damage the cell, tissue, or organ against which the immune response is mounted.

[00083] As used herein, an “autoimmune response” refers to one or more immune system reaction(s) to a self-antigen, e.g., produced by a subject’s own cells, tissues, or organs. The autoimmune response may result in increased production or activity of immune system cells, such as, but not limited to T cells, B cells, natural killer cells, monocytes, neutrophils, eosinophils, basophils, mast cells, erythrocytes, dendritic cells, antigen presenting cells, macrophages, or phagocytes as compared to a suitable control, e.g., a healthy control. The autoimmune response may result in inflammation, e.g., prolonged inflammation, or lead to an autoimmune disease. The autoimmune response may target, attack, remove, or neutralize the self-antigen produced by the subject’s own cells, tissues, or organs, which may lead to an autoimmune disease.

[00084] As used herein, “suppressing” an immune response(s) refers to decreasing or inhibiting the level of one or more immune system reaction(s), e.g., the production or activity of the immune system cells compared to a suitable control, e.g., not treated with or prior to treatment with the engineered T cell described herein. “Suppressing” an immune response(s) may refer to decreased production or activity of the immune system cells compared to a suitable control, e.g., not treated with or prior to treatment with the engineered T cell described herein. “Suppressing” an immune response may refer to increasing immune tolerance. For example, production or activity of the immune system cells may be measured by cell count, e.g., lymphocyte count or spleen cell count; cell activity, e.g., T cell assay; or gene or protein expression, e.g., biomarker expression; wherein the production or activity is decreased by 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or below the level of detection of the assay compared to a suitable control, e.g., not treated with or prior to treatment with the engineered T cell described herein.

[00085] As used herein, an “autoimmune disease” or “autoimmune disorder” refers to a condition characterized by pathological immune responses to a subject’s own antigens, cells, tissues, or organs. Examples of autoimmune diseases and disorders include, but are not limited to: ulcerative colitis, Crohn’s disease, rheumatoid arthritis, psoriasis, multiple sclerosis, systemic lupus erythematosus, and type 1 diabetes. In some embodiments, the engineered T cells have autologous or allogenic use.

[00086] As used herein, an “immune disorder” is understood as a disease or condition characterized by a pathological or undesired immune response in a subject. In certain embodiments, an immune disorder is an autoimmune disease. In certain embodiments, an immune disorder is GvHD. In certain embodiments, a subject with an immune disorder is in need of suppression of an immune response. In certain embodiments, a subject with an immune disorder is in need of an increase in immune tolerance.

[00087] A “T cell” plays a central role in the immune response following exposure to an antigen. T cells can be naturally occurring or non-natural, e.g., when T cells are formed by engineering, e.g., from a stem cell or by transdifferentiation, e.g., reprogramming a somatic cell. T cells can be distinguished from other lymphocytes by the presence of a T cell receptor on the cell surface. Included in this definition are conventional adaptive T cells, which include helper CD4⁺ T cells, cytotoxic CD8⁺ T cells, memory T cells, and regulatory CD4⁺ T cells, and innate-like T cells including natural killer T cells, mucosal associated invariant T cells, and gamma delta T cells. In some embodiments, T cells are CD4⁺. In some embodiments, T cells are CD3⁺/CD4⁺. In some embodiments, T cells are CD8⁺. In some embodiments, T cells may be further characterized as stem-cell like memory T cells (Tscm) (; CD62L+CD45RO⁻), central memory T cells (Tcm;) (CD62L+CD45RO⁺), effector memory T cells (Tem;) (CD62L-CD45RO⁺), and terminal effector T cells (Tte;) (CD62L-CD45RO⁻); a population of T cells may comprise any one or more of these T cell subtypes.

[00088] A “regulatory T cell” or “Treg” refers to a specialized T cell that plays a central role in suppressing excessive immune response(s), including inflammation and autoimmunity. Tregs can be naturally occurring (referred to herein as natural Tregs (“nTregs”) and thymic Tregs (“tTregs”) interchangeably) or non-natural, e.g., when Tregs are formed by engineering, including by insertion of a sequence encoding a dmTGFB1 molecule. Tregs when formed by engineering can include further modifications e.g., by modifications, e.g., knockdowns, of endogenous nucleic acid sequences encoding TGFBR2, IFNG, and TNFA and insertion of at least one sequence(s) encoding a regulatory T cell promoting molecule. A naturally occurring Treg or natural Treg or nTreg, also sometimes referred to as a thymus Treg or tTreg, is a specialized T cell that typically develops in the thymus gland and functions by suppressing excessive immune response(s). In some embodiments, a cell such as a conventional T cell or population of conventional T cells, e.g., a population of T cells not enriched for the presence of nTreg cells, may be engineered by insertion of a sequence encoding a dmTGFB1 molecule, and optionally further modifying endogenous nucleic sequences encoding, e.g., TGFBR2, TNFA, and IFNG, e.g., knocking down nucleic sequences encoding TGFBR2, TNFA, and IFNG, and insertion of sequence(s) encoding a regulatory T cell promoting molecule into the cell to exhibit the phenotypic characteristics and suppressive functions of a regulatory T cell, and these may be referred to as transduced or “engineered” T cells. In some embodiments, an engineered T cell comprises insertion of a sequence encoding a dmTGFB1 molecule; a modification of an endogenous nucleic acid sequence encoding an TGFBR2, a modification of an endogenous nucleic acid sequence encoding an IFNG, and a modification of an endogenous nucleic acid sequence encoding a TNFA; and insertion of a heterologous regulatory T cell promoting molecules such as IL10 and CTLA4. The modification of an endogenous nucleic acid sequence, e.g., a modification knocks down expression of an

endogenous gene, may comprise or consist of one or more indel or substitution mutations in the genomic sequence.

[00089] As used herein, a dmTGFB1 is a mutant TGFB1 based on a wild type TGFB1 from any species including, for example, human, mouse, rat, or cynomolgus TGFB1. In certain embodiments, the dmTGFB1 is human dmTGFB1, i.e., a mutant TGFB1 relative to a wild type human TGFB1. Coding and amino acid sequences for wild type TGFB1 are readily available in sequence databases, including NCBI, and exemplary sequences can be found under accession numbers NM_000660.7 and NP_000651.3 (human), NM_011577.2 and NP_035707.1 (mouse), NM_021578.2 and NP_067589.1 (rat), and XM_005589339.3 and XP_005589396.1 (cynomolgus). Each accession number is incorporated by reference in the version available as of the date of filing of the instant application. The ability to map mutations onto wild type sequences is well within the ability of those of skill in the art.

[00090] Transforming growth factor beta-1 (TGFB1) is synthesized as a large precursor molecule. TGFB1 preprotein contains a signal peptide of 29 amino acids that is proteolytically cleaved. TGFB1 is further cleaved after amino acid 278 to form latency-associated peptide (LAP) and active TGFB1. LAP dimerizes with interchain disulfide links at C223 and C225. TGFB1 can be secreted as an inactive small latent complex that consists of a mature TGF- β 1 homodimer non-covalently associated with an LAP homodimer at LAP residues I53-L59. LAP shields the type II receptor binding sites in the mature TGFB1. Most cells secrete TGFB1 as a large latent complex (LLC) of TGF- β 1/LAP covalently bound between C33 in the LAP chains and latent TGFB-binding protein (LTBP). LTBP facilitates TGFB1 folding, secretion, and possibly targeting to the extracellular matrix. Activation of the LLC occurs via the N-terminal domain of LTBP binding to the extracellular matrix.

[00091] Camurati-Engelmann Disease (CED) results from domain-specific heterozygous mutations in the transforming growth factor-beta-1 gene (TGFB1; OMIM entry 190180, incorporated by reference in the version available on the date of the filing of the application) on chromosome 19q13. Mutations reported in Camurati-Engelmann Disease families include (from Janssen et al., 2006. J Med Genet.43:1.):

Exon	DNA mutation	Protein mutation
1	28_36 duplication	L10-L12 duplication
1	241T $\rightarrow$ C	Y81H
2	463C $\rightarrow$ T	R156C
4	652C $\rightarrow$ T	R218C
4	653G $\rightarrow$ A	R218H
4	664C $\rightarrow$ G	H222D
4	?	C223S
4	667T $\rightarrow$ C	C223R
4	667T $\rightarrow$ G	C223G

4	673T → C	C225R
---	----------	-------

[00092] As shown in the table, the majority of pathogenic variants in individuals with CED result in single amino-acid substitutions in the carboxy terminus of TGFB1 latency-associated peptide (LAP). The substitutions are near the site of interchain disulfide bonds between the LAP homodimers. These pathogenic variants disrupt dimerization of LAP and binding to active TGFB1 (Walton et al., 2010. J Biol Chem. 285:17029–37), leading to increased active TGFB1 release from the cell. Walton et al. further demonstrated that the stability of the resultant large latent complex is dependent upon covalent dimerization of LAP, which is facilitated by key residues (F198, D199, V200, L208, F217, and L219) at the dimer interface. R218H mutated fibroblasts from individuals with CED showed increased active TGFB1 in the cell media compared to normal fibroblasts (Saito et al., 2001. J Biol Chem. 276:11469–72). In vitro analysis of R218C, H222D, and C225R mutated constructs also showed increased active TGF-β1 in the medium of transfected cells. In contrast, the Leu11_Leu13dup and Tyr81His pathogenic variants caused a decrease in the amount of TGFB1 secreted. However, in a luciferase reporter assay specific for TGFB1-induced transcriptional response, the mutated cells showed increased luciferase activity, suggesting intracellular activation of the receptor (Janssens et al., 2003. J Biol Chem. 278:7718–24).

[00093] As used herein, in certain embodiments, a dmTGFB1 is a TGFB1 comprising mutations at two, or more, amino acid positions relative to a wild type TGFB1 that reduce the stability of the resultant large latent complex that is dependent upon covalent dimerization of LAP relative to each TGFB1 single mutant alone. Mutations at two or more amino acid positions can include mutations at positions selected from F198, D199, V200, L208, F217, L219, R218, H222, C223, and C225 relative to a wild type human TGFB1.

[00094] In certain embodiments, a dmTGFB1 is a TGFB1 comprising mutations at two, or more, amino acid positions relative to a wild type TGFB1 at positions selected from F198, D199, V200, L208, F217, L219, R218, H222, C223, and C225 relative to a wild type human TGFB1 wherein when the dmTGFB1 is produced it is secreted from a cell in which TGFB1 is typically expressed. In some embodiments, the dmTGFB1 comprises mutations that are naturally occurring, e.g., in CED. In some embodiments, the dmTGFB1 comprises mutations that are non-naturally occurring, e.g., not naturally occurring in CED.

[00095] In certain embodiments, a human dmTGFB1 comprises mutations at 2, or more, amino acid positions selected from R218, H222, C223, and C225, relative to a wild type human TGFB1.

[00096] In certain embodiments, a human dmTGFB1 comprises 2, or more, mutations selected from R218C/H, H222D, C223S/R/G, and C225R, relative to a wild type human TGFB1.

[00097] In certain embodiments, a human dmTGFB1 comprises 2, or more, mutations selected from R218C/H and C225R, relative to a wild type human TGFB1, optionally R218H and C225R.

[00098] The dmTGFB1 provided herein has more free, active dmTGFB1 than either of the single mutants alone. In certain embodiments, the increase in active dmTGFB1 is at least additive as compared to the activity of the single mutants alone. In certain embodiments, the increase in active dmTGFB1 is significantly more than additive as compared to the activity of the single mutants alone. Methods to determine levels of total TGFB1 and active TGFB1 are known in the art using commercially available kits (e.g., LEGEND MAX Total TGF- β 1 ELISA kit (BioLegend, Cat. 436707), and a LEGEND MAX Free Active TGF- β 1 ELISA kit) and as provided in the Examples below.

[00099] As used herein, “regulatory T cell promoting molecules” refer to molecules that promote the conversion of conventional T cells to regulatory T cells including immunosuppressive molecules and Treg transcription factors. Further, regulatory T cell promoting molecules refer also to molecules that endow conventional T cells with regulatory activity, including Treg-associated immunosuppressive molecules and transcription factors. Regulatory T cell promoting molecules can be used in conjunction with dmTGFB1 promote the conversion of conventional T cells to regulatory T cells or to that endow conventional T cells with regulatory activity. Examples of immunosuppressive molecules to be used in combination with dmTGFB1 include, but are not limited to, interleukin-10 (IL10), cytotoxic T-lymphocyte associated protein 4 (CTLA4), T Cell immunoreceptor with Ig And ITIM domains (TIGIT), indoleamine 2,3-dioxygenase 1 (IDO1), ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), 5'-nucleotidase ecto (NT5E), interleukin-22 (IL22), amphiregulin (AREG), interleukin-35 (IL35), leucine rich repeat containing 32 (GARP), CD274 molecule (CD274), forkhead box P3 (FOXP3), IKAROS family zinc finger 2 (IKZF2), eosinophilia familial (EOS), interferon regulatory factor 4 (IRF4), lymphoid enhancer binding factor 1 (LEF1), BTB domain and CNC homolog 2 (BACH2), and interleukin 2 receptor subunit alpha (IL2RA, CD25). In some embodiments, regulatory T cell promoting molecules may be used in specific combinations, e.g., IL10 and CTLA4, ENTPD1 and NT5E, and IL22 and AREG. In particular, an IL10 and CTLA4 combination for use in conjunction with dmTGFB1 is provided herein. In some embodiments, the expression of immunosuppressive molecules may be promoted by the expression of transcription factors such as FoxP3, Helios, Eos, IRF4, Lef1, or BACH2.

[000100] In some embodiments, a conventional T cell may be engineered to modify, insert, or delete sequences in the genome, and the “engineered” T cell exhibits one or more phenotypic characteristics and suppressive functions of a natural regulatory T cell. For example, the “engineered” T cell exhibits suppressive activity in a mixed lymphocyte reaction assay as provided in Example 2.4 below, or may be capable of inhibiting graft versus host disease in the mouse model

presented in Example 3.2 below, e.g., in a statistically significant manner (see also, e.g., Parmar et al., Ex vivo fucosylation of third-party human regulatory T cells enhances anti-graft-versus-host disease potency in vivo, Blood 125(9) (2015)). In some embodiments, the “engineered” T cell is a conventional T cell that has been modified with the insertion of coding sequences for regulatory T cell promoting molecules, and with modification, e.g., knockdown, of expression of pro-inflammatory cytokines, e.g., TNFA in combination with one or both of IFNG and IL17A. In some embodiments, the starting T cell population for engineering is not enriched for the presence of natural Tregs, e.g., the starting T cell population has less than 20% natural Tregs.

[000101] As used herein, a “pro-inflammatory” molecule, e.g., cytokine, increases an immune response as described herein, e.g., reduces the efficacy of a Treg in the mouse model of graft-versus-host disease presented in Example 3.2 in a dose responsive manner. Examples of pro-inflammatory molecules include, but are not limited to, IFNG, TNFA, IL17A, IL6, IL2, perforin 1 (PRF1), granzyme A (GZMA), granzyme B (GZMB), natural killer cell granule protein 7 (NKG7), Fas ligand (FasL, NF superfamily, member 6), ryanodine receptor 2 (RYR2), and colony stimulating factor 2 (CSF2).

[000102] As used herein, “targeting receptor” refers to a receptor present on the surface of a cell, e.g., a T cell, to permit binding of the cell to a target site, e.g., a specific cell or tissue in an organism. Targeting receptors include, but are not limited to a chimeric antigen receptor (CAR), a T-cell receptor (TCR), and a receptor for a cell surface molecule operably linked through at least a transmembrane domain in an internal signaling domain capable of activating a T cell upon binding of the extracellular receptor portion of a protein, e.g., mucosal addressin cell adhesion molecule-1 (MADCAM-1), TNFA, CD70, CEA cell adhesion molecule 6 (CEACAM6), vascular cell adhesion molecule 1 (VCAM1), citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNF (ligand) super family member 7 (TNFSF7), TNF receptor superfamily member 17 (TNFRSF17), dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), and MHC class I HLA-A (HLA-A*02).

[000103] As used herein, a “chimeric antigen receptor” refers to an extracellular antigen recognition domain, e.g., an scFv, VHH, nanobody; operably linked to an intracellular signaling domain, which activates the T cell when an antigen is bound. CARs are composed of four regions: an antigen recognition domain, an extracellular hinge region, a transmembrane domain, and an intracellular T-cell signaling domain. Such receptors are well known in the art (see, e.g., WO2020092057, WO2019191114, WO2019147805, WO2018208837, the corresponding portions of the contents of each of which are incorporated herein by reference). A reversed universal CAR that promotes binding of an immune cell to a target cell through an adaptor molecule (see, e.g.,

WO2019238722, the contents of which are incorporated herein in their entirety) is also contemplated. CARs can be targeted to any antigen to which an antibody can be developed and are typically directed to molecules displayed on the surface of a cell or tissue to be targeted. In some embodiments, the CAR is capable of targeting engineered T cells to the gastrointestinal tract, e.g., the CAR targets MAdCAM-1. In some embodiments, the CAR is capable of targeting engineered T cells to activated immune cells (e.g., T cells or B cells expressing an upregulated target), e.g., the CAR targets CD70. In some embodiments, the CAR is capable of targeting engineered T cells to tissues comprising endothelial cells, e.g., the CAR targets VCAM-1, e.g., for suppressing immune responses in disorders such as Crohn's disease and multiple sclerosis. In some embodiments, the CAR is capable of targeting engineered T cells to endothelial cells, e.g., the CAR targets CEACAM6, e.g., for suppressing immune responses in disorders such as Crohn's disease. In some embodiments, the CAR is capable of targeting engineered T cells to pre-B cells, e.g., the CAR targets CD19, e.g., for suppressing immune responses in disorders such as multiple sclerosis and systemic lupus erythematosus. In some embodiments, the CAR is capable of targeting engineered T cells to B lymphocytes, e.g., the CAR targets CD20, e.g., for suppressing immune responses in disorders such as multiple sclerosis and systemic lupus erythematosus. In some embodiments, the CAR is capable of targeting engineered T cells to an inflammatory tissue, e.g., the CAR targets TNFA, e.g., for suppressing immune responses in disorders such as rheumatoid arthritis, inflammatory bowel disease, ulcerative colitis, or Crohn's disease. In some embodiments, the CAR is capable of targeting engineered T cells to an inflammatory tissue, e.g., the CAR targets TGF- β 1 e.g., for suppressing immune responses in disorders such as inflammatory bowel disease, ulcerative colitis, or Crohn's disease. In some embodiments, the CAR is capable of targeting engineered T cells to a neurological tissue, e.g., the CAR targets MBP, MOG, or PLP1 e.g., for suppressing immune responses in disorders such as multiple sclerosis. In some embodiments, the CAR is capable of targeting engineered T cells to immune cells expressing TNFSF7 including but not limited to activated T lymphocytes, B lymphocytes, dendritic cells, and/or NK cells, e.g., the CAR targets TNFSF7, e.g., for suppressing immune responses in disorders such as Crohn's disease, ulcerative colitis, systemic lupus erythematosus, or multiple sclerosis. In some embodiments, the CAR is capable of targeting engineered T cells to tissues comprising mature B lymphocytes, e.g., the CAR targets TNFRSF17, e.g., for suppressing immune responses in disorders such as systemic lupus erythematosus. In some embodiments, the CAR is capable of targeting engineered T cells to synovial tissue, e.g., the CAR targets citrullinated vimentin e.g., for suppressing immune responses in disorders such as rheumatoid arthritis. In some embodiments, the CAR targets dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demogelin 3 (DSG3), or MHC class I HLA-A (HLA-A*02). Additional CAR targets, e.g., inflammatory antigens, are known in the art. *See, e.g.,* WO2020092057A1, the contents of which are incorporated herein in their entirety.

[000104] As used herein, “treatment” refers to any administration or application of a therapeutic for disease or disorder in a subject, and includes inhibiting the disease, arresting its development, relieving one or more symptoms of the disease, curing the disease, preventing one or more symptoms of the disease, or preventing reoccurrence of one or more symptoms of the disease. Treating an autoimmune or inflammatory response or disorder may comprise alleviating the inflammation associated with the specific disorder resulting in the alleviation of disease-specific symptoms. Treatment with the engineered T cells described herein may be used before, after, or in combination with additional therapeutic agents, e.g., anti-inflammatory agents, immunosuppressive agents, or biologics for treatment of autoimmune disorders, e.g., Remicade, Humira.

[000105] A “promoter” refers to a regulatory region that controls the expression of a gene to which the regulatory region is linked.

[000106] “Polynucleotide” and “nucleic acid” are used herein to refer to a multimeric compound comprising nucleosides or nucleoside analogs which have nitrogenous heterocyclic bases or base analogs linked together along a backbone, including conventional RNA, DNA, mixed RNA-DNA, and polymers that are analogs thereof. A nucleic acid “backbone” can be made up of a variety of linkages, including one or more of sugar-phosphodiester linkages, peptide-nucleic acid bonds (“peptide nucleic acids” or PNA; PCT No. WO 95/32305), phosphorothioate linkages, methylphosphonate linkages, or combinations thereof. Sugar moieties of a nucleic acid can be ribose, deoxyribose, or similar compounds with substitutions, e.g., 2' methoxy, 2' halide, or a 2'-O-(2-methoxyethyl) (2'-O-moe) substitutions. Nitrogenous bases can be conventional bases (A, G, C, T, U), analogs thereof (e.g., modified uridines such as 5-methoxyuridine, pseudouridine, or N1-methylpseudouridine, or others); inosine; derivatives of purines or pyrimidines (e.g., N⁴-methyl deoxyguanosine, deaza- or aza-purines, deaza- or aza-pyrimidines, pyrimidine bases with substituent groups at the 5 or 6 position (e.g., 5-methylcytosine), purine bases with a substituent at the 2, 6, or 8 positions, 2-amino-6-methylaminopurine, O⁶-methylguanine, 4-thio-pyrimidines, 4-amino-pyrimidines, 4-dimethylhydrazine-pyrimidines, and O⁴-alkyl-pyrimidines; US Pat. No. 5,378,825 and PCT No. WO 93/13121). For general discussion see *The Biochemistry of the Nucleic Acids* 5-36, Adams et al., ed., 11th ed., 1992). Nucleic acids can include one or more “abasic” residues where the backbone includes no nitrogenous base for position(s) of the polymer (US Pat. No. 5,585,481). A nucleic acid can comprise only conventional RNA or DNA sugars, bases and linkages, or can include both conventional components and substitutions (e.g., conventional bases with 2' methoxy linkages, or polymers containing both conventional bases and one or more base analogs). Nucleic acid includes “locked nucleic acid” (LNA), an analogue containing one or more LNA nucleotide monomers with a bicyclic furanose unit locked in an RNA mimicking sugar conformation, which enhance hybridization affinity toward complementary RNA and DNA sequences (Vester and Wengel, 2004, *Biochemistry* 43(42):13233-41). Nucleic acid includes

“unlocked nucleic acid” or UNA. RNA and DNA can have different sugar moieties and can differ by the presence of uracil or analogs thereof in RNA and thymine or analogs thereof in DNA.

[000107] “Polypeptide” as used herein refers to a multimeric compound comprising amino acid residues that can adopt a three-dimensional conformation. Polypeptides include but are not limited to enzymes, enzyme precursor proteins, regulatory proteins, structural proteins, receptors, nucleic acid binding proteins, antibodies, etc. Polypeptides may, but do not necessarily, comprise post-translational modifications, non-natural amino acids, prosthetic groups, and the like.

[000108] As used herein, “open reading frame” or “ORF” of a gene refers to a sequence consisting of a series of codons that specify the amino acid sequence of the protein that the gene codes for. The ORF generally begins with a start codon (e.g., ATG in DNA or AUG in RNA) and ends with a stop codon, e.g., TAA, TAG or TGA in DNA or UAA, UAG, or UGA in RNA.

[000109] “Guide RNA”, “gRNA”, and “guide” are used herein interchangeably to refer to either a crRNA (also known as CRISPR RNA), or the combination of a crRNA and a trRNA (also known as tracrRNA). The crRNA and trRNA may be associated as a single RNA molecule (single guide RNA, sgRNA) or in two separate RNA molecules (dual guide RNA, dgRNA). “Guide RNA” or “gRNA” refers to each type. The trRNA may be a naturally-occurring sequence, or a trRNA sequence with modifications or variations compared to naturally-occurring sequences.

[000110] As used herein, a “guide sequence” or “guide region” or “targeting sequence” or “spacer” or “spacer sequence” and the like refers to a sequence within a gRNA that is complementary to a target sequence and functions to direct a gRNA to a target sequence for binding or modification (e.g., cleavage) by an RNA-guided nickase. A guide sequence can be 20 nucleotides in length, e.g., in the case of *Streptococcus pyogenes* (i.e., Spy Cas9 (also referred to as SpCas9)) and related Cas9 homologs/orthologs. Shorter or longer sequences can also be used as guides, e.g., 15-, 16-, 17-, 18-, 19-, 21-, 22-, 23-, 24-, or 25-nucleotides in length. A guide sequence can be 20-25 nucleotides in length, e.g., in the case of Nme Cas9, e.g., 20-, 21-, 22-, 23-, 24- or 25-nucleotides in length. For example, a guide sequence of 24 nucleotides in length can be used with Nme Cas9, e.g., Nme2 Cas9.

[000111] In some embodiments, the target sequence is in a genomic locus or on a chromosome, for example, and is complementary to the guide sequence. In some embodiments, the degree of complementarity or identity between a guide sequence and its corresponding target sequence may be about 75%, 80%, 85%, 90%, 95%, or 100%. In some embodiments, the guide sequence and the target region may be 100% complementary or identical. In other embodiments, the guide sequence and the target region may contain at least one mismatch. For example, the guide sequence and the target sequence may contain 1, 2, 3, or 4 mismatches, where the total length of the target sequence is at least 17, 18, 19, 20 or more base pairs. In some embodiments, the guide sequence and the

target region may contain 1-4 mismatches where the guide sequence comprises at least 17, 18, 19, 20 or more nucleotides. In some embodiments, the guide sequence and the target region may contain 1, 2, 3, or 4 mismatches where the guide sequence comprises 20 nucleotides. In some embodiments, the degree of complementarity or identity between a guide sequence and its corresponding target sequence is at least 80%, 85%, 90%, or 95%, for example when, the guide sequence comprises a sequence 24 contiguous nucleotides. In some embodiments, the guide sequence and the target region may be 100% complementary or identical. In other embodiments, the guide sequence and the target region may contain at least one mismatch, i.e., one nucleotide that is not identical or not complementary, depending on the reference sequence. For example, the guide sequence and the target sequence may contain 1-2 mismatches, e.g., no more than 1 mismatch, where the total length of the target sequence is 19, 20, 21, 22, 23, or 24, nucleotides, or more. In some embodiments, the guide sequence and the target region may contain 1-2 mismatches where the guide sequence comprises at least 24 nucleotides, or more. In some embodiments, the guide sequence and the target region may contain 1-2 mismatches where the guide sequence comprises 24 nucleotides. Mismatch positions are known in the art as provided in, for example, PAM distal mismatches tend to be better tolerated than PAM proximal matches. Mismatch tolerances at other positions are known in the art (see, e.g., Sternberg et al., 2015, Nature:527:110-113)

[000112] As used herein, a “target sequence” or “genomic target sequence” refers to a sequence of nucleic acid in a target gene genomic locus, in either the positive or the negative strand, that has complementarity to the guide sequence of the gRNA, i.e., that is sufficiently complementary to the guide sequence of the gRNA to permit specific binding of the guide to the target sequence. The interaction of the target sequence and the guide sequence directs an RNA-guided DNA binding agent to bind, and potentially nick or cleave (depending on the activity of the agent), within the target sequence. The specific length of the target sequence and the number of mismatches possible between the target sequence and the guide sequence depend, for example, on the identity of the Cas9 nuclease being directed by the gRNA. Target sequences for Cas proteins include both the positive and negative strands of genomic DNA (i.e., the sequence given and the sequence’s reverse complement), as a nucleic acid substrate for a Cas protein is a double stranded nucleic acid. Accordingly, where a guide sequence is said to be “complementary to a target sequence,” it is to be understood that the guide sequence may direct an RNA-guided DNA binding agent (e.g., dCas9 or impaired Cas9) to bind to the reverse complement of a target sequence. Thus, in some embodiments, where the guide sequence binds the reverse complement of a target sequence, the guide sequence is identical to certain nucleotides of the target sequence (e.g., the target sequence not including the PAM) except for the substitution of U for T in the guide sequence.

[000113] Target sequences for RNA-guided DNA binding agents include both the positive and negative strands of genomic DNA (i.e., the sequence given and the sequence’s reverse

complement), as a nucleic acid substrate for an RNA-guided DNA-binding agent is a double stranded nucleic acid. Accordingly, where a guide sequence is said to be “complementary to a target sequence”, it is to be understood that the guide sequence may direct a guide RNA to bind to the reverse complement of a target sequence. Thus, in some embodiments, where the guide sequence binds the reverse complement of a target sequence, the guide sequence is identical to certain nucleotides of the target sequence (*e.g.*, the target sequence not including the PAM) except for the substitution of U for T in the guide sequence.

[000114] As used herein, an “RNA-guided DNA-binding agent” means a polypeptide or complex of polypeptides having RNA and DNA binding activity, or a DNA-binding subunit of such a complex, wherein the DNA binding activity is sequence-specific and depends on the sequence of the RNA. The term RNA-guided DNA-binding agent also includes nucleic acids encoding such polypeptides. Exemplary RNA-guided DNA-binding agents include Cas cleavases/nickases. Exemplary RNA-guided DNA-binding agents may include inactivated forms thereof (“dCas DNA-binding agents”), *e.g.*, if those agents are modified to permit DNA cleavage, *e.g.*, via fusion with a FokI cleavage domain. “Cas nuclease”, as used herein, encompasses Cas cleavases and Cas nickases. Cas cleavases and Cas nickases include a Csm or Cmr complex of a type III CRISPR system, the Cas10, Csm1, or Cmr2 subunit thereof, a Cascade complex of a type I CRISPR system, the Cas3 subunit thereof, and Class 2 Cas nucleases. As used herein, a “Class 2 Cas nuclease” is a single-chain polypeptide with RNA-guided DNA binding activity. Class 2 Cas nucleases include Class 2 Cas cleavases/nickases (*e.g.*, H840A, D10A, or N863A variants), which further have RNA-guided DNA cleavases or nickase activity, and Class 2 dCas DNA-binding agents, in which cleavage/nickase activity is inactivated), for example if those agents are modified to permit DNA cleavage, or with a C to T deaminase or A to G deaminase activity. Cas nickases include nucleases in which one of the RuvC or HNH domain of the Cas protein, such that only a single strand is cleaved by the nuclease. In some embodiments, the RNA-guided DNA-binding agent comprises a deaminase region and an RNA-guided DNA nickase, such as a Cas9 nickase. Class 2 Cas nucleases include, for example, Cas9, Cpf1, C2c1, C2c2, C2c3, HF Cas9 (*e.g.*, N497A, R661A, Q695A, Q926A variants), HypaCas9 (*e.g.*, N692A, M694A, Q695A, H698A variants), eSPCas9(1.0) (*e.g.*, K810A, K1003A, R1060A variants), and eSPCas9(1.1) (*e.g.*, K848A, K1003A, R1060A variants) proteins and modifications thereof. Cpf1 protein, Zetsche et al., *Cell*, 163: 1-13 (2015), also contains a RuvC-like nuclease domain. Cpf1 sequences of Zetsche are incorporated by reference in their entirety. *See, e.g.*, Zetsche, Tables S1 and S3. *See, e.g.*, Makarova et al., *Nat Rev Microbiol*, 13(11): 722-36 (2015); Shmakov et al., *Molecular Cell*, 60:385-397 (2015). As used herein, delivery of an RNA-guided DNA-binding agent (*e.g.*, a Cas nuclease, a Cas9 nuclease, or an *S. pyogenes* Cas9 nuclease or an *Neisseria meningitidis* Cas9 nuclease) includes delivery of the polypeptide or mRNA.

[000115] As used herein, the term “editor” or “base editor” refers to an agent comprising a polypeptide that is capable of making a modification to a base (e.g., A, T, C, G, or U) within a nucleic acid sequence (e.g., DNA or RNA). In some embodiments, the editor is capable of deaminating a base within a nucleic acid. In some embodiments, the editor is capable of deaminating a base within a DNA molecule. In some embodiments, the editor is capable of deaminating a cytosine (C) in DNA. In some embodiments, the editor is a fusion protein comprising an RNA-guided nickase fused to a cytidine deaminase domain. In some embodiments, the editor is a fusion protein comprising an RNA-guided nickase fused to an APOBEC3A deaminase (A3A). In some embodiments, the editor comprises a Cas9 nickase fused to an APOBEC3A deaminase (A3A). In some embodiments, the editor is a fusion protein comprising an enzymatically inactive RNA-guided DNA-binding proteins fused to a cytidine deaminase domain.

[000116] As used herein, “ribonucleoprotein” (RNP) or “RNP complex” refers to a guide RNA together with an RNA-guided DNA binding agent, such as a Cas nuclease, e.g., a Cas cleavase, Cas nickase, or dCas DNA binding agent (e.g., Cas9). In some embodiments, the guide RNA guides the RNA-guided DNA binding agent such as Cas9 to a target sequence, and the guide RNA hybridizes with the target sequence and the agent binds to the target sequence; in cases where the agent is a cleavase or nickase, binding can be followed by cleaving or nicking.

[000117] As used herein, the term “uracil glycosylase inhibitor”, “uracil-DNA glycosylase inhibitor” or “UGI” refers to a protein that is capable of inhibiting a uracil-DNA glycosylase (UDG) base-excision repair enzyme (e.g., UniPROT ID: P14739; MTNLSDIIEKETGKQLVIQESILMLPEEVEEVIGNKPESDILVHTAYDESTDENVMMLLTSDAP EYKPWALVIQDSNGENKIKMLSGGSKRTADGSEFESPKKKRKVE (SEQ ID NO: 226); or TNLSDIIEKETGKQLVIQESILMLPEEVEEVIGNKPESDILVHTAYDESTDENVMMLLTSDAPE YKPWALVIQDSNGENKIKML (SEQ ID NO: 227)).

[000118] Exemplary nucleotide and polypeptide sequences of Cas9 molecules are provided below. Methods for identifying alternate nucleotide sequences encoding Cas9 polypeptide sequences, including alternate naturally occurring variants, are known in the art. Sequences with at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to any of the Cas9 nucleic acid sequences or nucleic acid sequences encoding the amino acid sequences provided herein are also contemplated. In certain embodiments, the nucleotide sequence encoding the Cas9 amino acid sequence is not a naturally occurring Cas9 nucleotide sequence. Sequences with at least 95%, 96%, 97%, 98%, or 99% identity to any of the Cas9 amino acid sequences provided herein are also contemplated. In certain embodiments, the Cas9 amino acid sequence is not a naturally occurring Cas9 sequence.

[000119] Exemplary open reading frame for Spy Cas9

AUGGACAAGAAGUACUCCAUCGGCCUGGACAUCGGCACCAACUCCGUGGGCUGGGCC
GUGAUCACCGACGAGUACAAGGUGCCCUCCAAGAAGUUCAAGGUGCUGGGCAACAC
CGACCGGCACUCCAUCAAGAAGAACCUGAUCGGCGCCUGCUGUUCGACUCCGGCGA
GACCGCCGAGGCCACCCGGCUGAAGCGGACCGCCCGGCGGCGGUACACCCGGCGGAA
GAACCGGAUCUGCUACCUGCAGGAGAUCUUCUCCAACGAGAUGGCCAAGGUGGACG
ACUCCUUCUUCACCGGCUGGAGGAGUCCUUCUGGUGGAGGAGGACAAGAAGCAC
GAGCGGCACCCCAUCUUCGGCAACAUCGUGGACGAGGUGGCCUACCACGAGAAGUAC
CCCACCAUCUACCACCUGCGGAAGAAGCUGGUGGACUCCACCGACAAGGCCGACCUG
CGGCUGAUCUACCUGGCCCUUGGCCACAUGAUCUAGUUCGGGGCCACUCCUGAUC
GAGGGCGACCUGAACCCCGACAACUCCGACGUGGACAAGCUGUUCAUCCAGCUGGUG
CAGACCUACAACCAGCUGUUCGAGGAGAACCCCAUCAACGCCUCCGGCGUGGACGCC
AAGGCCAUCCUGUCCGCCCGGCUGUCCAAGUCCCGGCGGCUGGAGAACCUGAUCGCC
CAGCUGCCCGGCGAGAAGAAGAACGGCCUGUUCGGCAACCUGAUCGCCUGUCCUG
GGCCUGACCCCAACUUCAAGUCCAACUUCGACCUGGCCGAGGACGCCAAGCUGCAG
CUGUCCAAGGACACCUACGACGACGACCUGGACAACCUGCUGGCCCAGAUCGGCGAC
CAGUACGCCGACCUGUUCUGGCCGCCAAGAACCUGUCCGACGCCAUCCUGCUGUCC
GACAUCUGCGGGUGAACACCGAGAUACCAAGGCCCCCUGUCCGCCUCCAUGAUC
AAGCGGUACGACGAGCACCACCAGGACCUGACCCUGCUGAAGGCCUGGUGCGGCAG
CAGCUGCCCGAGAAGUACAAGGAGAUUCUUCUUCGACCAGUCCAAGAACGGCUACGCC
GGCUACAUCGACGGCGGCGCCUCCAGGAGGAGUUCUACAAGUUCAUCAAGCCCAUC
CUGGAGAAGAUGGACGGCACCGAGGAGCUGCUGGUGAAGCUGAACCAGGAGGACCU
GCUGCGGAAGCAGCGGACCUUCGACAACGGCUCCAUCCCCACCAGAUCACCUGGG
CGAGCUGCACGCCAUCCUGCGGCGGCAGGAGGACUUCUACCCCUCCUGAAGGACAA
CCGGGAGAAGAUCGAGAAGAUCUGACCUUCCGGAUCCCUACUACGUGGGCCCCCU
GGCCCGGGGCAACUCCCGGUUCGCCUGGAUGACCCGGAAGUCCGAGGAGACCAUCAC
CCCCUGGAACUUCGAGGAGGUGGUGGACAAGGGCGCCUCCGCCAGUCCUUAUCGA
GCGGAUGACCAACUUCGACAAGAACCUGCCCAACGAGAAGGUGCUGCCCAAGCACUC
CCUGCUGUACGAGUACUUCACCGUGUACAACGAGCUGACCAAGGUGAAGUACGUGA
CCGAGGGCAUGCGGAAGCCCGCCUCCUGUCCGGCGAGCAGAAGAAGGCCAUCGUGG
ACCUGCUGUUAAGACCAACCGGAAGGUGACCGUGAAGCAGCUGAAGGAGGACUAC
UUCAAGAAGAUCGAGUGCUUCGACUCCGUGGAGAUCUCCGGCGUGGAGGACCGGU
CAACGCCUCCUGGGCACCUACCACGACCUGCUGAAGAUAUCAAGGACAAGGACU
CCUGGACAACGAGGAGAACGAGGACAUCUGGAGGACAUCGUGCUGACCCUGACCC
UGUUCGAGGACCGGGAGAUGAUCGAGGAGCGGCUGAAGACCUACGCCACCUGUUC
GACGACAAGGUGAUGAAGCAGCUGAAGCGGCGGCGGUACACCGGCUGGGGCCGGCU
GUCCCGGAAGCUGAUAACGGCAUCCGGGACAAGCAGUCCGGCAAGACCAUCCUGGA
CUUCCUGAAGUCCGACGGCUUCGCCAACCAGAACUUAUGCAGCUGAUCCACGACGA

CUCCCUGACCUUCAAGGAGGACAUCCAGAAGGCCAGGUGUCCGGCCAGGGCGACUC
CCUGCACGAGCACAUCCGCAACCUGGCCGGCUCCCCGCCAUCAAGAAGGGCAUCCU
GCAGACCGUGAAGGUGGUGGACGAGCUGGUGAAGGUGAUGGGCCGGCACAAGCCCG
AGAACAUCGUGAUCGAGAUGGCCCGGGAGAACCAGACCACCCAGAAGGGCCAGAAG
AACUCCCCGGGAGCGGAUGAAGCGGAUCGAGGAGGGCAUCAAGGAGCUGGGCUCCCA
GAUCCUGAAGGAGCACCCCGUGGAGAACACCCAGCUGCAGAACGAGAAGCUGUACC
UGUACUACCUGCAGAACGGCCGGGACAUGUACGUGGACCAGGAGCUGGACAUCAAC
CGGCUGUCCGACUACGACGUGGACCACAUCGUGCCCCAGUCCUCCUGAAGGACGAC
UCCAUCGACAACAAGGUGCUGACCCGGUCCGACAAGAACCAGGGGCAAGUCCGACAAC
GUGCCCUCCGAGGAGGUGGUGAAGAAGAUGAAGAACUACUGGCGGCAGCUGCUGAA
CGCCAAGCUGAUCACCCAGCGGAAGUUCGACAACCUGACCAAGGCCGAGCGGGGCGG
CCUGUCCGAGCUGGACAAGGCCGGCUUCAUCAAGCGGCAGCUGGUGGAGACCCGGCA
GAUCACCAAGCACGUGGCCCAGAUCUCCUGGACUCCCGGAUGAACACCAAGUACGACGA
GAACGACAAGCUGAUCCGGGAGGUGAAGGUGAUCACCCUGAAGUCCAAGCUGGUGU
CCGACUUCGGGAAGGACUUCAGUUCUACAAGGUGCGGGAGAUCAACAACUACCACC
ACGCCCACGACGCCUACCUGAACGCCGUGGUGGGCACCGCCCUGAUCAAGAAGUACC
CCAAGCUGGAGUCCGAGUUCGUGUACGGCGACUACAAGGUGUACGACGUGCGGAAG
AUGAUCGCCAAGUCCGAGCAGGAGAUCGGCAAGGCCACCGCCAAGUACUUCUUCUAC
UCCAACAUAUGAACUUCUUAAGACCGAGAUCACCCUGGCCAACGGCGAGAUCCGG
AAGCGGCCCCUGAUCGAGACCAACGGCGAGACCGGCGAGAUCGUGUGGGACAAGGG
CCGGGACUUCGCCACCGUGCGGAAGGUGCUGUCCAUGCCCCAGGUGAACAUUGUGAA
GAAGACCGAGGUGCAGACCGGCGGCUUCUCCAAGGAGUCCAUCCUGCCCAAGCGGAA
CUCCGACAAGCUGAUCGCCCCGGAAGAAGGACUGGGACCCCAAGAAGUACGGCGGCU
UCGACUCCCCCACCUGGGCCUACUCCGUGCUGGUGGUGGCCAAGGUGGAGAAGGGCA
AGUCCAAGAAGCUGAAGUCCGUGAAGGAGCUGCUGGGCAUCACCAUCAUGGAGCGG
UCCUCCUUCGAGAAGAACCCCAUCGACUUCUGGAGGCCAAGGGCUACAAGGAGGU
GAAGAAGGACCUGAUAUCAAGCUGCCCAAGUACUCCUGUUCGAGCUGGAGAACG
GCCGGAAGCGGAUGCUGGCCUCCGCCGGCGAGCUGCAGAAGGGCAACGAGCUGGGCC
UGCCCUCCAAGUACGUGAACUUCUGUACCUGGCCUCCACUACGAGAAGCUGAAGG
GCUCCCCCGAGGACAACGAGCAGAAGCAGCUGUUCGUGGAGCAGCACAAGCACUACC
UGGACGAGAUAUCGAGCAGAUCUCCGAGUUCUCCAAGCGGGUGAUCCUGGCCGAC
GCCAACCUGGACAAGGUGCUGUCCGCCUACAACAAGCACCGGGACAAGCCCAUCCGG
GAGCAGGCCGAGAACAUAUCCACCUGUUCACCCUGACCAACCUGGGCGCCCCGCC
GCCUUAAGUACUUCGACACCACCAUCGACCGGAAGCGGUACACCUCCACCAAGGAG
GUGCUGGACGCCACCCUGAUCCACCAGUCCAUCACCGGCCUGUACGAGACCCGGAUC
GACCUGUCCAGCUGGGCGGCGACGGCGGGCUGCCCCCAAGAAGAAGCGGAAGGUG
UGA (SEQ ID NO: 218)

[000120] Exemplary amino acid sequence for Spy Cas9

MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEA
TRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSSFFHRLEESFLVEEDKKHERHPIFGNIV
DEVAYHEKYPTIYHLRKKLV DSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVKL
FIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIASLG
LTPNFKSNFDLAEDAKLQLSKD TYDDDLNLLAQIGDQYADLFLAAKNLSDAILLSDILRV
NTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQ
EEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPF
LKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEVVVDKGASQSFIER
MTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFK
TNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDIL
EDIVLTLTLFEDREMIEERLKYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGK
TILDFLKSDGFANRNFMLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIIKKGILQ
TVKVVDLVKVMGRHKPENIVIAMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHP
VENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRS
DKNRGKSDNVPSEEVVKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKR
QLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSCLVSDFRKDFQFYKVREINN
YHHAHDAYLNAVVGTAIIKKYPKLESEFVYGDYKVYDVRKMIKSEQEIGKATAKYFFYS
NIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVL SMPQVNIVKKTEVQT
GGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKEVGKSKKLKSVKE
LLGITIMERSSSFENPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGN
ELALPSKYVNFLYLASHYEKLKGS PEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADAN
LDKVL SAYNKH RDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVL DATLI
HQSITGLYETRIDLSQLGGDGGGSPKKKRKV (SEQ ID NO: 219)

[000121] Exemplary open reading frame for Spy Cas9

AUGGACAAGAAGUACAGCAUCGGACUGGACAUCGGAACAAACAGCGUCGGAUGGGC
AGUCAUCACAGACGAAUACAAGGUCCCGAGCAAGAAGUUAAGGUCCUGGGAAACA
CAGACAGACACAGCAUCAAGAAGAACCUGAUCGGAGCACUGCUGUUCGACAGCGGA
GAAACAGCAGAAGCAACAAGACUGAAGAGAACAGCAAGAAGAAGAUACACAAGAAG
AAAGAACAGAAUCUGCUACCUGCAGGAAAUCUUCAGCAACGAAAUGGCAAAGGUCG
ACGACAGCUUCUCCACAGACUGGAAGAAAGCUUCCUGGUCGAAGAAGACAAGAAG
CACGAAAGACACCCGAUCUUCGGAACAUCGUCGACGAAGUCGAUACCACGAAAA
GUACCCGACAAUCUACCACCUGAGAAAGAAGCUGGUCGACAGCACAGACAAGGCAG
ACCUGAGACUGAUCUACCUGGCACUGGCACACAUGAUGAAGUUCAGAGGACACUUC
CUGAUCGAAGGAGACCUGAACCCGACAACAGCGACGUCGACAAGCUGUUCAUCCA
GCUGGUCCAGACAUAACAACCAGCUGUUCGAAGAAAACCCGAUCAACGCAAGCGGAG

UCGACGCAAAGGCAAUCCUGAGCGCAAGACUGAGCAAGAGCAGAAGACUGGAAAAC
CUGAUCGCACAGCUGCCGGGAGAAAAGAAGAACGGACUGUUCGGAAACCUGAUCGC
ACUGAGCCUGGGACUGACACCGAACUUAAGAGCAACUUCGACCUGGCAGAAGACG
CAAAGCUGCAGCUGAGCAAGGACACAUAACGACGACGACCUGGACAACCUGCUGGCAC
AGAUCGGAGACCAGUACGCAGACCUGUUCUGGCAGCAAAGAACCUGAGCGACGCA
AUCCUGCUGAGCGACAUCUGAGAGUCAACACAGAAAUCACAAAGGCACCGCUGAG
CGCAAGCAUGAUAAGAGAUACGACGAACACCACCAGGACCUGACACUGCUGAAGG
CACUGGUCAGACAGCAGCUGCCGGAAAAGUACAAGGAAAUCUUCUUCGACCAGAGC
AAGAACGGAUACGCAGGAUACAUCGACGGAGGAGCAAGCCAGGAAGAAUUCUACAA
GUUCAUAAGCCGAUCCUGGAAAAGAUGGACGGAACAGAAGAACUGCUGGUCAAGC
UGAACAGAGAAGACCUGCUGAGAAAAGCAGAGAACAUCGACAACGGAAGCAUCCCG
CACCAGAUCCACCUGGGAGAACUGCACGCAAUCCUGAGAAGACAGGAAGACUUCUA
CCCGUUCUGAAGGACAACAGAGAAAAGAUUCGAAAAGAUCCUGACAUCAGAAUCC
CGUACUACGUCGGACCGCUGGCAAGAGGAAACAGCAGAUUCGCAUGGAUGACAAGA
AAGAGCGAAGAAACAAUCACACCGUGGAACUUCGAAGAAGUCGUCGACAAGGGAGC
AAGCGCACAGAGCUUCAUCGAAAGAAUGACAAACUUCGACAAGAACCUGCCGAACG
AAAAGGUCCUGCCGAAGCACAGCCUGCUGUACGAAUACUUCACAGUCUACAACGAA
CUGACAAAGGUCAAGUACGUCACAGAAGGAAUGAGAAAGCCGGCAUUCUGAGCGG
AGAACAGAAGAAGGCAAUCGUCGACCUGCUGUUAAGACAAACAGAAAGGUCACAG
UCAAGCAGCUGAAGGAAGACUACUUAAGAAGAUCGAAUGCUUCGACAGCGUCGAA
AUCAGCGGAGUCGAAGACAGAUUCAACGCAAGCCUGGGAACAUAACCACGACCUGCU
GAAGAUCAUCAAGGACAAGGACUUCUGGACAACGAAGAAAACGAAGACAUCUGG
AAGACAUCGUCCUGACACUGACACUGUUCGAAGACAGAGAAAUGAUCGAAGAAAGA
CUGAAGACAUAACGCACACCUGUUCGACGACAAGGUCAUGAAGCAGCUGAAGAGAAG
AAGAUACACAGGAUGGGGAAGACUGAGCAGAAAGCUGAUAACGGAAUCAGAGACA
AGCAGAGCGGAAAGACAAUCCUGGACUUCUGAAGAGCGACGGAUUCGCAAACAGA
AACUUCAUGCAGCUGAUCCACGACGACAGCCUGACAUCUUAAGGAAGACAUCAGAA
GGCACAGGUCAGCGGACAGGGAGACAGCCUGCACGAACACAUCGCAAACCUGGCAG
GAAGCCCCGGCAAUCAAGAAGGGAAUCCUGCAGACAGUCAAGGUCGUCGACGAACUG
GUCAAGGUCAUGGGAAGACACAAGCCGGAAAACAUCGUCAUCGAAAUGGCAAGAGA
AAACCAGACAACACAGAAGGGACAGAAGAACAGCAGAGAAAGAAUGAAGAGAAUCG
AAGAAGGAAUCAAGGAACUGGGAAGCCAGAUCCUGAAGGAACACCCGGUCGAAAAC
ACACAGCUGCAGAACGAAAAGCUGUACCUGUACUACCUGCAGAACGGAAGAGACAU
GUACGUCGACCAGGAACUGGACAUAACAGACUGAGCGACUACGACGUCGACCACA
UCGUCCCCGAGAGCUUCUGAAGGACGACAGCAUCGACAACAAGGUCCUGACAAGA
AGCGACAAGAACAGAGGAAAGAGCGACAACGUCCCCGAGCGAAGAAGUCGUCAAGAA
GAUGAAGAACUACUGGAGACAGCUGCUGAACGCAAAGCUGAUCACACAGAGAAAGU

UCGACAACCUGACAAAGGCAGAGAGAGGAGGACUGAGCGAACUGGACAAGGCAGGA
 UUCAUCAAGAGACAGCUGGUCGAAACAAGACAGAUACAAAAGCACGUCGCACAGAU
 CCUGGACAGCAGAAUGAACACAAAGUACGACGAAAACGACAAGCUGAUCAGAGAAG
 UCAAGGUCAUCACACUGAAGAGCAAGCUGGUCAGCGACUUCAGAAAGGACUCCAG
 UUCUACAAGGUCAGAGAAAUCAACAACUACCACCACGCACACGACGCAUACCUGAAC
 GCAGUCGUCGGAACAGCACUGAUCAAGAAGUACCCGAAGCUGGAAAGCGAAUUCGU
 CUACGGAGACUACAAGGUCUACGACGUCAGAAAGAUGAUCGCAAAGAGCGAACAGG
 AAUUCGGAAGGCAACAGCAAAGUACUUCUUCUACAGCAACAUAUGAACUUCUUC
 AAGACAGAAAUCACACUGGCAAACGGAGAAAUCAGAAAGAGACCGCUGAUCGAAAC
 AAACGGAGAAACAGGAGAAAUCGUCUGGGACAAGGGAAGAGACUUCGCAACAGUCA
 GAAAGGUCCUGAGCAUGCCGCAGGUCAACAUCGUCAAGAAGACAGAAGUCCAGACA
 GGAGGAUUCAGCAAGGAAAGCAUCCUGCCGAAGAGAAACAGCGACAAGCUGAUCGC
 AAGAAAGAAGGACUGGGACCCGAAGAAGUACGGAGGAUUCGACAGCCCGACAGUCG
 CAUACAGCGUCCUGGUCGUCGCAAAGGUCGAAAAGGGAAAGAGCAAGAAGCUGAAG
 AGCGUCAAGGAACUGCUGGGAAUCACAAUCAUGGAAAGAAGCAGCUUCGAAAAGAA
 CCCGAUCGACUUCUGGAAGCAAAGGGAUACAAGGAAGUCAAGAAGGACCUGAUA
 UCAAGCUGCCGAAGUACAGCCUGUUCGAACUGGAAAACGGAAGAAAGAGAAUGCUG
 GCAAGCGCAGGAGAACUGCAGAAGGGAAACGAACUGGCACUGCCGAGCAAGUACGU
 CAACUUCUGUACCUGGCAAGCCACUACGAAAAGCUGAAGGGAAGCCCGGAAGACA
 ACGAACAGAAGCAGCUGUUCGUCGAACAGCACAAAGCACUACCUGGACGAAAUCAUC
 GAACAGAUACAGCGAAUUCAGCAAGAGAGUCAUCCUGGCAGACGCAAACCUGGACAA
 GGUCCUGAGCGCAUACAACAAGCACAGAGACAAGCCGAUCAGAGAACAGGCAGAAA
 ACAUCAUCCACCUGUUCACACUGACAAACCUGGGAGCACCGGCAGCAUUAAGUACU
 UCGACACAACAUCGACAGAAAGAGAUACACAAGCACAAAGGAAGUCCUGGACGCA
 ACACUGAUCCACCAGAGCAUACAGGACUGUACGAAACAAGAAUCGACCUGAGCCA
 GCUGGGAGGAGACGGAGGAGGAAGCCCGAAGAAGAAGAGAAAGGUCUAG (SEQ ID
 NO: 116)

[000122] Exemplary open reading frame for Spy Cas9 with Hibt tag

AUGGACAAGAAGUACUCCAUCGGCCUGGACAUCGGCACCAACUCCUGGGCUGGGCC
 GUGAUCACCGACGAGUACAAGGUGCCCUCCAAGAAGUUAAGGUGCUGGGCAACAC
 CGACCGGCACUCCAUAAGAAGAACCUGAUCGGCGCCCGUCUGUUCGACUCCGGCGA
 GACCGCCGAGGCCACCCGGCUGAAGCGGACCGCCCGGCGGCGGUACCCCGGCGGAA
 GAACCGGAUCUGCUACCUGCAGGAGAUUCUCCAACGAGAUGGCCAAGGUGGACG
 ACUCCUUCUUCACCAGGUGGAGGAGUCCUUCUGGUGGAGGAGGACAAGAAGCAC
 GAGCGGCACCCCAUCUUCGGCAACAUCGUGGACGAGGUGGCCUACCACGAGAAGUAC
 CCCACCAUCUACCACCUGCGGAAGAAGCUGGUGGACUCCACCGACAAGGCCGACCUG

CGGCUGAUCUACCUGGGCCCUGGGCCACAUGAUAAGUUCGGGGCCACUUCCUGAUC
GAGGGCGACCUGAACCCCGACAACUCCGACGUGGACAAGCUGUUCAUCCAGCUGGUG
CAGACCUACAACCAGCUGUUCGAGGAGAACCCCAUCAACGCCUCCGGCGUGGACGCC
AAGGCCAUCCUGUCCGCCCAGCUGUCCAAGUCCCGGCGGCUGGAGAACCUGAUCGCC
CAGCUGCCCCGGCGAGAAGAAGAACGGCCUGUUCGGCAACCUGAUCGCCCUGUCCUG
GGCCUGACCCCCAACUUAAGUCCAACUUCGACCUGGGCCGAGGACGCCAAGCUGCAG
CUGUCCAAGGACACCUACGACGACGACCUGGACAACCUGCUGGGCCCAGAUCGGCGAC
CAGUACGCCGACCUGUUCUGGGCCGCAAGAACCUGUCCGACGCCAUCCUGCUGUCC
GACAUCUGCGGGUGAACACCGAGAUCACCAAGGCCCCCUGUCCGCCUCCAUGAUC
AAGCGGUACGACGAGCACCACCAGGACCUGACCCUGCUGAAGGCCCUGGUGCGGCAG
CAGCUGCCCCGAGAAGUACAAGGAGAUUUUUCGACCAGUCCAAGAACGGCUACGCC
GGCUACAUCGACGGCGGGCGCCUCCAGGAGGAGUUCUACAAGUUAUCAAGCCCAUC
CUGGAGAAGAUGGACGGCACCGAGGAGCUGCUGGUGAAGCUGAACCAGGAGGACCU
GCUGCGGAAGCAGCGGACCUUCGACAACGGCUCCAUCCCCACCAGAUCACCUGGG
CGAGCUGCACGCCAUCCUGCGGGCGGAGGAGGACUUCUACCCCUUCCUGAAGGACAA
CCGGGAGAAGAUCGAGAAGAUCUGACCUUCCGGAUCCCUACUACGUGGGCCCCCU
GGCCCGGGGCAACUCCCGGUUCGCCUGGAUGACCCGGAAGUCCGAGGAGACCAUCAC
CCCCUGGAACUUCGAGGAGGUGGUGGACAAGGGCGCCUCCGCCAGUCCUUAUCGA
GCGGAUGACCAACUUCGACAAGAACCUGCCCAACGAGAAGGUGCUGCCCAAGCACUC
CCUGCUGUACGAGUACUUCACCGUGUACAACGAGCUGACCAAGGUGAAGUACGUGA
CCGAGGGCAUGCGGAAGCCCGCCUCCUGUCCGGCGAGCAGAAGAAGGCCAUCGUGG
ACCUGCUGUUAAGACCAACCGGAAGGUGACCGUGAAGCAGCUGAAGGAGGACUAC
UUAAGAAGAUCGAGUGCUUCGACUCCGUGGAGAUCUCCGGCGUGGAGGACCGGUU
CAACGCCUCCUGGGCACCUACCACGACCUGCUGAAGAUAUCAAGGACAAGGACUUC
CCUGGACAACGAGGAGAACGAGGACAUCUGGAGGACAUCGUGCUGACCCUGACCC
UGUUCGAGGACCGGGAGAUGAUCGAGGAGCGGCUGAAGACCUACGCCACCUGUUC
GACGACAAGGUGAUGAAGCAGCUGAAGCGGCGGGCGGUACACCGGCUGGGGCCGGCU
GUCCCGGAAGCUGAUAACGGCAUCCGGGACAAGCAGUCCGGCAAGACCAUCCUGGA
CUUCCUGAAGUCCGACGGCUUCGCCAACCAGAACUUAUGCAGCUGAUCCACGACGA
CUCCUGACCUUAAGGAGGACAUCAGAAAGGCCAGGUGUCCGGCCAGGGCGACUC
CCUGCACGAGCACAUCGCCAACCUGGCCGGCUCCCCCGCCAUAAGAAGGGCAUCCU
GCAGACCGUGAAGGUGGUGGACGAGCUGGUGAAGGUGAUGGGCCGGCACAAGCCCG
AGAACAUCGUGAUCGAGAUGGCCCCGGGAGAACCAGACCACCAGAAAGGGCCAGAAG
AACUCCCGGGAGCGGAUGAAGCGGAUCGAGGAGGGCAUCAAGGAGCUGGGCUCCCA
GAUCCUGAAGGAGCACCCCGUGGAGAACACCCAGCUGCAGAACGAGAAGCUGUACC
UGUACUACCUGCAGAACGGCCGGGACAUGUACGUGGACCAGGAGCUGGACAUAAC
CGGCUGUCCGACUACGACGUGGACCACAUCGUGCCCCAGUCCUCCUGAAGGACGAC

UCCAUCGACAACAAGGUGCUGACCCGGUCCGACAAGAACCGGGGCAAGUCCGACAAC
 GUGCCCUCCGAGGAGGUGUGAAGAAGAUGAAGAACUACUGGCGGCAGCUGCUGAA
 CGCCAAGCUGAUCACCCAGCGGAAGUUCGACAACCUGACCAAGGCCGAGCGGGGCGG
 CCUGUCCGAGCUGGACAAGGCCGGCUUCAUCAAGCGGCAGCUGGUGGAGACCCGGCA
 GAUCACCAAGCACGUGGCCCAGAUAUCCUGGACUCCCGGAUGAACACCAAGUACGACGA
 GAACGACAAGCUGAUAUCCGGGAGGUGAAGGUGAUAUCCCGAAGUCCAAGCUGGUGU
 CCGACUUCGGGAAGGACUUCAGUUCUACAAGGUGCGGGAGAUAACAACUACCACC
 ACGCCACGACGCCUACCUGAACGCCGUGGUGGGCACCGCCUGAUAAGAAGUACC
 CCAAGCUGGAGUCCGAGUUCGUGUACGGCGACUACAAGGUGUACGACGUGCGGAAG
 AUGAUCGCCAAGUCCGAGCAGGAGAUCGGCAAGGCCACCGCCAAGUACUUCUUCUAC
 UCCAACAUAUGAACUUCUUAAGACCGAGAUAUCCUGGCCAACGGCGAGAUCGCG
 AAGCGGCCCCUGAUCGAGACCAACGGCGAGACCGGCGAGAUCGUGUGGGACAAGGG
 CCGGGACUUCGCCACCGUGCGGAAGGUGCUGUCCAUGCCCCAGGUGAACAUUGUGAA
 GAAGACCGAGGUGCAGACCGGGCGGCUUCUCCAAGGAGUCCAUCCUGGCCAAGCGGAA
 CUCCGACAAGCUGAUCGCCCGGAAGAAGGACUGGGACCCCAAGAAGUACGGCGGCU
 UCGACUCCCCACCGUGGCCUACUCCGUGCUGGUGGUGGCCAAGGUGGAGAAGGGCA
 AGUCCAAGAAGCUGAAGUCCGUGAAGGAGCUGCUGGGCAUCACCAUCAUGGAGCGG
 UCCUCCUUCGAGAAGAACCCCAUCGACUUCUGGAGGCCAAGGGCUACAAGGAGGU
 GAAGAAGGACCUGAUAUCAAGCUGCCCAAGUACUCCUGUUCGAGCUGGAGAACG
 GCCGGAAGCGGAUGCUGGCCUCCGCCGGCGAGCUGCAGAAGGGCAACGAGCUGGCCC
 UGCCCUGCAAGUACGUGAACUUCUGUACCUGGCCUCCACUACGAGAAGCUGAAGG
 GCUCCCCCGAGGACAACGAGCAGAAGCAGCUGUUCGUGGAGCAGCACAAGCACUACC
 UGGACGAGAUAUCGAGCAGAUAUCCGAGUUCUCCAAGCGGGUGAUAUCCUGGCCGAC
 GCCAACCGGACAAGGUGCUGUCCGCCUACAACAAGCACCGGGACAAGCCCAUCCGG
 GAGCAGGCCGAGAACAUAUCCACCUGUUCACCCUGACCAACCUGGGCGCCCCCGCC
 GCCUUAAGUACUUCGACACCACCAUCGACCGGAAGCGGUACACCUCCACCAAGGAG
 GUGCUGGACGCCACCCUGAUCCACCAGUCCAUAUCCGGCCUGUACGAGACCCGGAUC
 GACCUGUCCAGCUGGGCGGCGACGGCGGGCUGCCCCCAAGAAGAAGCGGAAGGUG
 UCCGAGUCCGCCACCCCGAGUCCGUGUCCGGCUGGCGGCUGUUAAGAAGAUCUCC
 UGA (SEQ ID NO: 117)

[000123] Exemplary amino acid sequence for Spy Cas9 with Hbit tag

MDKKYSIGLDIGTNSVGWAVITDEYKVPSSKFKVLGNTDRHSIKKNLIGALLFDSGETAEA
 TRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHRLLESFLVEEDKKHERHPIFGNIV
 DEVAYHEKYPTIYHLRKKLVDSTDKADLRILIYLAHMIKFRGHFLIEGDLNPDNSDVKL
 FIQLVQTYNQLFEENPINASGVDAKILSARLSKSRLENLIAQLPGEKKNGFLGNLIALSLG
 LTPNFKSNFDLAEDAKLQLSKDQYDDDLNLLAQIGDQYADLFLAAKNLSDAILLSDILRV

NTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQ
 EEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPF
 LKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIER
 MTNFDKNLPNEKVLPKHSLLEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFK
 TNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDIL
 EDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGK
 TILDFLKSDGFANRNFMLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIAKKGILQ
 TVKVDELVKVMGRHKPENIVIAMARENQTTQKGQKNSRERMKRIEELGKELGSQILKEHP
 VENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRS
 DKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKR
 QLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSCLVSDFRKDFQFYKVINN
 YHHAHDAYLNAVVGTAIIKKYPKLESEFVYGDYKVYDVRKMIKSEQEIGKATAKYFFYS
 NIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSPQVNVKKTEVQT
 GGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVAKVEKGKSKKLKSVKE
 LLGITIMERSSEKPNIDFLEAGYKEVKDLIIKLPKYSLEFENGRKRLASAGELQKGN
 ELALPSKYVNFLYLASHYEKLKGSPEDEQKQLFVEQHKHYLDEIIEQISEFSKRVLADAN
 LDKVLSAYNKHDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDTLI
 HQSITGLYETRIDLSQLGGDGGGSPKKKRKVSESATPESVSGWRLFKKIS (SEQ ID NO: 118)

[000124] Exemplary amino acid sequence for Spy Cas9 nickase

MDKKYSIGLAIGTNSVGWAVITDEYKVPSSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEA
 TRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHRLSESLVEEDKKHERHPIFGNIV
 DEVAYHEKYPTIYHLRKKLVDDSTDKADLRLIYLALAHMIKFRGHFLIEGDLTGGGGSVDVN
 TEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQE
 EEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPF
 KDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERM
 TNFDKNLPNEKVLPKHSLLEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKT
 NRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILE
 DIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKT
 ILDFLKSDGFANRNFMLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIAKKGILQT
 VKVDELVKVMGRHKPENIVIAMARENQTTQKGQKNSRERMKRIEELGKELGSQILKEHPV
 ENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSD
 KNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQ
 LVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSCLVSDFRKDFQFYKVINN
 YHHAHDAYLNAVVGTAIIKKYPKLESEFVYGDYKVYDVRKMIKSEQEIGKATAKYFFYSN
 IMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSPQVNVKKTEVQTG
 GFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVAKVEKGKSKKLKSVKEL

LGITIMERSSEFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNE
LALPSKYVNFYLYASHYEKLKGSPEQNEQKQLFVEQHKHYLDEIIEQISEFSKRVLADANL
DKVLSAYNKHRRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDTLIH
QSITGLYETRIDLSQLGGDGGGSPKKKRKV (SEQ ID NO: 220)

[000125] Exemplary open reading frame for Nme2Cas9

ATGGACGGCTCCGGCGGCGGCTCCCCCAAGAAGAAGCGGAAGGTGGAGGACAAGCGG
CCCGCCGCCACCAAGAAGGCCGGCCAGGCCAAGAAGAAGAAGGGCGGCTCCGGCGGC
GGCGCCGCTTCAAGCCCAACCCCATCAACTACATCCTGGGCCTGGACATCGGCATCG
CCTCCGTGGGCTGGGCCATGGTGGAGATCGACGAGGAGGAGAACCCCATCCGGCTGAT
CGACCTGGGCGTGCGGGTGTTTCGAGCGGGCCGAGGTGCCCAAGACCGGCGACTCCCTG
GCCATGGCCCGGCGGCTGGCCCGGTCCGTGCGGCGGCTGACCCGGCGGCGGGCCACC
GGCTGCTGCGGGCCCGGCGGCTGCTGAAGCGGGAGGGCGTGCTGCAGGCCGCCGACTT
CGACGAGAACGGCCTGATCAAGTCCCTGCCCAACACCCCTGGCAGCTGCGGGCCGCC
GCCCTGGACCGGAAGCTGACCCCCCTGGAGTGGTCCGCCGTGCTGCTGCACCTGATCA
AGCACCGGGGCTACCTGTCCCAGCGGAAGAACGAGGGCGAGACCGCCGACAAGGAGC
TGGGCGCCCTGCTGAAGGGCGTGGCCAACAACGCCACGCCCTGCAGACCGGCGACTT
CCGGACCCCGCCGAGCTGGCCCTGAACAAGTTCGAGAAGGAGTCCGGCCACATCCGG
AACCAGCGGGGCGACTACTCCCACACCTTCTCCCGGAAGGACCTGCAGGCCGAGCTGA
TCCTGTGTTCGAGAAGCAGAAGGAGTTCGGCAACCCCCACGTGTCCGGCGGCCTGAA
GGAGGGCATCGAGACCCTGCTGATGACCCAGCGGCCCGCCCTGTCCGGCGACGCCGTG
CAGAAGATGCTGGGCCACTGCACCTTCGAGCCCGCCGAGCCCAAGGCCGCCAAGAACA
CCTACACCGCCGAGCGGTTTCATCTGGCTGACCAAGCTGAACAACCTGCGGATCCTGGA
GCAGGGCTCCGAGCGGCCCTGACCGACACCGAGCGGGCCACCCTGATGGACGAGCCC
TACCGGAAGTCCAAGCTGACCTACGCCAGGCCCGGAAGCTGCTGGGCCTGGAGGACA
CCGCCTTCTTCAAGGGCCTGCGGTACGGCAAGGACAACGCCGAGGCCTCCACCCTGAT
GGAGATGAAGGCCTACCACGCCATCTCCCGGGCCCTGGAGAAGGAGGGCCTGAAGGA
CAAGAAGTCCCCCTGAACCTGTCTCCGAGCTGCAGGACGAGATCGGCACCGCCTTC
TCCCTGTTCAAGACCGACGAGGACATCACCGGCCGGCTGAAGGACCGGGTGCAGCCCG
AGATCCTGGAGGCCCTGCTGAAGCACATCTCCTTCGACAAGTTCGTGCAGATCTCCCTG
AAGGCCCTGCGGCGGATCGTGCCCTGATGGAGCAGGGCAAGCGGTACGACGAGGCCT
GCGCCGAGATCTACGGCGACCACTACGGCAAGAAGAACAACCGAGGAGAAGATCTACC
TGCCCCCATCCCCGCCGACGAGATCCGGAACCCCGTGGTGCTGCGGGCCCTGTCCCA
GGCCCGGAAGGTGATCAACGGCGTGGTGCGGCGGTACGGCTCCCCCGCCCGGATCCAC
ATCGAGACCGCCCGGGAGGTGGGCAAGTCCTTCAAGGACCGGAAGGAGATCGAGAAG
CGGCAGGAGGAGAACCGGAAGGACCGGGAGAAGGCCGCCGCCAAGTTCCGGGAGTAC
TTCCCCAACTTCGTGGGCGAGCCCAAGTCCAAGGACATCCTGAAGCTGCGGCTGTACG

AGCAGCAGCACGGCAAGTGCCTGTACTCCGGCAAGGAGATCAACCTGGTGCGGCTGAA
CGAGAAGGGCTACGTGGAGATCGACCACGCCCTGCCCTTCTCCCGGACCTGGGACGAC
TCCTTCAACAACAAGGTGCTGGTGCTGGGCTCCGAGAACCAGAACAAAGGGCAACCAGA
CCCCCTACGAGTACTTCAACGGCAAGGACAACCTCCCGGGAGTGGCAGGAGTTCAAGGC
CCGGGTGGAGACCTCCCGGTTCCCCCGGTCCAAGAAGCAGCGGATCCTGCTGCAGAAG
TTCGACGAGGACGGCTTCAAGGAGTGCAACCTGAACGACACCCGGTACGTGAACCGGT
TCCTGTGCCAGTTCGTGGCCGACCACATCCTGCTGACCGGCAAGGGCAAGCGGCGGGT
GTTTCGCTCCAACGGCCAGATACCAACCTGCTGCGGGGCTTCTGGGGCCTGCGGAAG
GTGCGGGCCGAGAACGACCGGCACCACGCCCTGGACGCCGTGGTGGTGGCCTGCTCCA
CCGTGGCCATGCAGCAGAAGATACCCGGTTCGTGCGGTACAAGGAGATGAACGCCTT
CGACGGCAAGACCATCGACAAGGAGACCGGCAAGGTGCTGCACCAGAAGACCCACTT
CCCCAGCCCTGGGAGTTCTTCGCCCAGGAGGTGATGATCCGGGTGTTGGCAAGCCC
GACGGCAAGCCCAGTTTCGAGGAGGCCGACACCCCCGAGAAGCTGCGGACCCCTGCTG
GCCGAGAAGCTGTCCTCCCGCCCCGAGGCCGTGCACGAGTACGTGACCCCCCTGTTTCG
TGTCCTCCGGGCCCCAACCAGGAAGATGTCCGGCGCCCAAGGACACCCTGCGGTCCGC
CAAGCGGTTCGTGAAGCACAACGAGAAGATCTCCGTGAAGCGGGTGTGGCTGACCGAG
ATCAAGCTGGCCGACCTGGAGAACATGGTGAACCTACAAGAACGGCCGGGAGATCGAG
CTGTACGAGGCCCTGAAGGCCCGGTGGAGGCCTACGGCGGCAACGCCAAGCAGGCCT
TCGACCCCAAGGACAACCCCTTCTACAAGAAGGGCGGCCAGCTGGTGAAGGCCGTGCG
GGTGGAGAAGACCCAGGAGTCCGGCGTGCTGCTGAACAAGAAGAAGCCTACACCAT
CGCCGACAACGGCGACATGGTGCGGGTGGACGTGTTCTGCAAGGTGGACAAGAAGGG
CAAGAACCAGTACTTCATCGTGCCCATCTACGCCTGGCAGGTGGCCGAGAACATCCTG
CCCGACATCGACTGCAAGGGCTACCGGATCGACGACTCCTACACCTTCTGCTTCTCCCT
GCACAAGTACGACCTGATCGCCTTCCAGAAGGACGAGAAGTCCAAGGTGGAGTTCGCC
TACTACATCAACTGCGACTCCTCCAACGGCCGGTTCTACCTGGCCTGGCACGACAAGG
GCTCCAAGGAGCAGCAGTTCCGGATCTCCACCCAGAACCTGGTGCTGATCCAGAAGTA
CCAGGTGAACGAGCTGGGCAAGGAGATCCGGCCCTGCCGGCTGAAGAAGCGGCCCCC
CGTGCGGTAG (SEQ ID NO: 221)

[000126] Exemplary mRNA for Nme2Cas9 nickase

GGGAAGCUCAGAAUAAACGCUCAACUUUGGCCGGAUCUGCCACCAUGGACGGCUCC
GGCGGCGGCUCCCCAAGAAGAAGCGGAAGGUGGAGGACAAGCGGCCCCGCCACC
AAGAAGGCCGGCCAGGCCAAGAAGAAGAAGGGCGGCUCCGGCGGGCGGCCGCCUUC
AAGCCCAACCCCAUCAACUACAUCCUGGGCCUGGACAUCGGCAUCGCCUCCGUGGGC
UGGGCCAUGGUGGAGAUCGACGAGGAGGAGAACCCCAUCCGGCUGAUCGACCUGGG
CGUGCGGGUGUUCGAGCGGGCCGAGGUGCCCAAGACCGGCGACUCCCUGGCCAUGGC
CCGGCGGCUUGCCCCGUCCGUGCGGCGGCUGACCCGGCGGCGGGCCACCGGCUGCU

GCGGGCCCGCGGCUGCUGAAGCGGGAGGGCGUGCUGCAGGCCGCCGACUUCGACGA
GAACGGCCUGAUC AAGUCCCUGCCCCAACCCCCUGGCAGCUGCGGGCCGCCGCCCU
GGACCGGAAGCUGACCCCCUGGAGUGGUCCGCCGUGCUGCUGCACCUGAUC AAGCA
CCGGGGCUACCUGUCCCAGCGGAAGAACGAGGGCGAGACCGCCGACAAGGAGCUGG
GCGCCCUGCUGAAGGGCGUGGCCAACAAACGCCACGCCUGCAGACCGGCGACUUC
GGACCCCCGCCGAGCUGGCCUGAACAAGUUCGAGAAGGAGUCCGGCCACAUCGGA
ACCAGCGGGGCGACUACUCCCACACCUUCUCCCCGGAAGGACCUGCAGGCCGAGCUGA
UCCUGCUGUUCGAGAAGCAGAAGGAGUUCGGCAACCCCCACGUGUCCGGCGGCCUGA
AGGAGGGCAUCGAGACCCUGCUGAUGACCCAGCGGCCCGCCCUGUCCGGCGACGCCG
UGCAGAAGAUGCUGGGCCACUGCACCUUCGAGCCCCGCCGAGCCCAAGGCCGCCAAGA
ACACCUACACCGCCGAGCGGUUCAUCUGGCUGACCAAGCUGAACAACCUUGC GAUCC
UGGAGCAGGGCUCCGAGCGGCCCCUGACCGACACCGAGCGGGCCACCCUGAUGGACG
AGCCCUACCGGAAGUCCAAGCUGACCUACGCCCAGGCCCGGAAGCUGCUGGGCCUGG
AGGACACCGCCUUCUUCAAGGGCCUGCGGUACGGCAAGGACAACGCCGAGGCCUCCA
CCCUGAUGGAGAUGAAGGCCUACCACGCCAUCUCCCGGGGCCUGGAGAAGGAGGGCC
UGAAGGACAAGAAGUCCCCCUGAACCUGUCCUCCGAGCUGCAGGACGAGAUCGGCA
CCGCCUUCUCCUGUUCAAGACCGACGAGGACAUCACCGGCCGGCUGAAGGACCGGG
UGCAGCCCGAGAUCUGGAGGCCUGCUGAAGCACAUCUCCUUCGACAAGUUCGUGC
AGAUCUCCUGAAGGCCUGCGGGCGGAUCGUGCCCCUGAUGGAGCAGGGCAAGCGG
UACGACGAGGCCUGCGCCGAGAUCUACGGCGACCACUACGGCAAGAAGAACACCGAG
GAGAAGAUCUACCUGCCCCCAUCCCCGCCGACGAGAUCCGGAACCCCGUGGUGCUG
CGGGCCCUGUCCAGGCCCGGAAGGUGAUAACGGCGUGGUGCGGCGGUACGGCUCC
CCCCGCCGGAUCCACAUCGAGACCGCCCGGGAGGUGGGCAAGUCCUUCAAGGACCGG
AAGGAGAUCGAGAAGCGGCAGGAGGAGAACCGGAAGGACCGGGAGAAGGCCGCCG
CAAGUUCGGGAGUACUCCCCAACUUCGUGGGCGAGCCCAAGUCCAAGGACAUCCU
GAAGCUGCGGCUGUACGAGCAGCAGCACGGCAAGUGCCUGUACUCCGGCAAGGAGA
UCAACCUGGUGCGGCUGAACGAGAAGGGCUACGUGGAGAUCGACCACGCCUGCCCU
UCUCCCGGACCUGGGACGACUCCUUAACAACAAGGUGCUGGUGCUGGGCUCCGAGA
ACCAGAACAAGGGCAACCAGACCCCCUACGAGUACUUAACGGCAAGGACAACUCC
GGGAGUGGCAGGAGUUAAGGCCCGGGUGGAGACCUCCCGGUUCCCCCGGUCCAAG
AAGCAGCGGAUCCUGCUGCAGAAGUUCGACGAGGACGGCUUAAGGAGUGCAACCU
GAACGACACCCGGUACGUGAACCGGUUCCUGUGCCAGUUCGUGGCCGACCACAUCCU
GCUGACCGGCAAGGGCAAGCGGCGGGUGUUCGCCUCCAACGGCCAGAUCACCAACCU
GCUGCGGGGCUUCUGGGGCCUGCGGAAGGUGCGGGCCGAGAACGACCGGCACCACGC
CCUGGACGCCGUGGUGGUGGCCUGCUCCACCGUGGCCAUGCAGCAGAAGAUCACCCG
GUUCGUGCGGUACAAGGAGAUGAACGCCUUCGACGGCAAGACCAUCGACAAGGAGA
CCGGCAAGGUGCUGCACCAGAAGACCCACUCCCCCAGCCUGGGAGUUCUUCGCCC

AGGAGGUGAUGAUCCGGGUGUUCGGCAAGCCCGACGGCAAGCCCGAGUUCGAGGAG
 GCCGACACCCCCGAGAAGCUGCGGACCCUGCUGGGCCGAGAAGCUGUCCUCCCGGCC
 GAGGCCGUGCACGAGUACGUGACCCCCCUGUUCGUGUCCCGGGCCCCCAACCGGAAG
 AUGUCCGGCGCCACAAGGACACCCUGCGGUCCGCCAAGCGGUUCGUGAAGCACAAC
 GAGAAGAUCUCCGUGAAGCGGGUGUGGCUGACCGAGAUAAGCUGGCCGACCUGGA
 GAACAUGGUGAACUACAAGAACGGCCGGGAGAUCGAGCUGUACGAGGCCUGAAGG
 CCCGGCUGGAGGCCUACGGCGGCAACGCCAAGCAGGCCUUCGACCCCAAGGACAACC
 CCUUCUACAAGAAGGGCGGCCAGCUGGUGAAGGCCGUGCGGGUGGAGAAGACCCAG
 GAGUCCGGCGUGCUGCUGAACAAGAAGAACGCCUACACCAUCGCCGACAACGGCGAC
 AUGGUGCGGGUGGACGUGUUCUGCAAGGUGGACAAGAAGGGCAAGAACCAGUACUU
 CAUCGUGCCCAUCUACGCCUGGCAGGUGGCCGAGAACAUCUGCCCCGACAUCGACUG
 CAAGGGCUACCGGAUCGACGACUCCUACACCUUCUGCUUCUCCUGCACAAGUACGA
 CCUGAUCGCCUUCAGAAAGGACGAGAAGUCCAAGGUGGAGUUCGCCUACUACAUC
 ACUGCGACUCCUCCAACGGCCGGUUCUACCUGGCCUGGCACGACAAGGGCUCCAAGG
 AGCAGCAGUUCGGGAUCUCCACCCAGAACCUGGUGCUGAUCCAGAAGUACCAGGUG
 AACGAGCUGGGCAAGGAGAUCCGGCCUGCCGGCUGAAGAAGCGGCCCCCCCGUGCGG
 UAGCUAGCACCAGCCUCAAGAACACCCGAAUGGAGUCUCUAAGCUACAUAUACCA
 ACUUACACUUUACAAAAUGUUGUCCCCCAAAUGUAGCCAUUCGUAUCUGCUCCUA
 AUAAAAAGAAAGUUUCUUCACAUUCUCUCGAGAAAAAAAAAAAAAUGGAAAAAAAAAA
 AAACGGAAAAAAAAAAAAAGGUAAAAAAAAAAAAAUAAAAAAAAAAAAACAUAAAA
 AAAAAAACGAAAAAAAAAAAAACGUAAAAAAAAAAAAACUAAAAAAAAAAAAAGUA
 AAAAAAAAACCUAAAAAAAAAAAAUGUAAAAAAAAAAAAAGGGAAAAAAAAAAAA
 ACGCAAAAAAAAAAAAAACAAAAAAAAAAAAAUGCAAAAAAAAAAAAAUCGAAAAAAA
 AAAAAUCUAAAAAAAAAAAAACGAAAAAAAAAAAAACCCAAAAAAAAAAAAAGACAAAA
 AAAAAAAUAGAAAAAAAAAAAAAGUUAAAAAAAAAAAAACUGAAAAAAAAAAAAUUU
 AAAAAAAAAAAAUUCUAG (SEQ ID NO: 222)

[000127] Exemplary amino acid sequence for Nme2Cas9

MDGSGGGSPKKKRKVEDKRPAATKKAGQAKKKKGGSGGGAAFKPNPINYLGLDIGIASV
 GWAMVEIDEEENPIRLIDLGVRFERAEVPKTGDSLAMARRLARSVRRLTRRRAHRLRLAR
 RLLKREGVLQAADFDEGLIKSLPNTPWQLRAAALDRKLTPLEWSAVLLHLIKHRGYLSQ
 RKNEGETADKELGALLKGVANNAHALQTGDFRTPAELALNKFESGHIRNQRGDYSHTF
 SRKDLQAEILLFEKQKEFGNPHVSGGLKEGIETLLMTQRPALSGDAVQKMLGHCTFEPAE
 PKAAKNTYTAERFIWLTKLNNLRILEQGSERPLTDTERATLMDEPYRKSKLTYAQARKLLG
 LEDTAFFKGLRYGKDNEASTLMEMKAYHAISRALEKEGLKDKKSPLNLSSELQDEIGTAF
 SLFKTDEDITGRLKDRVQPEILEALLKHISFDKFVQISLKALRRIVPLMEQGKRYDEACAEIY
 GDHYGKKNTEEKIYLPPIPADEIRNPVVLRLALSQARKVINGVVRRYGSPARIHIETAREVGK

SFKDRKEIEKRQEENRKDREKAAAKFREYFPNFVGEPKSKDILKLRLYEQQHGKCLYSGKE
INLVRLENEKGYVEIDHALPFSRTWDDSFNNKVLVLGSENQNKGNQTPYEYFNGKDNSREW
QEFKARVETSRFPRSKKQRILLQKFDEDDGFKECNLNDTRYVNRFLCQFVADHILLTGKGKR
RVFASNGQITNLLRGFWGLRKVRAENDRHHALDAVVVACSTVAMQOKITRFVRYKEMNA
FDGKTIDKETGKVLHQKTHFPQPWEFFAQEVMIRVFGKPDGKPEFEEADTPEKLRTLLAEK
LSSRPEAVHEYVTPLFVSRAPNRKMSGAHKDTLRSKRFBVKHNEKISVKRVWLTEIKLADL
ENMVNYKNGREIELYEALKARLEAYGGNAKQAFDPKDNPFYKKGGLVKAVRVEKTQES
GVLLNKKNAYTIADNGDMVRVDVFCVKDKKGNQYFIVPIYAWQVAENILPDIDCKGYRI
DDSYTFCFSLHKYDLIAFQKDEKSKVEFAYYINCDSSNGRFYLAWHDKGSKEQQFRISTQN
LVLIQKYQVNELGKEIRPCRLKKRPPVR (SEQ ID NO: 223)

[000128] Exemplary mRNA encoding UGI

GGGAGACCCAAGCUGGCUAGCUCCCGCAGUCGGCGUCCAGCGGCUCUGCUUGUUCGU
GUGUGUGUCGUUGCAGGCCUUAUUCGGAUCCGCCACCAUGGGACCGAAGAAGAAGA
GAAAGGUCGGAGGAGGAAGCACAAACCUGUCGGACAUCAUCGAAAAGGAAACAGGA
AAGCAGCUGGUCAUCCAGGAAUCGAUCCUGAUGCUGCCGGAAGAAGUCGAAGAAGU
CAUCGGAAACAAGCCGGAUUCGGACAUCUGGUCCACACAGCAUACGACGAAUCGAC
AGACGAAAACGUCAUGCUGCUGACAUCGGACGCACCGGAUACAAGCCGUGGGCAC
UGGUCAUCCAGGACUCGAACGGAGAAAACAAGAUCAAGAUGCUGUGAUAGUCUAGA
CAUCACAUUUAAAAGCAUCUCAGCCUACCAUGAGAAUAAGAGAAAGAAAAUGAAGA
UCAAUAGCUUAUUCUUCUUUUUCUUUUUCGUUGGUGUAAAAGCCAACACCCUGUC
UAAAAACAUAUUUUUCUUUUUAUCAUUUUUGCCUCUUUUCUCUGUCUCAAUUAAU
AAAAAAUGGAAAGAACCUCGAGUCUAG (SEQ ID NO: 224)

[000129] Open reading frame for UGI

AUGGGACCGAAGAAGAAGAGAAAGGUCGGAGGAGGAAGCACAAACCUGUCGGACA
CAUCGAAAAGGAAACAGGAAAGCAGCUGGUCAUCCAGGAAUCGAUCCUGAUGCUGC
CGGAAGAAGUCGAAGAAGUCAUCGAAACAAGCCGGAUUCGGACAUCUGGUCCAC
ACAGCAUACGACGAAUCGACAGACGAAAACGUCAUGCUGCUGACAUCGGACGCACC
GGAAUACAAGCCGUGGGCACUGGUCAUCCAGGACUCGAACGGAGAAAACAAGAUCA
AGAUGCUGUGA (SEQ ID NO: 225)

[000130] Exemplary amino acid sequence for UGI

MTNLSDIIEKETGKQLVIQESILMLPEEVVEEVIGNKPESDILVHTAYDESTDENVMMLTSDAP
EYKPWALVIQDSNGENKIKMLSGGSKRTADGSEFESPKKKRKVE (SEQ ID NO: 226)

[000131] Exemplary amino acid sequence for UGI

TNLSDIIEKETGKQLVIQESILMLPEEVEEVIGNKPESDILVHTAYDESTDENVMLLTSDAPE
YKPWALVIQDSNGENKIKML (SEQ ID NO: 227)

[000132] As used herein, “ribonucleoprotein” (RNP) or “RNP complex” refers to a guide RNA together with an RNA-guided DNA binding agent, such as a Cas nuclease, *e.g.*, a Cas cleavase, Cas nickase, or dCas DNA binding agent (*e.g.*, Cas9). In some embodiments, the guide RNA guides the RNA-guided DNA-binding agent such as Cas9 to a target sequence, and the guide RNA hybridizes with and the agent binds to the target sequence; in cases where the agent is a cleavase or nickase, binding can be followed by double-stranded DNA cleavage or single-stranded DNA cleavage.

[000133] As used herein, a first sequence is considered to “comprise a sequence with at least X% identity to” a second sequence if an alignment of the first sequence to the second sequence shows that X% or more of the positions of the second sequence in its entirety are matched by the first sequence. For example, the sequence AAGA comprises a sequence with 100% identity to the sequence AAG because an alignment would give 100% identity in that there are matches to all three positions of the second sequence. The differences between RNA and DNA (generally the exchange of uridine for thymidine or vice versa) and the presence of nucleoside analogs such as modified uridines do not contribute to differences in identity or complementarity among polynucleotides as long as the relevant nucleotides (such as thymidine, uridine, or modified uridine) have the same complement (*e.g.*, adenosine for all of thymidine, uridine, or modified uridine; another example is cytosine and 5-methylcytosine, both of which have guanosine or modified guanosine as a complement). Thus, for example, the sequence 5'-AXG where X is any modified uridine, such as pseudouridine, N1-methyl pseudouridine, or 5-methoxyuridine, is considered 100% identical to AUG in that both are perfectly complementary to the same sequence (5'-CAU). Exemplary alignment algorithms are the Smith-Waterman and Needleman-Wunsch algorithms, which are well-known in the art. One skilled in the art will understand what choice of algorithm and parameter settings are appropriate for a given pair of sequences to be aligned; for sequences of generally similar length and expected identity >50% for amino acids or >75% for nucleotides, the Needleman-Wunsch algorithm with default settings of the Needleman-Wunsch algorithm interface provided by the EBI at the www.ebi.ac.uk web server is generally appropriate.

[000134] As used herein, a first sequence is considered to be “X% complementary to” a second sequence if X% of the bases of the first sequence base pairs with the second sequence. For example, a first sequence 5'AAGA3' is 100% complementary to a second sequence 3'TTCT5', and the second sequence is 100% complementary to the first sequence. In some embodiments, a first sequence 5'AAGA3' is 100% complementary to a second sequence 3'TTCTGTGA5', whereas the second sequence is 50% complementary to the first sequence.

[000135] As used herein, “mRNA” is used herein to refer to a polynucleotide that is entirely or predominantly RNA or modified RNA and comprises an open reading frame that can be translated into a polypeptide (*i.e.*, can serve as a substrate for translation by a ribosome and amino-acylated tRNAs). mRNA can comprise a phosphate-sugar backbone including ribose residues or analogs thereof, *e.g.*, 2'-methoxy ribose residues. In some embodiments, the sugars of an mRNA phosphate-sugar backbone consist essentially of ribose residues, 2'-methoxy ribose residues, or a combination thereof.

[000136] As used herein, “indel” refers to insertion/deletion mutations consisting of a number of nucleotides that are either inserted or deleted at the site of a double-stranded break (DSB) in a target nucleic acid. As used herein, when indel formation that results in an insertion, the insertion is a random insertion at the site of a double stranded break and is not directed by or based on a template sequence.

[000137] As used herein, a “target sequence” refers to a sequence of nucleic acid in a target gene that has complementarity to the guide sequence of the gRNA. The interaction of the target sequence and the guide sequence directs an RNA-guided DNA-binding agent to bind, and potentially nick or cleave (depending on the activity of the agent), within the target sequence.

[000138] As used herein, “polypeptide” refers to a wild-type or variant protein (*e.g.*, mutant, fragment, fusion, or combinations thereof). A variant polypeptide may possess at least or about 5%, 10%, 15%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% functional activity of the wild-type polypeptide. In some embodiments, the variant is at least 70%, 75%, 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the sequence of the wild-type polypeptide. In some embodiments, a variant polypeptide may be a hyperactive variant. In certain instances, the variant possesses between about 80% and about 120%, 140%, 160%, 180%, 200%, 300%, 400%, 500%, or more of a functional activity of the wild-type polypeptide. As used herein, a “heterologous gene” refers to a gene that has been introduced as an exogenous source within a cell (*e.g.*, inserted at a genomic locus such as a safe harbor locus including a TCR gene locus). That is, the introduced gene is heterologous with respect to its insertion site. A polypeptide expressed from such heterologous gene is referred to as a “heterologous polypeptide.” The heterologous gene can be naturally-occurring or engineered, and can be wild-type or a variant. The heterologous gene may include nucleotide sequences other than the sequence that encodes the heterologous polypeptide (*e.g.*, an internal ribosomal entry site). The heterologous gene can be a gene that occurs naturally in the genome, as a wild-type or a variant (*e.g.*, mutant). For example, although the cell contains the gene of interest (as a wild-type or as a variant), the same gene or variant thereof can be introduced as an exogenous source for, *e.g.*, expression at a locus that is highly expressed. The heterologous gene can also be a gene that is not naturally occurring in the genome, or that expresses a heterologous polypeptide that does not

naturally occur in the genome. “Heterologous gene”, “exogenous gene”, and “transgene” are used interchangeably. In some embodiments, the heterologous gene or transgene includes an exogenous nucleic acid sequence, *e.g.*, a nucleic acid sequence is not endogenous to the recipient cell. In some embodiments, the heterologous gene or transgene includes an exogenous nucleic acid sequence, *e.g.*, a nucleic acid sequence that does not naturally occur in the recipient cell. For example, a heterologous gene a heterologous gene may be heterologous with respect to its insertion site and with respect to its recipient cell.

[000139] A “safe harbor” locus is a locus within the genome wherein a gene may be inserted without significant deleterious effects on the cell. Non-limiting examples of safe harbor loci that are targeted by nuclease(s) for use herein include AAVS1 (PPP1 R12C), TCR, B2M, and any of the loci targeted for knockdown described herein, *e.g.*, TNFA, IFNG, IL17A, and IL6 genomic loci. In some embodiments, insertions at a locus or loci targeted for knockdown such as a TRC gene, *e.g.*, TRAC gene, is advantageous for allogenic cells. Other suitable safe harbor loci are known in the art.

II. Compositions

A. Targeting Receptor

[000140] In some embodiments, the engineered T cells comprising a heterologous sequence(s) encoding dmTGFB1 molecule and a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2, further comprise insertion into the cell of heterologous sequence(s) encoding a targeting receptor, optionally, further comprising a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TNFA, a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding an IFNG, and/or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule, each under control of a promoter sequence. The sequence(s) encoding the targeting receptor is under the control of a promoter sequence, *e.g.*, an endogenous promoter or a heterologous promoter. In certain embodiments, the regulatory T cell promoting molecule comprises IL10 or CTLA4. In certain embodiments, the regulatory T cell promoting molecule comprises IL10 and CTLA4.

[000141] In some embodiments, the targeting receptor is a chimeric antigen receptor (CAR), a T-cell receptor (TCR), or a receptor for a cell surface molecule operably linked through at least a transmembrane domain in an internal signaling domain capable of activating a T cell upon binding of the extracellular receptor portion. In some embodiments, the targeting receptor may be a receptor present on the surface of a cell, *e.g.*, a T cell, to permit binding of the cell to a target site, *e.g.*, a specific cell or tissue in an organism. The targeting receptor need not be an antigen receptor, *e.g.*, the targeting receptor may be an RGD peptide that is capable of targeting an integrin. In some embodiments, the targeting receptor targets a molecule selected from the group consisting of

MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), and MHC class I HLA-A (HLA-A*02).

[000142] In some embodiments, the engineered T cells comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG, insertion of sequences encoding dmTGFB1, IL10, CTLA4, and a targeting receptor, e.g., a CAR, e.g., a CAR targeting MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02).

[000143] In some embodiments, the sequence(s) encoding the targeting receptor is incorporated into an expression construct. In some embodiments, the expression construct comprising the sequence(s) encoding the targeting receptor further comprises sequence(s) encoding a regulatory T cell promoting molecule, e.g., the sequence(s) encoding the targeting receptor and the sequence(s) encoding the regulatory T cell promoting molecule are incorporated into the same expression construct. In some embodiments, the expression construct comprising the sequence(s) encoding the targeting receptor does not further comprise sequence(s) encoding a regulatory T cell promoting molecule, e.g., the sequence(s) encoding the regulatory T cell promoting molecule are incorporated into a separate expression construct. In some embodiments, the expression construct comprising the sequence(s) encoding the targeting receptor is an episomal expression construct. In some embodiments, the sequence(s) encoding the targeting receptor is inserted into the genome, e.g., a targeted or an untargeted insertion.

[000144] In some embodiments, the sequence(s) encoding the targeting receptor may be inserted into a site selected from a TCR gene locus, e.g., TRAC locus, a TNF gene locus, an IFNG gene locus, IL17A locus, a IL6 locus, an IL2 locus, or an adeno-associated virus integration site 1 (AAVS1) locus.

[000145] In some embodiments, the engineered T cells comprise an insertion of sequence(s) encoding a targeting receptor by gene editing, e.g., as assessed by sequencing, e.g., NGS.

[000146] In some embodiments, a population of T cells comprises T cells that are engineered to comprise insertion of sequences encoding a dmTGFB1, a modification, e.g., knockdown, of an

endogenous nucleic acid sequence encoding a TGFBR2, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion of sequences encoding a regulatory T cell promoting molecule, and insertion of sequence(s) encoding a targeting receptor, e.g., a CAR. In some embodiments, at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the T cells in the population of T cells are engineered to comprise insertion of sequence(s) encoding the targeting receptor, e.g., as assessed by sequencing, e.g., NGS. It is understood that a T cell population can be enriched for a population of cells having a targeting receptor using selection methods known in the art.

[000147] In some embodiments, disclosed herein are T cells engineered by introducing or inserting a targeting receptor, e.g., a CAR, nucleic acid within a T cell, e.g., within a genomic locus of a T cell or a population of T cells using a guide RNA with an RNA-guided DNA binding agent, and a construct (*e.g.*, donor construct or template) comprising a targeting receptor, e.g., a CAR, nucleic acid, e.g., to make an engineered T cell. In some embodiments, disclosed herein are T cells engineered by expressing a targeting receptor, e.g., a CAR, from the genomic locus of a T cell or a population of T cells, *e.g.*, using a guide RNA with an RNA-guided DNA-binding agent and a construct (*e.g.*, donor) comprising a targeting receptor, e.g., a CAR, nucleic acid. In some embodiments, disclosed herein are T cells engineered by inducing a break (*e.g.*, double-stranded break (DSB) or single-stranded break (nick)) within the genome of a T cell or a population of T cells for inserting the targeting receptor, e.g., a CAR, *e.g.*, using a guide RNA with an RNA-guided DNA-binding agent (*e.g.*, a CRISPR/Cas system). Cells and cell populations made by the methods are also provided.

[000148] In some embodiments, the targeting receptor, e.g., a CAR, is capable of conferring target specificity to the engineered T cell comprising the targeting receptor, e.g., a CAR, e.g., to particular cells, tissues, or organs.

[000149] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to the gastrointestinal system, e.g., the targeting receptor is a CAR targeting MAdCAM-1, e.g., for suppressing immune responses in disorders such as inflammatory bowel disease, ulcerative colitis, or Crohn's disease.

[000150] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to an inflammatory tissue, e.g., the targeting receptor is a CAR targeting TNFA, e.g., for suppressing immune responses in disorders such as inflammatory bowel disease, ulcerative colitis, or Crohn's disease.

[000151] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to an inflammatory tissue and/or activated immune cells, e.g., the targeting

receptor is a CAR targeting CD70, e.g., for suppressing immune responses in disorders such as inflammatory bowel disease, ulcerative colitis, or Crohn's disease.

[000152] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to endothelial cells, e.g., the targeting receptor is a CAR targeting CEACAM6, e.g., for suppressing immune response(s), including inflammation, in disorders such as Crohn's disease.

[000153] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to tissues comprising endothelial cells, e.g., the targeting receptor is a CAR targeting VCAM-1, e.g., for suppressing immune responses in disorders such as Crohn's disease and multiple sclerosis.

[000154] In some embodiments, the CAR is capable of targeting engineered T cells to synovial tissue, e.g., the targeting receptor is a CAR targeting citrullinated vimentin e.g., for suppressing immune responses in disorders such as rheumatoid arthritis.

[000155] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to a neurological tissue, e.g., the targeting receptor is a CAR targeting MBP, MOG, or PLP1, e.g., for suppressing immune responses in disorders such as multiple sclerosis.

[000156] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to B cells, e.g., the targeting receptor is a CAR targeting CD19, e.g., for suppressing immune responses in disorders such as multiple sclerosis and systemic lupus erythematosus.

[000157] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to B cells, e.g., the targeting receptor is a CAR targeting CD20, e.g., for suppressing immune responses in disorders such as multiple sclerosis and systemic lupus erythematosus.

[000158] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to immune cells expressing TNFSF7 including but not limited to activated T lymphocytes, B lymphocytes, dendritic cells, and/or NK cells, e.g., the CAR targets TNFSF7, e.g., for suppressing immune responses in disorders such as Crohn's disease, ulcerative colitis, systemic lupus erythematosus, or multiple sclerosis.

[000159] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to tissues comprising mature B lymphocytes, e.g., the targeting receptor is a CAR targeting TNFRSF17, e.g., for suppressing immune responses in disorders such as systemic lupus erythematosus.

[000160] In some embodiments, the targeting receptor, e.g., a CAR, targets SCL2A2. In some embodiments, the targeting receptor, e.g., a CAR, targets DPP6. In some embodiments, the targeting receptor, e.g., a CAR, targets GAD2. In some embodiments, the targeting receptor, e.g., a CAR, targets DSG3. In some embodiments, the targeting receptor, e.g., a CAR, targets MHC class I HLA-A (HLA-A*02).

[000161] Additional CAR targets, e.g., inflammatory antigens, are known in the art. *See, e.g.,* WO2020092057A1, the contents of which are incorporated herein in their entirety. In some embodiments, the insertion can be assessed by detecting the amount of protein or mRNA in an engineered T cell, population of engineered T cells, tissue, body fluid of interest, or tissue culture media comprising the engineered T cells. In some embodiments, the insertion by gene editing can be assessed by sequence, e.g., next generation sequencing (NGS). Assays for protein and mRNA expression of the targeting receptor, e.g., a CAR, are described herein and known in the art.

[000162] In embodiments disclosed herein in which the target of the targeting receptor, e.g., a CAR, is also present on the surface of a cell, e.g. an immune cell or a T-cell, the engineered T cells or population of T cells may further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding the target.

[000163] In certain embodiments, the engineered T cells or population of T cells comprise two or more heterologous coding sequences, e.g., a dmTGFB1, a regulatory T cell promoting molecule, and a targeting receptor; under the control of a promoter. In certain embodiments, each heterologous coding sequence is under control of a separate promoter. In certain embodiments, two heterologous coding sequences are under control of the same promoter. In certain embodiments, two or more heterologous coding sequences are under control of the same promoter. In certain embodiments, when the engineered T cells or population of T cells comprise three or more heterologous coding sequences, e.g., a dmTGFB1, a regulatory T cell promoting molecule(s), and a targeting receptor; under the control of a promoter, each heterologous sequence is each independently under control of a separate promoter or under control of a promoter that controls expression of more than one heterologous coding sequence.

[000164] In some embodiments, the engineered T cells or population of T cells do not include a heterologous targeting receptor.

B. Engineered T Cells

[000165] Provided herein are T cells and populations of T cells engineered to comprise a modification comprising insertion into the cell of heterologous sequence encoding a dmTGFB1 that is under the control of a promoter and a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an TGFBR2.

[000166] In some embodiments, the T cells comprising heterologous sequence encoding a dmTGFB1 under the control of a promoter and a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an TGFBR2 are further engineered to comprise a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA, and/or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under the control of a promoter sequence, as well as compositions and uses thereof. In some embodiments, the regulatory T cell promoting molecule is selected from IL10, CTLA4, TIGIT, IDO1, ENTPD1, NT5E, IL22, AREG, IL35, GARP, CD274, FOXP3, IKZF2, EOS, IRF4, LEF1, BACH2, and IL2RA.

[000167] In some embodiments, the T cells or population of T cells comprising heterologous sequence encoding a dmTGFB1 molecule under the control of a promoter is further engineered to comprise a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an TGFBR2, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA, and insertion into the cell of heterologous sequences encoding two or more regulatory T cell promoting molecules each under the control of a promoter sequence. For example, the engineered T cell comprises a first heterologous sequence encoding a first regulatory T cell promoting molecule that is under the control of a first promoter and a second heterologous sequence encoding a second regulatory T cell promoting molecule that is under the control of a second promoter. The first promoter and the second promoter may be the same promoter or different promoters. In certain embodiments, the heterologous sequence encoding the dmTGFB1 molecule is under the control of promoter sequence that controls expression of a regulatory T cell promoting molecule. In certain embodiments, the heterologous sequence encoding the dmTGFB1 molecule is not under the control of promoter sequence that controls expression of a regulatory T cell promoting molecule. In certain embodiments, the heterologous sequence encoding the dmTGFB1 molecule is under the control of promoter sequence that controls expression of a targeting receptor. In certain embodiments, the heterologous sequence encoding the dmTGFB1 molecule is not under the control of promoter sequence that controls expression of a targeting receptor.

[000168] In some embodiments, the T cells or population of T cells comprising heterologous sequence encoding a dmTGFB1 under the control of a promoter and a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an TGFBR2 is further engineered to comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion into the cell of heterologous sequence(s) encoding IL10 that is under the control of

a promoter; and insertion into the cell of heterologous sequence(s) encoding CTLA4 that is under the control of a promoter.

[000169] In some embodiments, the T cells or population of T cells comprising heterologous sequence encoding a dmTGFB1 under the control of a promoter and a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an TGFBR2 is further engineered to comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion into the cell of heterologous sequence(s) encoding IL10 that is under the control of a promoter; and insertion into the cell of heterologous sequence(s) encoding CTLA4 that is under the control of a promoter, and exhibits at least one suppressive activity of a naturally occurring regulatory T cell (nTreg), e.g., suppression of an immune response or biomarker in an in vitro or in vivo assay, e.g., an animal model of GvHD.

[000170] In some embodiments, the heterologous sequence(s) encoding dmTGFB1 or one or more regulatory T cell promoting molecule is incorporated into an expression construct. In some embodiments, heterologous sequences encoding two or more molecules may be incorporated into two or more separate expression constructs. For example, a first heterologous sequence encoding a dmTGFB1 is provided in a first expression construct, a second heterologous sequence encoding a regulatory T cell promoting molecule is provided in a second, separate expression construct, and a third heterologous sequence encoding a regulatory T cell promoting molecule is provided in a third, separate expression construct. In an alternative example, a first heterologous sequence encoding a dmTGFB1 is provided in the same expression construct, as a second heterologous sequence encoding a regulatory T cell promoting molecule and a third heterologous sequence encoding a regulatory T cell promoting molecule. In some embodiments, one or more expression construct is an episomal expression construct. In some embodiments, one or more heterologous sequence is inserted into the genome, e.g., a targeted or an untargeted insertion.

[000171] In certain embodiments, modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2 includes modification of TGFBR2. In certain embodiments, modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG includes modification of IFNG. In certain embodiments, modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA includes modification of TNFA.

[000172] In some embodiments, the sequence(s) encoding dmTGFB1 or the regulatory T cell promoting molecule may be inserted into a site selected from a TCR gene locus, e.g., TRAC locus; a TNF gene locus, an IFNG gene locus, a IL17A locus, a IL6 locus, an IL2 locus, or an adeno-associated virus integration site 1 (AAVS1) locus.

[000173] In some embodiments, the engineered T cells or population of T cells engineered to comprise a modification, e.g., an insertion of a sequence encoding a dmTGFB1, e.g., by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion of a sequence encoding a dmTGFB1 molecule.

[000174] In some embodiments, the population of engineered T cells comprise a modification of an insertion of a sequence encoding a dmTGFB1 generated e.g., by gene editing, and e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion of a sequence encoding a dmTGFB1 molecule, further comprises a modification, e.g., knockdown, in a TGFBR2 sequence by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion, deletion, or substitution in the endogenous TGFBR2 sequence. In some embodiments, the expression of TGFBR2 (full-length, wild-type protein or mRNA) is decreased by at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the TGFBR2 gene has not been modified as determined, e.g., by ELISA or flow cytometry. Assays for TGFBR2 protein and mRNA expression, e.g., in the population of T cells, are known in the art.

[000175] In some embodiments, the population of engineered T cells comprise a modification of an insertion of a sequence encoding a dmTGFB1 generated e.g., by gene editing, and e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion of a sequence encoding a dmTGFB1 molecule, further comprises a modification, e.g., knockdown, in a TNFA sequence by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion, deletion, or substitution in the endogenous TNFA sequence. In some embodiments, the expression of TNFA (full-length, wild-type protein or mRNA) is decreased by at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the TNFA gene has not been modified as determined, e.g., by ELISA or flow cytometry. Assays for TNFA protein and mRNA expression, e.g., in the population of T cells, are known in the art. In certain embodiments, knockdown of TNFA results in a TNFA level of 2500 pg/ml or less as determined, for example, using a custom U-PLEX Biomarker kit (Meso Scale Diagnostics, Cat. K15067L-2), according to manufacturer's instructions.

[000176] In some embodiments, the population of engineered T cells comprise a modification, e.g., an insertion of a sequence encoding a dmTGFB1, e.g., by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%,

80%, 85%, 90%, or 95% of cells comprise an insertion of a sequence encoding a dmTGFB1 molecule, further comprises a modification, e.g., knockdown, in an IFNG sequence by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion, deletion, or substitution in the endogenous IFNG sequence. In some embodiments, the expression of IFNG (full-length, wild-type protein or mRNA) is decreased by at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the IFNG gene has not been modified as determined, e.g., by ELISA or flow cytometry. Assays for IFNG protein and mRNA expression, e.g., in the population of T cells, are known in the art. In certain embodiments, knockdown of IFNG results in an IFNG level of 300,000 pg/ml or less as determined, for example, using a custom U-PLEX Biomarker kit (Meso Scale Diagnostics, Cat. K15067L-2), according to manufacturer's instructions.

[000177] In some embodiments, the modification that knocks down expression of a gene, e.g., TGFB2, TNFA, or IFNG, is one or more of an insertion, a deletion, or a substitution.

[000178] In some embodiments, the engineered T cells or population of T cells comprise a modification, e.g., an insertion of a sequence encoding a dmTGFB1, e.g., by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion of a sequence encoding a dmTGFB1 molecule, further comprise an insertion of sequence(s) encoding a regulatory T cell promoting molecule, e.g., by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion of a sequence encoding a regulatory T cell promoting molecule. In some embodiments, the inserted regulatory T cell promoting molecule, e.g., IL10, results in statistically significantly increased expression of protein or mRNA as compared to a suitable control, e.g., wherein the regulatory T cell promoting molecule gene has not been inserted as determined, e.g., by ELISA or flow cytometry. In some embodiments, the engineered T cells comprise an insertion of sequence(s) encoding IL10 by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion or a sequence encoding IL10. In some embodiments, the inserted sequence(s) encoding IL10 results in statistically significantly increased expression of protein or mRNA as compared to a suitable control, e.g., wherein the regulatory T cell promoting molecule. Assays for IL10 protein and mRNA expression, e.g., in the population of T cells, are known in the art, e.g., ELISA and flow cytometry. In certain embodiments, the level of IL10 is at least 300 pg/ml as determined, for example, using a custom U-PLEX Biomarker kit (Meso Scale Diagnostics, Cat. K15067L-2), according to manufacturer's instructions.

[000179] In some embodiments, the engineered T cells or population of T cells comprise a modification, e.g., an insertion of a sequence encoding a dmTGFB1, e.g., by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion of a sequence encoding a dmTGFB1 molecule, further comprise an insertion of sequence(s) encoding CTLA4 e.g., by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion or a sequence encoding CTLA4. In some embodiments, the inserted sequence(s) encoding CTLA4 results in statistically significantly increased expression of protein or mRNA as compared to a suitable control, e.g., wherein the regulatory T cell promoting molecule. Assays for CTLA4 protein and mRNA expression, e.g., in the population of T cells, are described herein and known in the art, e.g., ELISA and flow cytometry.

[000180] In some embodiments, a population of T cells comprises T cells engineered to comprise a modification, e.g., an insertion of a sequence encoding a dmTGFB1, e.g., by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion of a sequence encoding a dmTGFB1 molecule, are further engineered to comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion of sequences encoding a regulatory T cell promoting molecule. In some embodiments, at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (e.g., within the detection limits of the assay used) of the T cells in the population of T cells are engineered to comprise a heterologous regulatory T cell promoting molecule, e.g., as assessed by sequencing, e.g., NGS. In some embodiments, at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the T cells in the population of T cells are engineered to comprise a modification, e.g., knockdown, of sequence(s) encoding TGFB2, e.g., as assessed by sequencing, e.g., NGS. In some embodiments, at least 50%, 55%, 60%, 65%, at least 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the T cells in the population of T cells are engineered to comprise a modification, e.g., knockdown, of sequence(s) encoding TNFA, e.g., as assessed by sequencing, e.g., NGS. In some embodiments, at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the T cells in the population of T cells are engineered to comprise a modification, e.g., knockdown, of sequence(s) encoding IFNG, e.g., as assessed by sequencing, e.g., NGS. In some embodiments, at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the T cells in the population of T cells are engineered to comprise insertion of sequences encoding a regulatory T cell promoting molecule, e.g., as assessed by sequencing, e.g., NGS. In some embodiments, at least

30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the T cells in the population of T cells are engineered to comprise insertion of sequence(s) encoding IL10, e.g., as assessed by sequencing, e.g., NGS. In some embodiments, at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the T cells in the population of T cells are engineered to comprise insertion of sequence(s) encoding CTLA4, e.g., as assessed by sequencing, e.g., NGS.

[000181] In some embodiments, the engineered T cells or population of T cells engineered to comprise a modification, e.g., an insertion of a sequence encoding a dmTGFB1, e.g., by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion of a sequence encoding a dmTGFB1 molecule, further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence, further comprise a modification of an endogenous nucleic acid sequence encoding an interleukin 17A (IL17A), an interleukin 6 (IL6), interleukin 2 (IL2), a perforin 1 (PRF1), a granzyme A (GZMA), a granzyme B (GZMB), natural killer cell granule protein 7 (NKG7), Fas ligand (FasL, NF superfamily, member 6), ryanodine receptor 2 (RYR2), and colony stimulating factor 2 (CSF2) wherein the modification knocks down expression of the IL17A, the IL6, the IL2, the PRF1, the GZMA, the GZMB, the NKG7, the FASL, the RYR2, or the CSF2, respectively the further modification of an endogenous nucleic acid sequence comprises modification of IL17A.

[000182] In some embodiments, the T cells or population of T cells are engineered using a gene editing system, e.g., using an RNA-guided DNA binding agent. In some embodiments, the T cells are engineered using a CRISPR/Cas gene editing system. In some embodiments, the T cells are engineered using a CRISPR/Cas type II gene editing system, e.g., using Cpf1. In some embodiments, the T cells are engineered using a CRISPR/Cas9 gene editing system, e.g., using SpyCas9. Exemplary Cas9 sequences are provided herein.

[000183] In some embodiments, the T cells or population of T cells are engineered using guide RNAs that specifically target sites within the TGFB2, IFNG, and TNFA genes to provide knockdown of the of TGFB2, IFNG and TNFA genes. Exemplary sequences are provided in Tables 1 and 2 for knockdown of IFNG and TNFA, respectively, as are genomic coordinates of the target of each listed guide sequence. Exemplary sequences are provided in **Table 3** for knockdown of TGFB2 as are genomic coordinates of the target of each listed guide sequence.

[000184] In some embodiments, the engineered T cells or population of T cells comprise TGFBR2, IFNG, and TNFA genes that are knocked down using a guide RNA disclosed herein with an RNA-guided DNA binding agent. In some embodiments, disclosed herein are T cells engineered by inducing a break (*e.g.*, double-stranded break (DSB) or single-stranded break (nick)) within the TGFBR2, IFNG, and TNFA genes of a T cell, *e.g.*, using a guide RNA disclosed herein with an RNA-guided DNA-binding agent (*e.g.*, a CRISPR/Cas system). The methods may be used in vitro or ex vivo, *e.g.*, in the manufacture of cell products for suppressing immune response(s), including inflammation and autoimmunity. In some embodiments, the guide RNAs disclosed herein mediate a target-specific cutting by an RNA-guided DNA-binding agent (*e.g.*, Cas nuclease) at a site described herein within a TGFBR2 gene. In some embodiments, the guide RNAs disclosed herein mediate a target-specific cutting by an RNA-guided DNA-binding agent (*e.g.*, Cas nuclease) at a site described herein within an IFNG gene. In some embodiments, the guide RNAs disclosed herein mediate a target-specific cutting by an RNA-guided DNA-binding agent (*e.g.*, Cas nuclease) at a site described herein within a TNFA gene. It will be appreciated that, in some embodiments, the guide RNAs comprise guide sequences that bind to, or are capable of binding to, said regions.

[000185] Engineered T cells or population of T cells comprising a genetic modification at genomic coordinates chosen from those listed in Table 1 are provided, *e.g.*, cells comprising an indel or substitution mutation within any of the listed genomic ranges within IFNG. Engineered T cells comprising a genetic modification at genomic coordinates chosen from those listed in Table 2 are also provided, *e.g.*, cells comprising an indel or substitution mutation within any of the listed genomic ranges within TNFA. In some embodiments, the engineered T cell will comprise a modification within a genomic coordinate region chosen from Table 1 and a modification with a genomic coordinate region chosen from Table 2. Engineered T cells or population of T cells comprising a genetic modification at genomic coordinates chosen from those listed in **Table 3** are provided, *e.g.*, cells comprising an indel or substitution mutation within any of the listed genomic ranges within TGFBR2.

[000186] In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is 95%, 90%, 85%, 80%, or 75% identical to a sequence selected from the group of sequences in Table 1, Table 2, or **Table 3**. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is 95%, 90%, 85%, 80%, or 75% identical to a sequence selected from the group of sequences in Table 1, Table 2, or **Table 3**.

[000187] In some embodiments, the guide RNAs disclosed herein comprise a guide sequence having at least 15, 16, 17, 18, 19, or 20 contiguous nucleotides of a sequence selected from the group consisting of a sequence that is 95%, 90%, 85%, 80%, or 75% identical to a sequence selected from the group of sequences in Table 1. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence at least 17, 18, 19, or 20 contiguous nucleotides of a sequence

selected from the group of sequences in Table 1. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is 95%, 90%, 85%, 80%, or 75% identical to a sequence selected from the group of sequences in Table 1. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is 17, 18, 19, or 20 contiguous nucleotides of a sequence selected from the group of sequences in Table 1. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is selected from the group of sequences in Table 1.

[000188] In some embodiments, the guide RNAs disclosed herein comprise a guide sequence having at least 15, 16, 17, 18, 19, or 20 contiguous nucleotides of a sequence selected from the group consisting of a sequence that is 95%, 90%, 85%, 80%, or 75% identical to a sequence selected from the group of sequences in Table 2. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence at least 17, 18, 19, or 20 contiguous nucleotides of a sequence selected from the group of sequences in Table 2. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is 95%, 90%, 85%, 80%, or 75% identical to a sequence selected from the group of sequences in Table 2. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is 17, 18, 19, or 20 contiguous nucleotides of a sequence selected from the group of sequences in Table 2. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is selected from the group of sequences in Table 2.

[000189] In some embodiments, the guide RNAs disclosed herein comprise a guide sequence having at least 15, 16, 17, 18, 19, or 20 contiguous nucleotides of a sequence selected from the group consisting of a sequence that is 95%, 90%, 85%, 80%, or 75% identical to a sequence selected from the group of sequences in **Table 3**. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence at least 17, 18, 19, or 20 contiguous nucleotides of a sequence selected from the group of sequences in **Table 3**. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is 95%, 90%, 85%, 80%, or 75% identical to a sequence selected from the group of sequences in Table 2. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is 17, 18, 19, or 20 contiguous nucleotides of a sequence selected from the group of sequences in **Table 3**. In some embodiments, the guide RNAs disclosed herein comprise a guide sequence that is selected from the group of sequences in **Table 3**.

[000190] Genomic coordinates throughout are according to human reference genome hg38 unless otherwise noted.

[000191] In certain embodiments a guide RNA comprising a guide sequence targeting IFNG and a guide RNA comprising a guide sequence targeting TNFA are included.

[000192] In some embodiments, the engineered T cells may be engineered autologous T cells or engineered allogenic T cells. In autologous T cell therapy, T cells may be engineered from T cells that are derived from the same patient who will be treated by the engineered T cells. In some

embodiments, engineered autologous T cells may be generated by extracting T cells from a patient and modifying the T cells according to the modifications described herein, and the engineered autologous T cells are reintroduced in the same patient. In some embodiments, engineered autologous T cells may be generated for each individual patient and administered on a patient-by-patient basis.

[000193] In some embodiments, the engineered T cells may be engineered allogeneic T cells. In allogeneic T cell therapy, T cells may be engineered from a donor cells, and then administered to one or more patients, using an “off-the-shelf” approach.

Engineered Allogeneic T cells

[000194] In some embodiments, the engineered T cell described herein is an engineered allogeneic T cell. Accordingly, provided herein are compositions and methods for altering, e.g., inhibiting or reducing, the expression and/or function (e.g., the level of expression of a functional version) of a gene target or a protein encoded by a gene target, thereby improving the efficacy (for example, by reducing or eliminating undesirable immunogenicity (such as a host versus graft response or a graft versus host response)), function, proliferation, stimulation or survival of a transplanted cell, for example an engineered T cell as described herein, e.g., an allogeneic engineered T cell for immunotherapy, to generate an “off the shelf” T cell.

[000195] “Off the shelf” therapies using donor (allogeneic) cells obviate the need to recover and modify the patient’s own lymphocytes. However, allogeneic T cells may provoke an undesirable immune response or otherwise be short-lived. Often, immune rejection of allogeneic cells results from a mismatching of major histocompatibility complex (MHC) molecules between the donor and recipient. Slight differences, e.g., in MHC alleles between individuals can cause the T-cells in a recipient to become activated. During T-cell development, an individual’s T-cell repertoire is tolerized to one’s own MHC molecules, but T-cells that recognize another individual’s MHC molecules may persist in circulation and are referred to as alloreactive T-cells. Alloreactive T-cells can become activated e.g., by the presence of another individual’s cells expressing MHC molecules in the body, causing e.g., graft versus host disease and transplant rejection. See also e.g., WO/2023/245108, the contents of which are hereby incorporated by reference in its entirety.

[000196] In some embodiments, the gene targets are allogeneic T-cell proteins such as an HLA-A, HLA-B, TRAC, or CIITA. Without being bound by theory it is believed that inhibition or elimination of the level of an allogeneic T-cell target or level of expression of an allogeneic T-cell target gene target (e.g., via alteration of the gene) may improve the function of a cell, e.g., a transplanted e.g., an engineered allogeneic T cell, by reducing or eliminating a graft vs. host

response, a host vs. graft response, or will render said transplanted cell resistant to immunosuppressant therapy.

[000197] In some embodiments, compositions and methods described herein can be used to improve cell, *e.g.*, an allogeneic engineered T-cell described herein, function (for example, by reducing or eliminating undesirable immunogenicity (such as a host versus graft response or a graft versus host response)), survival, proliferation and/or efficacy by altering the gene of a component of the major histocompatibility complex, *e.g.*, an HLA protein, *e.g.*, HLA-A and/or HLA-B. While not wishing to be bound by theory, it is considered that reduced or absent expression of a mismatch (*e.g.*, one that does not match the type of the subject receiving the cell therapy) HLA protein (or component) reduces or eliminates host vs. graft disease by eliminating host T-cell receptor recognition of and response to mismatched (*e.g.*, allogeneic) graft tissue. This approach, therefore, could be used to generate “off the shelf” T-cells (Torikai et al., 2012 Blood 119, 5697-5705).

[000198] In some embodiments, compositions and methods described herein can be used to improve cell, *e.g.*, an allogeneic engineered T-cell described herein, function (for example, by reducing or eliminating undesirable immunogenicity (such as a host versus graft response or a graft versus host response)), survival, proliferation and/or efficacy by altering the gene of a component of the T-cell receptor (TCR), *e.g.*, TRAC. While not wishing to be bound by theory, it is considered that reduced or absent expression of functional T-cell receptor components reduces or eliminates the presence of TCR on the surface of said cell, thereby reducing or preventing graft vs. host disease by eliminating T-cell receptor recognition of and response to host tissues. This approach, therefore, could be used to generate “off the shelf” T-cells.

[000199] In some embodiments, compositions and methods described herein can be used to improve cell, *e.g.*, an allogeneic engineered T-cell described herein, function (for example, by reducing or eliminating undesirable immunogenicity (such as a host versus graft response or a graft versus host response)), survival, proliferation and/or efficacy by altering the gene encoding a protein that regulates expression of one or more components of the major histocompatibility complex, *e.g.*, CIITA. While not wishing to be bound by theory, it is believed that reducing or eliminating the expression of a regulator of MHC class II expression, *e.g.*, CIITA, will reduce or eliminate the expression of MHC class II molecules on the allogeneic cell, thereby reducing or eliminating expression of a mismatch (*e.g.*, one that does not match the type of the subject receiving the cell therapy) MHC class II protein (or component), thereby reducing or eliminating host vs. graft disease by, *e.g.*, eliminating host T-cell receptor recognition of and response to mismatched (*e.g.*, allogeneic) graft tissue, *e.g.*, an allogeneic engineered T-cell, as described herein. This approach, therefore, could be used to generate “off the shelf” T-cells.

[000200] In some embodiments, it may be beneficial to reduce or eliminate expression of one or more MHC class I molecules and one or more MHC II molecules, *e.g.*, in a T-cell, *e.g.*, in an allogeneic engineered T-cell as described herein, to further reduce or eliminate the host versus graft disease response upon administration of the cell. Thus, in embodiments of the cells and methods of the present disclosure, cells may be contacted with a composition of the present disclosure (*e.g.*, a composition comprising a gRNA and a Cas9 molecule) comprising a gRNA molecule, *e.g.*, as described herein, to HLA-A and/or HLA-B (*e.g.*, such that expression of one or more MHC class I molecules is reduced or eliminated in said cell) and a composition of the present disclosure (*e.g.*, a composition comprising a gRNA and a Cas9 molecule) comprising a gRNA molecule, *e.g.*, as described herein, to CIITA (*e.g.*, such that expression of one or more MHC class II molecules is reduced or eliminated). In embodiments of the cells and methods of the present disclosure, the cell may also be contacted with a composition of the present disclosure (*e.g.*, a composition comprising a gRNA and a Cas9 molecule) comprising a gRNA molecule, *e.g.*, as described herein, to a component of the TCR, *e.g.*, to TRAC (*e.g.*, such that expression of the T-cell receptor, *e.g.*, one or more components of the TCR is reduced or eliminated). In some embodiments, a cell of the present disclosure has reduced or eliminated expression of TCR (*e.g.*, as detected by flow cytometry), reduced or eliminated expression of one or more MHC class I molecules (*e.g.*, as detected by flow cytometry), and reduced or eliminated expression of one or more MHC class II molecules (*e.g.*, as detected by flow cytometry).

[000201] In some embodiments, the “off the shelf” allogeneic T cells and populations of allogeneic T cells are engineered to comprise a modification comprising: an insertion into the cell of heterologous sequence(s) encoding IL10 that is under the control of a promoter; an insertion into the cell of heterologous sequence(s) encoding CTLA4 that is under the control of a promoter; a modification *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding an IFNG; a modification *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding an HLA-A; a modification *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding an HLA-B; a modification *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a CIITA; and a modification *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TRAC. In some embodiments, the “off the shelf” allogeneic T cells and populations of allogeneic T cells further comprise a targeting receptor, as described herein.

[000202] In some embodiments, the “off the shelf” allogeneic T cells and populations of allogeneic T cells are engineered to comprise a modification comprising: an insertion into the cell of heterologous sequence(s) encoding dmTGFB1 that is under the control of a promoter; an insertion into the cell of heterologous sequence(s) encoding IL10 that is under the control of a promoter; an insertion into the cell of heterologous sequence(s) encoding CTLA4 that is under the control of a

promoter; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an HLA-A; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an HLA-B; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a CIITA; and a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a TRAC. In some embodiments, the “off the shelf” allogeneic T cells and populations of allogeneic T cells further comprise a targeting receptor, as described herein.

[000203] In some embodiments, the “off the shelf” allogeneic T cells and populations of allogeneic T cells are engineered to comprise a modification comprising: an insertion into the cell of heterologous sequence(s) encoding dmTGFB1 that is under the control of a promoter; an insertion into the cell of heterologous sequence(s) encoding IL10 that is under the control of a promoter; an insertion into the cell of heterologous sequence(s) encoding CTLA4 that is under the control of a promoter; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an HLA-A; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an HLA-B; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a CIITA; and a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a TRAC. In some embodiments, the “off the shelf” allogeneic T cells and populations of allogeneic T cells further comprise a targeting receptor, as described herein.

[000204] In some embodiments, the “off the shelf” allogenic T cells and populations of allogenic T cells are CD3+ cells. In some embodiments, the “off the shelf” of allogenic CD3+ T cells and populations of allogenic CD3+ T cells may firstly be engineered to comprise a modification comprising one or more of: a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an HLA-A; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an HLA-B; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a CIITA; and a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a TRAC, which are then selected for CD4+ T cells or CD8+ T cells, and then further modified to generate the engineered T cells described herein. The order in which different modifications are engineered may be varied, for example, such that the “off the shelf” allogenic CD3+ T cells and populations of allogenic CD3+ T cells are engineered to comprise a modification such as:

(a) an insertion into the cell of heterologous sequence(s) encoding IL10 that is under the control of a promoter; an insertion into the cell of heterologous sequence(s) encoding CTLA4 that is

under the control of a promoter; and a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG;

(b) an insertion into the cell of heterologous sequence(s) encoding dmTGFB1 that is under the control of a promoter; an insertion into the cell of heterologous sequence(s) encoding IL10 that is under the control of a promoter; an insertion into the cell of heterologous sequence(s) encoding CTLA4 that is under the control of a promoter; and a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; or

(c) an insertion into the cell of heterologous sequence(s) encoding dmTGFB1 that is under the control of a promoter; an insertion into the cell of heterologous sequence(s) encoding IL10 that is under the control of a promoter; an insertion into the cell of heterologous sequence(s) encoding CTLA4 that is under the control of a promoter; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; and a modification e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG,

and subsequently engineered to comprise a targeting receptor.

In other embodiments, the “off the shelf” allogenic CD3+ T cells and populations of allogenic CD3+ T cells are engineered to comprise a targeting receptor, and then subsequently engineered to comprise modifications (a), (b), or (c) described above.

Table 1: Human guide sequences and chromosomal coordinates for knockdown of IFNG

Exemplary Genomic Coordinates (hg38)	Exon	Strand	Guide Sequence (5' to 3')	SEQ ID NO
chr12:68155336-68155356	4	+	GCAGGCAGGACAACCAUAC	1
chr12:68155337-68155357	4	+	CAGGCAGGACAACCAUACU	2
chr12:68155374-68155394	4	-	AGGAGUCAGAUGCUGUUUCG	3
chr12:68155394-68155414	4	-	CUAAAACAGGGAAGCGAAAA	4
chr12:68155398-68155418	4	+	CGCUUCCCUGUUUAGCUGC	5
chr12:68155406-68155426	4	-	UGUCGCCAGCAGCUAAAACA	6
chr12:68155407-68155427	4	-	CUGUCGCCAGCAGCUAAAAC	7
chr12:68155420-68155440	4	+	GCGACAGUUCAGCCAUCACU	8

Exemplary Genomic Coordinates (hg38)	Exon	Strand	Guide Sequence (5' to 3')	SEQ ID NO
chr12:68155435-68155455	4	-	ACAUGAACUCAUCCAAGUGA	9
chr12:68155447-68155467	4	+	GUUCAUGUAUUGCUUUGCGU	10
chr12:68157972-68157992	3	+	GACAUUCAUGUCUCCUUGA	11
chr12:68157989-68158009	3	+	UGAUGGUCUCCACACUCUUU	12
chr12:68157989-68158009	3	-	AAAGAGUGUGGAGACCAUCA	13
chr12:68158001-68158021	3	-	CCAGAGCAUCCAAAAGAGUG	14
chr12:68158215-68158235	2	-	GAUAAUGGAACUCUUUUCUU	15
chr12:68158230-68158250	2	-	CAUUCAGAUGUAGCGGAUAA	16
chr12:68158237-68158257	2	-	UGCAGGUCAUUCAGAUGUAG	17
chr12:68159524-68159544	1	+	CUUCUUUUACAUAUGGGUCC	18
chr12:68159559-68159579	1	-	UGCAUCGUUUUGGGUUCUCU	19
chr12:68159568-68159588	1	-	UUUCAGCUCUGCAUCGUUUU	20
chr12:68159569-68159589	1	-	UUUUCAGCUCUGCAUCGUUUU	21

Table 2: Human guide sequences and chromosomal coordinates for knockdown of TNFA

Exemplary Genomic Coordinates (hg38)	Exon	Strand	Guide sequence (5' to 3')	SEQ ID NO
chr6:31575742-31575762	1	+	UGAGCACUGAAAGCAUGAUC	22
chr6:31575749-31575769	1	+	UGAAAGCAUGAUCCGGGACG	23
chr6:31575755-31575775	1	+	CAUGAUCCGGGACGUGGAGC	24
chr6:31575781-31575801	1	-	CUUCUUGGGGAGCGCCUCCU	25
chr6:31575783-31575803	1	+	GAGGCGCUCCCCAAGAAGAC	26
chr6:31575784-31575804	1	+	AGGCGCUCCCCAAGAAGACA	27
chr6:31575785-31575805	1	+	GGCGCUCCCCAAGAAGACAG	28
chr6:31575786-31575806	1	+	GCGCUCCCCAAGAAGACAGG	29

Exemplary Genomic Coordinates (hg38)	Exon	Strand	Guide sequence (5' to 3')	SEQ ID NO
chr6:31575794-31575814	1	+	CAAGAAGACAGGGGGGCCCC	30
chr6:31575794-31575814	1	-	GGGGCCCCCUGUCUUCUUG	31
chr6:31575796-31575816	1	-	CUGGGGCCCCCUGUCUUCU	32
chr6:31575813-31575833	1	-	AAGCACCGCCUGGAGCCCUG	33
chr6:31575814-31575834	1	-	CAAGCACCGCCUGGAGCCCU	34
chr6:31575823-31575843	1	-	GCUGAGGAACAAGCACCGCC	35
chr6:31575842-31575862	1	+	CCUCUUCUCCUUCUGAUCG	36
chr6:31575846-31575866	1	+	UUCUCCUUCUGAUCGUGGC	37
chr6:31575853-31575873	1	-	GGCGCCUGCCACGAUCAGGA	38
chr6:31575857-31575877	1	-	UGGUGGCGCCUGCCACGAUC	39
chr6:31575888-31575908	1	+	CUGCUGCACUUUGGAGUGAU	40
chr6:31575890-31575910	1	-	CGAUCACUCCAAAGUGCAGC	41
chr6:31575898-31575918	1	+	UUGGAGUGAUCGGCCCCCAG	42
chr6:31575899-31575919	1	+	UGGAGUGAUCGGCCCCCAGA	43
chr6:31575917-31575937	1	-	AGGCACUCACCUCUCCCCUC	44
chr6:31576538-31576558	2	-	UGAUUAGAGAGAGGUCCCUG	45
chr6:31576539-31576559	2	-	CUGAUUAGAGAGAGGUCCCU	46
chr6:31576540-31576560	2	-	GCUGAUUAGAGAGAGGUCCC	47
chr6:31576544-31576564	2	+	CCUCUCUCUAAUCAGCCCUC	48
chr6:31576547-31576567	2	-	CCAGAGGGCUGAUUAGAGAG	49
chr6:31576550-31576570	2	+	UCUAAUCAGCCCUCUGGCCC	50
chr6:31576562-31576582	2	-	UACUGACUGCCUGGGCCAGA	51
chr6:31576563-31576583	2	-	UUACUGACUGCCUGGGCCAG	52
chr6:31576570-31576590	2	-	GAGACACUUACUGACUGCCU	53
chr6:31576571-31576591	2	-	GGAGACACUUACUGACUGCC	54

Exemplary Genomic Coordinates (hg38)	Exon	Strand	Guide sequence (5' to 3')	SEQ ID NO
chr6:31576785-31576805	3	-	GGGCUACAGGCUUGUCACUC	55
chr6:31576786-31576806	3	-	UGGGCUACAGGCUUGUCACU	56
chr6:31576798-31576818	3	-	UUACCUACAACAUGGGCUAC	57
chr6:31576805-31576825	3	-	AGAGCUCUUACCUACAACAU	58
chr6:31576806-31576826	3	-	CAGAGCUCUUACCUACAACA	59
chr6:31577110-31577130	4	+	UCCAGCAAACCCUCAAGCUG	60
chr6:31577112-31577132	4	+	CAGCAAACCCUCAAGCUGAG	61
chr6:31577122-31577142	4	-	GGAGCUGCCCCUCAGCUUGA	62
chr6:31577123-31577143	4	-	UGGAGCUGCCCCUCAGCUUG	63
chr6:31577136-31577156	4	+	AGCUCCAGUGGCUGAACCGC	64
chr6:31577137-31577157	4	+	GCUCCAGUGGCUGAACCGCC	65
chr6:31577143-31577163	4	-	UGGCCCCGGCGGUUCAGCCAC	66
chr6:31577152-31577172	4	+	CCGCCGGGCCAAUGCCCUC	67
chr6:31577155-31577175	4	-	CCAGGAGGGCAUUGGCCCCG	68
chr6:31577159-31577179	4	+	GCCAAUGCCCUCUGGCCAA	69
chr6:31577163-31577183	4	-	GCCAUUGGCCAGGAGGGCAU	70
chr6:31577164-31577184	4	+	UGCCCUCCUGGCCAAUGGCG	71
chr6:31577170-31577190	4	-	GCUCCACGCCAUUGGCCAGG	72
chr6:31577173-31577193	4	-	UCAGCUCCACGCCAUUGGCC	73
chr6:31577178-31577198	4	-	AUCUCUCAGCUCCACGCCAU	74
chr6:31577185-31577205	4	+	GGAGCUGAGAGAUAAACCAGC	75
chr6:31577188-31577208	4	+	GCUGAGAGAUAAACCAGCUGG	76
chr6:31577200-31577220	4	+	CCAGCUGGUGGUGCCAUCAG	77
chr6:31577201-31577221	4	+	CAGCUGGUGGUGCCAUCAGA	78
chr6:31577216-31577236	4	-	AUGAGGUACAGGCCCCUCUGA	79

Exemplary Genomic Coordinates (hg38)	Exon	Strand	Guide sequence (5' to 3')	SEQ ID NO
chr6:31577227-31577247	4	-	CCUGGGAGUAGAUGAGGUAC	80
chr6:31577237-31577257	4	+	UACUCCCAGGUCCUCUCAA	81
chr6:31577245-31577265	4	-	CUUGGCCCCUUGAAGAGGACC	82
chr6:31577295-31577315	4	-	GGCGAUGCGGCUGAUGGUGU	83
chr6:31577296-31577316	4	-	CGGCGAUGCGGCUGAUGGUG	84
chr6:31577301-31577321	4	-	GGAGACGGCGAUGCGGCUGA	85
chr6:31577308-31577328	4	-	UCUGGUAGGAGACGGCGAUG	86
chr6:31577311-31577331	4	+	CGCCGUCUCCUACCAGACCA	87
chr6:31577322-31577342	4	-	GAGGUUGACCUUGGUCUGGU	88
chr6:31577331-31577351	4	-	GGCAGAGAGGAGGUUGACCU	89
chr6:31577349-31577369	4	+	CCAUCAAGAGCCCCUGCCAG	90
chr6:31577350-31577370	4	+	CAUCAAGAGCCCCUGCCAGA	91
chr6:31577362-31577382	4	-	CUGGGGUCUCCCUCUGGCAG	92
chr6:31577363-31577383	4	-	UCUGGGGUCUCCCUCUGGCA	93
chr6:31577397-31577417	4	-	GAUGGGCUCAUACCAGGGCU	94
chr6:31577401-31577421	4	+	CUGGUAUGAGCCCAUCUAUC	95
chr6:31577402-31577422	4	-	AGAUAGAUGGGCUCAUACCA	96
chr6:31577402-31577422	4	+	UGGUAUGAGCCCAUCUAUCU	97
chr6:31577403-31577423	4	-	CAGAUAGAUGGGCUCAUACC	98
chr6:31577406-31577426	4	+	AUGAGCCCAUCUAUCUGGGA	99
chr6:31577407-31577427	4	+	UGAGCCCAUCUAUCUGGGAG	100
chr6:31577414-31577434	4	-	AAGACCCCUCCCAGAUAGAU	101
chr6:31577437-31577457	4	-	GUCGGUCACCCUUCUCCAGC	102
chr6:31577454-31577474	4	+	GACUCAGCGCUGAGAUCAAU	103
chr6:31577455-31577475	4	-	GAUUGAUCUCAGCGCUGAGU	104

Exemplary Genomic Coordinates (hg38)	Exon	Strand	Guide sequence (5' to 3')	SEQ ID NO
chr6:31577480-31577500	4	-	UCGGCAAAGUCGAGAUAGUC	105
chr6:31577481-31577501	4	-	CUCGGCAAAGUCGAGAUAGU	106
chr6:31577483-31577503	4	+	UAUCUCGACUUUGCCGAGUC	107
chr6:31577484-31577504	4	+	AUCUCGACUUUGCCGAGUCU	108
chr6:31577488-31577508	4	+	CGACUUUGCCGAGUCUGGGC	109
chr6:31577498-31577518	4	+	GAGUCUGGGCAGGUCUACUU	110
chr6:31577499-31577519	4	+	AGUCUGGGCAGGUCUACUUU	111
chr6:31577499-31577519	4	-	AAAGUAGACCUGCCCAGACU	112
chr6:31577532-31577552	4	-	GGAUGUUCGUCCUCCUCACA	113

Table 3: Human guide sequences and chromosomal coordinates for knockdown of TGFBR2

Exemplary Genomic Coordinates (hg38)	Guide sequence (5' to 3')	SEQ ID NO
chr3:30672267-30672287	ACAGUGAUCACACUCCAUGU	235
chr3:30644743-30644763	UAUCAUGUCGUUAUUAACUG	236
chr3:30671764-30671784	GCAGAAGCUGAGUUCAACCU	237
chr3:30672177-30672197	ACCUACAGGAGUACCUGACG	238
chr3:30606958-30606978	CCCGACUUCUGAACGUGCGG	239
chr3:30606870-30606890	AGCGGGGUCUGCCAUGGGUC	240
chr3:30606961-30606981	UCACCCGACUUCUGAACGUG	241
chr3:30606911-30606931	GGCCGCUGCACAUCGUCCUG	242
chr3:30606909-30606929	GGACGAUGUGCAGCGGCCAC	243
chr3:30606954-30606974	CCCACCGCACGUUCAGAAGU	244
chr3:30606865-30606885	CGAGCAGCGGGGUCUGCCA	245
chr3:30606947-30606967	AACGUGCGGUGGGAUCGUGC	246
chr3:30606864-30606884	ACGAGCAGCGGGGUCUGCCA	247
chr3:30606916-30606936	GUCCACAGGACGAUGUGCAG	248
chr3:30606871-30606891	GCGGGGUCUGCCAUGGGUCG	249
chr3:30606930-30606950	UGCUGGCGAUACGCGUCCAC	250
chr3:30606957-30606977	CCGACUUCUGAACGUGCGGU	251
chr3:30623239-30623259	UGGGCAGUCCUAUUACAGCU	252
chr3:30623238-30623258	GGGCAGUCCUAUUACAGCUG	253
chr3:30623240-30623260	AUGGGCAGUCCUAUUACAGC	254
chr3:30623263-30623283	GAUACUUUACUAUGUCUCAG	255
chr3:30644843-30644863	AGUUGCUCUAUGCAGGAUUUC	256
chr3:30644757-30644777	AUGAUAGUCACUGACAACAA	257
chr3:30650418-30650438	AUUGCACUCAUCAGAGCUAC	258
chr3:30650323-30650343	CCCCUACCAUGACUUUAUUC	259

chr3:30650327-30650347	UCCAGAAUAAAGUCAUGGUA	260
chr3:30650317-30650337	AGUCAUGGUAGGGGAGCUUG	261
chr3:30650326-30650346	CCAGAAUAAAGUCAUGGUAG	262
chr3:30650318-30650338	AAGUCAUGGUAGGGGAGCUU	263
chr3:30650319-30650339	AAAGUCAUGGUAGGGGAGCU	264
chr3:30650393-30650413	CACAUGAAGAAAGUCUCACC	265
chr3:30672152-30672172	AUACCGCCUCCACGCCAA	266
chr3:30672410-30672430	GUGGAUGACCUGGCUAACAG	267
chr3:30672296-30672316	CUGUGCACGAUGGGCAUCUU	268
chr3:30671871-30671891	GUUGAUGUUGUUGGCACACG	269
chr3:30672193-30672213	AGCUGAUGACAUGCCGCGUC	270
chr3:30671791-30671811	CGGCAAGACGCGGAAGCUCA	271
chr3:30672024-30672044	CCAAGAGGCAUACUCCUCAU	272
chr3:30671784-30671804	CCGCGUCUUGCCGGUUUCCC	273
chr3:30672322-30672342	CGAGGAUAAUUGGAGCUCUUG	274
chr3:30672192-30672212	UGACGCGGCAUGUCAUCAGC	275
chr3:30672270-30672290	GUGAUCACACUCCAUGUGGG	276
chr3:30671941-30671961	UCGCUUUGCUGAGGUCUAUA	277
chr3:30671752-30671772	GCUUCUGCUGCCGGUUAACG	278
chr3:30672387-30672407	CAGAGUAGGGUCCAGACGCA	279
chr3:30671929-30671949	CAAAGCGACCUUCCCCACC	280
chr3:30672266-30672286	CACAGUGAUCACACUCCAUG	281
chr3:30672295-30672315	CAAGAUGCCCAUCGUGCACA	282
chr3:30672021-30672041	CCUAUGAGGAGUAUGCCUCU	283
chr3:30671932-30671952	GGGGAAAGGUCGCUUUGCUG	284
chr3:30671835-30671855	GCGGUCAUCUCCAGGAUGA	285
chr3:30672023-30672043	CAAGAGGCAUACUCCUCAUA	286
chr3:30671908-30671928	GCCCAUUGAGCUGGACACCC	287
chr3:30672212-30672232	UGGGAGGACCUGCGCAAGCU	288
chr3:30671854-30671874	ACGUGGAGCUGAUGUCAGAG	289
chr3:30671701-30671721	UGGCAACUCCCAGUGGUGGC	290
chr3:30672294-30672314	CCAAGAUGCCCAUCGUGCAC	291
chr3:30672193-30672213	GACGCGCAUGUCAUCAGCU	292
chr3:30671821-30671841	CGAGCACUGUGCCAUCAUCC	293
chr3:30672268-30672288	CACAUGGAGUGUGAUCACUG	294
chr3:30672250-30672270	UGUGGAGGUGAGCAAUCCCC	295
chr3:30672421-30672441	UUACCUGCCACUGUUAGCC	296
chr3:30672400-30672420	UACUCUGUCUGUGGAUGACC	297
chr3:30672249-30672269	GUGGAGGUGAGCAAUCCCCC	298
chr3:30672196-30672216	GCGGCAUGUCAUCAGCUGGG	299
chr3:30672340-30672360	AGGUUAGGUCGUUCUUCACG	300
chr3:30671842-30671862	UGUCAGAGCGGUCAUCUUC	301
chr3:30671739-30671759	UCUACUGCUACCGCGUUAAC	302
chr3:30674221-30674241	ACAUCUCGCUGUAAUGCAGU	303
chr3:30674178-30674198	GCAGACCGAUGUCUACUCCA	304
chr3:30674085-30674105	CUGUCUGUUUUGCUAUAGG	305
chr3:30674136-30674156	CCUAGAAUCCAGGAUGAAUU	306
chr3:30674220-30674240	GACAUCUCGCUGUAAUGCAG	307
chr3:30674184-30674204	CGAUGUCUACUCCAUGGCUC	308
chr3:30688451-30688471	GACAACGUGUUGAGAGAUCC	309
chr3:30688403-30688423	CGACCUUGGAACCAAAUGG	310
chr3:30688434-30688454	GUCCUUAUGCUUUCGACAC	311
chr3:30688432-30688452	CCUUAUGCUUUCGACACAG	312

chr3:30688402-30688422	UCCAUUUGGUUCCAAGGUGC	313
chr3:30688388-30688408	AAAGAUUAUGAGCCUCCAUI	314
chr3:30688429-30688449	CCCCUGUGUCGAAAGCAUGA	315
chr3:30688510-30688530	GUAAACACUCACUCCUUACC	316
chr3:30688416-30688436	ACAGGGGUGCUCCCGCACCU	317
chr3:30691489-30691509	GAAGCGUUCUGCCACACACU	318
chr3:30691522-30691542	AUCUGGACAGGCUCUCGGGG	319
chr3:30691427-30691447	UCAACGUCUCACACACCAUC	320
chr3:30691519-30691539	AGCAUCUGGACAGGCUCUCG	321
chr3:30691435-30691455	GUGAGACGUUGACUGAGUGC	322
chr3:30691594-30691614	CCUGCCCCAGAAGAGCUAUU	323
chr3:30691409-30691429	CCCGCUACAGGGCAUCCAGA	324
chr3:30691463-30691483	UGAGACGGGCCUCUGGGUCG	325
chr3:30606962-30606982	ACGUUCAGAAGUCGGGUGAG	326
chr3:30606974-30606994	CGGGUGAGUGGUCCCCAGCC	327
chr3:30606975-30606995	GGGUGAGUGGUCCCCAGCCC	328
chr3:30644885-30644905	GAAGCCACAGGAAGUCUGUG	329
chr3:30644893-30644913	AGGAAGUCUGUGUGGCUGUA	330
chr3:30650250-30650270	CCUAGAGUGAAGAGAUUCAU	331
chr3:30671618-30671638	UCUGAUGGGGAAACAAAACA	332
chr3:30671763-30671783	AGCAGAAGCUGAGUUCAACC	333
chr3:30671983-30672003	UUCAGAGCAGUUUGAGACAG	334
chr3:30672088-30672108	GAACAUACUCCAGUUCUGA	335
chr3:30672094-30672114	ACUCCAGUCCUGACGGCUG	336
chr3:30672099-30672119	AGUCCUGACGGCUGAGGAG	337
chr3:30674083-30674103	UAUAGCAAAAACAGACAGUG	338
chr3:30674198-30674218	UUCCCAGAGCACCAGAGCCA	339
chr3:30688476-30688496	GACCAGAAAUUCCCAGCUUC	340
chr3:30688481-30688501	AGCCAGAAGCUGGGAAUUUC	341
chr3:30688490-30688510	UGGUGGUUGAGCCAGAAGCU	342
chr3:30688491-30688511	CUGGUGGUUGAGCCAGAAGC	343
chr3:30688507-30688527	AACACUCACUCCUUACCUGG	344
chr3:30691396-30691416	UAGCGGGAAAGAGAUCCAAA	345
chr3:30691397-30691417	GUAGCGGGAAAGAGAUCCAA	346
chr3:30691412-30691432	CCAUCUGGAUGCCCUGUAGC	347
chr3:30691413-30691433	ACCAUCUGGAUGCCCUGUAG	348
chr3:30691582-30691602	GAGCUAUUUGGUAGUGUUUA	349
chr3:30691583-30691603	AGAGCUAUUUGGUAGUGUUU	350

[000205] A non-limiting modified guide sequence for knockdown of TNFA is shown below (hg38 coordinates chr12:68158001-68158021, G019757):

[000206] mC*mC*mA*GAGCAUCCAAAAGAGUGGUUUUAGAmGmCmUmAmGmAmA
mAmUmAmGmCAAGUUA AAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAm
AmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU (SEQ
ID NO: 119), wherein m is a 2'-OMe modified nucleotide/ nucleoside residue, * is indicative of a
phosphorothioate linkage between the residues, a capital letter indicates a residue, e.g., a residue
comprising a ribose sugar.

[000207] A non-limiting modified guide sequence for knockdown of IFNG is shown below (hg38 coordinates chr6:31576805-31576825, G019753):

[000208] mA*mG*mA*GCUCUUACCUACAACAUGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU (SEQ ID NO: 120).

[000209] A non-limiting modified guide sequence for knockdown of IL17A is shown below (hg38 coordinates chr6:52189069-52189089):

[000210] mU*mC*mA*CAGAGGGAUUCUCUCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU (SEQ ID NO: 217).

[000211] An exemplary modified mock guide is shown below (hg38 coordinates chr1:0-20):

[000212] mG*mA*mU*CACGUCGGCCGUUGGCGGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU (SEQ ID NO: 121).

[000213] A non-limiting modified guide sequence for knockdown of TGFBR2 is shown below (hg38 coordinates chr3:30671941-30671961):

[000214] mU*mC*mG*CUUUGCUGAGGUCUAUAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCACGAAAGGGCACCGAGUCGmU*mG*mC*mU (SEQ ID NO: 351).

[000215] A non-limiting modified guide sequence for knockdown of HLA-A is shown below (hg38 coordinates chr6:29942891-29942915, G034202):

[000216] mG*mC*mU*mCmUAUmCmCAmCGmGCGCCmCGCGmGCUmGUUGmUmAmGmCUCCcmUmGmAmAmAmCmCGUUmGmCUAmCAAUAAGmGmCCmGmUmCmGmAmAmAmGmAmUGUGCmCGmCAAmCGCUCUmGmCCmUmUmCmUGGCAUCG*mU*mU (SEQ ID NO: 366), wherein m is a 2'-OMe modified nucleotide/ nucleoside residue, * is indicative of a phosphorothioate linkage between the residues, a capital letter indicates a residue, e.g., a residue comprising a ribose sugar.

[000217] A non-limiting modified guide sequence for knockdown of HLA-B is shown below (hg38 coordinates chr6:31355222-31355246, G034208):

[000218] mU*mC*mU*mGmGGAmAmAGmGAmGGGAmAGAUmGAGmGUUGmUmAmGmCUCCcmUmGmAmAmAmCmCGUUmGmCUAmCAAUAAGmGmCCmGmUmCmGmA mAmAmGmAmUGUGCmCGmCAAmCGCUCUmGmCCmUmUmCmUGGCAUCG*mU*mU (SEQ ID NO: 367), wherein m is a 2'-OMe modified nucleotide/ nucleoside residue, * is indicative of a phosphorothioate linkage between the residues, a capital letter indicates a residue, e.g., a residue comprising a ribose sugar.

[000219] A non-limiting modified guide sequence for knockdown of CIITA is shown below (hg38 coordinates chr16:10906643-10906667, G034619):

[000220] mA*mG*mC*mUmGCCmGmUUmCmUUmGCCCmGUCCmGGGmGUUGmUmAmGmCUCCcmUmGmAmAmAmCmCGUUmGmCUAmCAAUAAGmGmCCmGmUmCmGmA mAmGmAmUGUGCmCGmCAAmCGCUCUmGmCCmUmUmCmUGGCAUCG*mU*mU (SEQ ID NO: 368), wherein m is a 2'-OMe modified nucleotide/ nucleoside residue, * is indicative of a phosphorothioate linkage between the residues, a capital letter indicates a residue, e.g., a residue comprising a ribose sugar.

[000221] A non-limiting modified guide sequence for knockdown of TRAC is shown below (hg38 coordinates Chr14: 22547524- 22547544, G025420):

[000222] mC*mU*mC*UCAGCUGGUACACGGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUA AAAUAAGGCUAGUCCGUUAUCACGAAAGGGCACCGAGUCG GmU*mG*mC*mU (SEQ ID NO: 369), wherein m is a 2'-OMe modified nucleotide/ nucleoside residue, * is indicative of a phosphorothioate linkage between the residues, a capital letter indicates a residue, e.g., a residue comprising a ribose sugar; or

[000223] mC*mU*mC*UCAGCUGGUACACGGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUA AAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU (SEQ ID NO: 370), wherein m is a 2'-OMe modified nucleotide/ nucleoside residue, * is indicative of a phosphorothioate linkage between the residues, a capital letter indicates a residue, e.g., a residue comprising a ribose sugar.

[000224] In some embodiments, disclosed herein are T cells engineered by introducing or inserting a heterologous dmTGFB1 nucleic acid within a genomic locus of a T cell or a population of T cells using a guide RNA with an RNA-guided DNA binding agent, and a construct (e.g., donor construct or template) comprising a heterologous dmTGFB1 nucleic acid, e.g., to make an engineered T cell. In some embodiments, disclosed herein are T cells engineered by expressing a heterologous dmTGFB1 from a genomic locus of a T cell or a population of T cells, e.g., using a guide RNA with an RNA-guided DNA-binding agent and a construct (e.g., donor) comprising a heterologous dmTGFB1 nucleic acid. In some embodiments, disclosed herein are T cells

engineered by inducing a break (*e.g.*, double-stranded break (DSB) or single-stranded break (nick)) within the genome of a T cell or a population of T cells for inserting the dmTGFB1 gene, *e.g.*, using a guide RNA with an RNA-guided DNA-binding agent (*e.g.*, a CRISPR/Cas system). Cells and cell populations made by the methods are also provided.

[000225] In some embodiments, disclosed herein are T cells engineered by introducing or inserting a heterologous dmTGFB1 nucleic acid within a genomic locus of a T cell, are further engineered by introducing or inserting a heterologous IL10 nucleic acid within a genomic locus of a T cell or a population of T cells using a guide RNA with an RNA-guided DNA binding agent, and a construct (*e.g.*, donor construct or template) comprising a heterologous IL10 nucleic acid, *e.g.*, to make an engineered T cell. In some embodiments, disclosed herein are T cells engineered by expressing a heterologous IL10 from a genomic locus of a T cell or a population of T cells, *e.g.*, using a guide RNA with an RNA-guided DNA-binding agent and a construct (*e.g.*, donor) comprising a heterologous IL10 nucleic acid. In some embodiments, disclosed herein are T cells engineered by inducing a break (*e.g.*, double-stranded break (DSB) or single-stranded break (nick)) within the genome of a T cell or a population of T cells for inserting the IL10 gene, *e.g.*, using a guide RNA with an RNA-guided DNA-binding agent (*e.g.*, a CRISPR/Cas system). Cells and cell populations made by the methods are also provided.

[000226] In some embodiments, disclosed herein are T cells engineered by introducing or inserting a heterologous dmTGFB1 nucleic acid within a genomic locus of a T cell, are further engineered by introducing or inserting a heterologous CTLA4 nucleic acid within a genomic locus of a T cell or a population of T cells using a guide RNA with an RNA-guided DNA binding agent, and a construct (*e.g.*, donor construct or template) comprising a heterologous CTLA4 nucleic acid, *e.g.*, to make an engineered T cell. In some embodiments, disclosed herein are T cells engineered by expressing a heterologous CTLA4 from the genomic locus of a T cell or a population of T cells, *e.g.*, using a guide RNA with an RNA-guided DNA-binding agent and a construct (*e.g.*, donor) comprising a heterologous CTLA4 nucleic acid. In some embodiments, disclosed herein are T cells engineered by inducing a break (*e.g.*, double-stranded break (DSB) or single-stranded break (nick)) within the genome of a T cell or a population of T cells for inserting the CTLA4 gene, *e.g.*, using a guide RNA with an RNA-guided DNA-binding agent (*e.g.*, a CRISPR/Cas system). Cells and cell populations made by the methods are also provided.

[000227] In some embodiments, disclosed herein are T cells engineered by introducing or inserting a heterologous dmTGFB1 nucleic acid within a genomic locus of a T cell, are further engineered by introducing or inserting a heterologous CTLA4 nucleic acid and a heterologous IL10 nucleic acid within a genomic locus of a T cell or a population of T cells using a guide RNA with an RNA-guided DNA binding agent, and one or more constructs (*e.g.*, donor construct or template) comprising a heterologous CTLA4 nucleic acid and a heterologous IL10 nucleic acid, *e.g.*, to make

an engineered T cell. In some embodiments, disclosed herein are T cells engineered by expressing a heterologous CTLA4 and a heterologous IL10 from the genomic locus of a T cell or a population of T cells, *e.g.*, using a guide RNA with an RNA-guided DNA-binding agent and one or more constructs (*e.g.*, donor construct or template) comprising a heterologous CTLA4 nucleic acid and a heterologous IL10 nucleic acid. In some embodiments, disclosed herein are T cells engineered by inducing a break (*e.g.*, double-stranded break (DSB) or single-stranded break (nick)) within the genome of a T cell or a population of T cells for inserting the CTLA4 gene and the IL10 gene, *e.g.*, using a guide RNA with an RNA-guided DNA-binding agent (*e.g.*, a CRISPR/Cas system). In some embodiments, the guide RNAs mediate a target-specific cutting by an RNA-guided DNA-binding agent (*e.g.*, Cas nuclease) at a site described herein for insertion of sequence(s) encoding two or more regulatory T cell promoting molecule, *e.g.*, IL10 and CTLA4. It will be appreciated that, in some embodiments, the guide RNAs comprise guide sequences that bind to, or are capable of binding to, said regions. Cells and cell populations made by the methods are also provided.

[000228] Exemplary nucleotide and polypeptide sequences of regulatory T cell promoting molecules are provided below. Methods for identifying alternate nucleotide sequences encoding polypeptide sequences, including alternate naturally occurring variants and non-human homologues, are known in the art. Exemplary nucleic acid sequences encoding dmTGFB1, IL10, and CTLA4 are provided below. Other suitable dmTGFB1, IL10, and CTLA4 sequences are known in the art or can be designed based on the disclosure provided herein. *See, e.g.*, Gorby et al., Engineered IL-10 variants elicit potent immuno-modulatory activities at therapeutic low ligand doses, BioRxiv (2020); Saxton et al. Structure-based decoupling of the pro- and anti-inflammatory functions of interleukin-10, Science, (2021)371:eabc8433 (doi: 10.1126/science.abc8433), WO2021243057; and Xu et al., Affinity and cross-reactivity engineering of CTLA4-Ig to modulate T cell costimulation, J Immunol (2012), the contents, variants (in particular IL-10 variants), and sequences of which are hereby incorporated by reference. Methods for identifying alternate IL10 and CTLA4 sequences are also known in the art. *See, e.g., id.* For example, Gorby discloses the anti-inflammatory and pro-cytotoxicity activities of IL-10. "In addition to its anti-inflammatory activities, recent studies have shown that IL-10 can increase the cytotoxic function of CD8 T cells, augmenting their ability to target tumors and boosting the anti-cancer response (Oft, 2019). This seems paradoxical as IL-10 in the tumor microenvironment is linked to tumor evasion of the immune response, most likely due to IL-10's inhibitory effects on antigen presentation (Mannino et al., 2015; Yue et al., 1997). Despite this paradox, several studies have elegantly demonstrated that IL-10 can improve production of the CD8 effector molecules granzyme B and interferon gamma both in vitro and in vivo (Emmerich et al., 2012; Mumm et al., 2011; Mumm and Oft, 2013). Currently there are several clinical trials testing the anti-tumor properties of IL-10, with already initial promising results (Naing et al., 2019). In these trials high doses of PEGylated IL-10 (Pegilodekakin) were used, which

resulted in prolonged IL-10 retention in the circulation to ensure efficacy, again highlighting that effective IL-10 in vivo responses need high concentrations and sustained levels of IL-10. Saxton et al., used yeast display-based directed evolution to engineer IL-10 variants. IL-10 is an immunoregulatory cytokine with both anti-inflammatory and immunostimulatory properties and is frequently dysregulated in disease. Mechanistically, IL-10 functions as a secreted homodimer that engages two copies of a heterodimeric receptor complex comprising the private receptor subunit, IL-10R α , and the shared subunit, IL-10R β . The IL-10-dependent dimerization of IL-10R α and IL-10R β in turn initiates activation of the transcription factor STAT3, which mediates the diverse biological effects of IL-10. Saxton et al. teach IL-10 variants with a range of IL-10R β binding strengths uncovered substantial differences in response thresholds across immune cell populations, providing a means of manipulating IL-10 cell type selectivity. A “super-10” variant (D25A/E96A based on amino acid numbering of SEQ ID NO: 231) with enhanced affinity for IL-10R β , enabling assembly of the hexameric IL-10–IL-10R α –IL-10R β complex was identified by Saxton et al. Further variants (e.g., D25K, D25A, N21A/R104A, and D25A/N21A/R104A based on amino acid numbering of SEQ ID NO: 231) displayed myeloid-biased activity by suppressing macrophage activation without stimulating inflammatory CD8⁺ T cells, thereby uncoupling the major opposing functions of IL-10. In certain embodiments, IL-10 variants, referred to herein as “suppressive IL-10 variants,” with impaired immunostimulatory properties, e.g. variants that retain myeloid-biased activity by suppressing macrophage activation and that display impaired stimulation of inflammatory CD8⁺ T cells, are used in the engineered T cells provided herein. In certain embodiments, suppressive IL-10 variants used in the engineered T cells provided herein include substitutions selected from D25K, D25A, D25A/E96A, N21A/R104A, and D25A/N21A/R104A, e.g., D25A/E96A. The results from Saxton et al. provide a mechanistic blueprint for tuning the pleiotropic actions of IL-10. Further suppressive IL-10 variants are provided in WO2021243057, which is incorporated herein by reference, which provides a number of IL-10 sequences, including polypeptides have an altered binding affinity for IL-10R β compared to binding affinity of a reference IL-10 polypeptide lacking the one or more amino acid substitution, and methods to characterize activity. Further variants, including suppressive IL-10 variants are disclosed and predicted to displayed myeloid-biased activity by suppressing macrophage activation without stimulating inflammatory CD8⁺ T cells, similar to the D25K, D25A, D25A/E96A, N21A/R104A, and D25A/N21A/R104A variants. Embodiments provided therein include suppressive IL-10 variants with one or more amino acid substitution is at a position corresponding to an amino acid residue selected from D25, H14, N18, R24, D28, E74, H90, N92, E96, T100, and R104, optionally including a further substitution at one or more amino acids selected from N21, M22, R32, and S93. In certain embodiments, D25 is substituted with an amino acid selected from K, A, N, H, I, K, or V. In certain embodiments, E96 is substituted with an amino acid selected from A, N, D, Q, H, K, or S. Exemplary combinations of substitutions can include a) N18Y/N92Q/T100D/R104W ; (b)

N18Y/N21H/N92Q/E96D/T100V/R104W; (c) N18Y/N21H/E96H/T100V/R104W ; (d) N18Y/D25A/N92Q/T100D/R104W; (e) N18Y/D25K/N92Q/T100D/R104W; and (f) N18Y/D25A/N92Q/E96A/T100D/R104W. Exemplary substitutions include (a) D25A; (b) D25K; (c) E96A; (d) E96K; (e) D25A/E96A; (f) N21A/R104A; (g) N21A/D25A; (h) N21A/D25A/E96A; and (i) N21A/M22A/D25A. Sequences with at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to any of the nucleic acid sequences, amino acid sequences, or nucleic acid sequences encoding the amino acid sequences described herein, e.g., due to mutations or truncations, are also contemplated. In some embodiments, a nucleic acid sequence encoding any of the amino acid sequences provided herein is also provided.

[000229] Non-limiting exemplary nucleic acid sequences encoding TGFB1 are provided:

[000230] Wild-type TGFB1

Atgccgcctccgggctgctggctgctgctgctacgcctgctgtggctactgtgctgacgcctggccgggcccgcgggacatc
cacctgcaagactatcgacatggagctgtgtaagcgggaagcgcacgcaggccatccgcggccagatcctgtccaagctcggctcgcagc
ccccgcagccagggggaggtgccgcccggcccgtgcccaggccgtgctgcacctgtacaacagcaccgcgaccgggtggccgggg
agagtgcagaaccggagcccgcgagcctgaggccgactactacgccaaggaggtcacccgcgtgctaattgttgaaaccacaaacgaatcta
tgacaagtccaagcagagtacacagcatatatatgttctcaacatcagagctccgagaagcggtaacctgaaccctgttctctcccggg
cagagctgcgtctgctgaggctcaagttaaaagtggagcagcacgtggagctgtaccagaatacagcaacaattcctggcgatacctcagca
accggctgctggcaccagcgactgccagagtggttatctttgatgtcaccggagttgtgcggcagtggttgagccgtggagggggaattga
ggcgtttgccttagcgcgccactgctcctgtgacagcagggataaacacactgcaagtggacatcaacgggttactaccggccgcccgcgaggtga
cctggccaccattcatggcatgaaccggcctttcctgcttctcatggccaccccgtggagaggcccagcatctgcaaagctcccggcaccgc
cgagccctggacaccaactattgcttcagctccacggagaagaactgctgcgtgcggcagctgtacattgacttccgaaggacctcggctgga
agtggatccacgagcccaagggtaccatgccaaactctgcctcgggcccctgcccctacatttgagcctggacacgcagtacagcaaggtcc
tggccctgtacaaccagcataaccggggcgccctcggcggcgccgtgctgcgtgcggcaggcgctggagccgctgcccctcatgtgtactacgtg
ggccgcaagcccaaggtggagcagctgtccaacatgatcgtgcgtcctctgcaagtgcgc (SEQ ID NO: 204)

[000231] Dual Mutant (dm) TGFB1 (R218H, C225R)

[illegible]

ctggaagtggatccacgagcccaagggtaccatgccaactctgcctcggggccctgcccctacattggagcctggacacgcagtacagcaa
 ggtcctggccctgtacaaccagcataaccggggcgctcgccggcgccgtgctgcgtgccgcaggcgctggagccgctgcccacgtgtact
 acgtggggcgcaagcccaagggtggagcagctgtccaacatgatcgtgcgtcctcctcaagtgcagc (SEQ ID NO: 208)

[000232] Non-limiting exemplary amino acid sequences of human TGFB1

[000233] Wild-type

MPPSGLRLLLLLLPLLWLLVLTTPGRPAAGLSTCKTIDMELVKKRIEAIKRGILSKRLASPP
 SQGEVPPGPLPEAVLALYNSTRDRVAGESAEPEPEPEADYYAKEVTRVLMVETHNEIYDKF
 KQSTHSIYMFFNTSELREAVPEPVLLSRAELRLLRLKLKVEQHVELYQKYSNNSWRYLSNR
 LLAPSDSPEWLSFDVTGVVRQWLSRGGEIEGFRLSAHCSDSRDNTLQVDINGFTTGRRGD
 LATIHGMNRPFLLLMATPLERAQHLQSSRHRRALDTNYCFSSTEKNCCVRQLYIDFRKDLG
 WKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVLALYNQHNPASAAAPCCVPQALEPLPIV
 YYVGRKPKVEQLSNMIVRSCKCS (SEQ ID NO: 210)

[000234] dmTGFB1 R218C, C225R

MPPSGLRLLLLLLPLLWLLVLTTPGRPAAGLSTCKTIDMELVKKRIEAIKRGILSKRLASPP
 SQGEVPPGPLPEAVLALYNSTRDRVAGESAEPEPEPEADYYAKEVTRVLMVETHNEIYDKF
 KQSTHSIYMFFNTSELREAVPEPVLLSRAELRLLRLKLKVEQHVELYQKYSNNSWRYLSNR
 LLAPSDSPEWLSFDVTGVVRQWLSRGGEIEGFHLSAHCSRDSRDNTLQVDINGFTTGRRGD
 LATIHGMNRPFLLLMATPLERAQHLQSSRHRRALDTNYCFSSTEKNCCVRQLYIDFRKDLG
 WKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVLALYNQHNPASAAAPCCVPQALEPLPIV
 YYVGRKPKVEQLSNMIVRSCKCS (SEQ ID NO: 214)

[000235] Non-limiting exemplary nucleic acid sequences encoding IL10 are provided:

[000236] Wild-type IL10:

ATGCACAGCTCAGCACTGCTCTGTTGCCTGGTCCTCCTGACTGGGGTGAGGGCCAGCCC
 AGGCCAGGGCACCCAGTCTGAGAACAGCTGCACCCACTTCCCAGGCAACCTGCCTAAC
 ATGCTTCGAGATCTCCGAGATGCCTTCAGCAGAGTGAAGACTTTCTTTCAAATGAAGGA
 TCAGCTGGACAACCTGTTGTTAAAGGAGTCCTTGCTGGAGGACTTTAAGGGTTACCTGG
 GTTGCCAAGCCTTGTCTGAGATGATCCAGTTTTACCTGGAGGAGGTGATGCCCCAAGCT
 GAGAACCAAGACCCAGACATCAAGGCGCATGTGAACCTCCCTGGGGGAGAACCTGAAG
 ACCCTCAGGCTGAGGCTACGGCGCTGTCATCGATTTCTTCCCTGTGAAAACAAGAGCA
 AGGCCGTGGAGCAGGTGAAGAATGCCTTTAATAAGCTCCAAGAGAAAGGCATCTACAA
 AGCCATGAGTGAGTTTGACATCTTCATCAACTACATAGAAGCCTACATGACAATGAAG
 ATACGAAAC (SEQ ID NO: 122)

[000237] High affinity IL10 (N36I, N110I, K117N, F129L):

ATGCACAGCTCAGCACTGCTCTGTTGCCTGGTCCTCCTGACTGGGGTGAGGGCCAGCCC
 AGGCCAGGGCACCCAGTCTGAGAACAGCTGCACCCACTTCCCAGGCATCCTGCCTAAC
 ATGCTTCGAGATCTCCGAGATGCCTTCAGCAGAGTGAAGACTTTCTTTCAAATGAAGGA
 TCAGCTGGACAACTTGTGTGTTAAAGGAGTCCTTGCTGGAGGACTTTAAGGGTTACCTGG
 GTTGCCAAGCCTTGTCTGAGATGATCCAGTTTTACCTGGAGGAGGTGATGCCCCAAGCT
 GAGAACCAAGACCCAGACATCAAGGCGCATGTG^{atc}TCCCTGGGGGAGAACCTGAATAC
 CCTCAGGCTGAGGCTACGGCGCTGTCATCGA^{ctc}CTTCCCTGTGAAAACAAGAGCAAGG
 CCGTGGAGCAGGTGAAGAATGCCTTTAATAAGCTCCAAGAGAAAGGCATCTACAAAGC
 CATGAGTGAGTTTGACATCTTCATCAACTACATAGAAGCCTACATGACAATGAAGATA
 CGAAAC (SEQ ID NO: 123)

[000238] Non-limiting exemplary amino acid sequences of IL10 are provided:

[000239] Wild-type IL10:

MHSSALLCCLVLLTGVRASPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQ
 LDNLLLKESLLEDFKGYLGQCQALSEMIQFYLEEVMPPQAENQDPDIKAHVNSLGENLKTLLR
 RLRRCHRLPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN (SEQ
 ID NO: 124)

[000240] High affinity IL10 (N36I, N110I, K117N, F129L):

MHSSALLCCLVLLTGVRASPGQGTQSENSCTHFPGILPNMLRDLRDAFSRVKTFFQMKDQL
 DNLLLKESLLEDFKGYLGQCQALSEMIQFYLEEVMPPQAENQDPDIKAHVISLGENLNTLLR
 LRRCHRLPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN (SEQ
 ID NO: 125)

[000241] Mature wild-type IL10

SPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYL
 GCQALSEMIQFYLEEVMPPQAENQDPDIKAHVNSLGENLKTLLRRLRRCHRLPCENKSKAV
 EQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN (SEQ ID NO: 231)

[000242] IL10 (D25K)

SPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYL
 GCQALSEMIQFYLEEVMPPQAENQDPDIKAHVNSLGENLKTLLRRLRRCHRLPCENKSKAV
 EQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN (SEQ ID NO: 232)

[000243] IL10 (D25E)

SPGQGTQSENSCTHFPGNLPNMLRELRLDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYLG
 CQALSEMIQFYLEEVMPPQAENQDPDIKAHVNSLGENLKTLLRRLRRCHRLPCENKSKAVE
 QVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN (SEQ ID NO: 233)

[000244] IL10 (D25K/E96A)

SPGQGTQSENSCTHFPGNLPNMLRKLRLDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYL
GCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGANLKTLLRLRLRRCHRFLPCENKSKA
VEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN (SEQ ID NO: 234)

[000245] Non-limiting exemplary nucleic acid sequences encoding TGFB1 are provided:

[000246] Wild-type TGFB1 (see paragraph [000145] and SEQ ID NO: 204 above)

[000247] Dual mutant TGFB1 (R218C, C225R)

[000248] Atgccgccctccgggctgcgggctgctgctgctgctgctaccgctgctgtggctactggctgacgcctggccggccgg
ccgcccggactatccacctgaagactatcgacatggagctggtgaagcgggaagcgcatcgaggccatccgcccggccagatcctgtccaagctg
cggctcgcgcagccccccgagccagggggaggtgcgcccggcccgtgcccaggccgtgctgcctgtacaacagcacccgcgaccg
ggtggccggggagagtgagaaaccggagcccagcctgaggccgactactacgccaaggaggtcacccgctgctaattggtgaaaccca
caacgaaatctatgacaagttaagcagagtacacacagcatatatgttcttaacacatcagagctccgagaagcgggtacctgaaccgctgtt
gtctccccgggagagctgcgtctgctgaggctcaagttaaaagtgagcagcacgtggagctgtaccagaaatacagcaacaattcctggcg
atacctcagcaaccggctgctggcaccagcgactcgccagagtgttatctttgatgtcaccggagttgtgcggcagtggtgagccgtgga
ggggaaattgagggctttTGCcttagcgcccactgctccAGAgacagcagggataacacactgcaagtggacatcaacgggttcactacc
ggccgcccagggtgacctggccaccattcatggcatgaaccggccttctctgtctcatggccaccccgctggagagggccagcatctgcaa
agctccccggcaccgcccagccctggacaccaactattgcttcagctccacggagaagaactgctgcgtgcggcagctgtacattgacttccgc
aaggacctcgctggaagtggatccagagcccaagggtaccatgccaactctgcctcgggccctgcccctacatttgagcctggacacg
cagtacagcaaggtcctgcccctgtacaaccagcataacccggcgccctcggcggcgccgtgctgcgtgcgcgagcgctggagccgctgc
ccatcgtgtactacgtggccgcaagccaaggtggagcagctgtccaacatgatcgtgcgctcctgcaagtgcagc (SEQ ID NO:
352)

[000249] Dual mutant TGFB1 (R218H, C225R) (see paragraph [000347] and SEQ ID NO:
[214] above).

[000250] Non-limiting exemplary nucleic acid sequences encoding CTLA4 are provided:

[000251] Wild-type CTLA4:

ATGGCCTGCTTGGGCTTCCAAAGGCATAAAGCCCAGCTTAATCTTGCTACTCGCACGTG
GCCCTGCACATTGCTCTTTTTCCTCCTGTTTCAATCCCGTGTTTTGCAAGGCGATGCATGT
GGCACAACCTGCCGTCGTTCTGGCATCATCAAGAGGTATTGCTAGCTTCGTTTGTGAGT
ACGCCTCCCCTGGAAAAGCGACGGAGGTGCGCGTCACTGTATTGCGGCAAGCCGACAG
CCAAGTTACTGAAGTCTGCGCGGCAACGTATATGATGGGCAATGAGCTGACATTCTT
GACGATTCAATCTGCACGGGAACAAGTAGTGGTAACCAGGTGAATCTCACTATTCAAG
GTCTGAGAGCCATGGACACCGGCCTCTACATTTGTAAGGTGGAGCTGATGTATCCTCCC
CCATATTATCTGGGGATCGGAAATGGGACACAGATATATGTTATTGATCCCGAGCCAT
GTCCCGATAGTGACTTCCTCTTGTGGATACTTGCCGCTGTGAGCAGTGGTTTGTGTTTTT

ATTCATTCCTCCTTACGGCAGTATCACTTTCAAAAATGCTCAAGAAGCGAAGTCCTTTG
ACAACCTGGCGTATATGTCAAAATGCCACCAACAGAGCCCGAATGTGAGAAACAGTTCC
AGCCGTACTTTATTCTATAAAC (SEQ ID NO: 126)

[000252] High affinity CTLA4 (belatacept; Binding domain: A29Y, L104E):

ATGGCCTGCTTGGGCTTCCAAAGGCATAAAGCCCAGCTTAATCTTGCTACTCGCACGTG
GCCCTGCACATTGCTCTTTTTCCTCCTGTTTCATTCCCGTGTTTTGCAAGGCGATGCATGT
GGCACAACCTGCCGTCGTTCTGGCATCATCAAGAGGTATTGCTAGCTTCGTTTGTGAGT
ACGCCTCCCCTGGAAAATACACGGAGGTGCGCGTCACTGTATTGCGGCAAGCCGACAG
CCAAGTTACTGAAGTCTGCGCGGCAACGTATATGATGGGCAATGAGCTGACATTCCTT
GACGATTCAATCTGCACGGGAACAAGTAGTGGTAACCAGGTGAATCTCACTATTCAAG
GTCTGAGAGCCATGGACACCGGCCTCTACATTTGTAAGGTGGAGCTGATGTATCCTCCC
CCATATTATGAGGGGATCGGAAATGGGACACAGATATATGTTATTGATCCCGAGCCAT
GTCCCGATAGTGACTTCCTCTTGTTGGATACTTGCCGCTGTGAGCAGTGTTTTGTTTTTTT
ATTCATTCCTCCTTACGGCAGTATCACTTTCAAAAATGCTCAAGAAGCGAAGTCCTTTG
ACAACCTGGCGTATATGTCAAAATGCCACCAACAGAGCCCGAATGTGAGAAACAGTTCC
AGCCGTACTTTATTCTATAAAC (SEQ ID NO: 127)

[000253] High affinity CTLA4 (Binding domain: A29H):

ATGGCCTGCTTGGGCTTCCAAAGGCATAAAGCCCAGCTTAATCTTGCTACTCGCACGTG
GCCCTGCACATTGCTCTTTTTCCTCCTGTTTCATTCCCGTGTTTTGCAAGGCGATGCATGT
GGCACAACCTGCCGTCGTTCTGGCATCATCAAGAGGTATTGCTAGCTTCGTTTGTGAGT
ACGCCTCCCCTGGAAAACATACGGAGGTGCGCGTCACTGTATTGCGGCAAGCCGACAG
CCAAGTTACTGAAGTCTGCGCGGCAACGTATATGATGGGCAATGAGCTGACATTCCTT
GACGATTCAATCTGCACGGGAACAAGTAGTGGTAACCAGGTGAATCTCACTATTCAAG
GTCTGAGAGCCATGGACACCGGCCTCTACATTTGTAAGGTGGAGCTGATGTATCCTCCC
CCATATTATCTGGGGATCGGAAATGGGACACAGATATATGTTATTGATCCCGAGCCAT
GTCCCGATAGTGACTTCCTCTTGTTGGATACTTGCCGCTGTGAGCAGTGTTTTGTTTTTTT
ATTCATTCCTCCTTACGGCAGTATCACTTTCAAAAATGCTCAAGAAGCGAAGTCCTTTG
ACAACCTGGCGTATATGTCAAAATGCCACCAACAGAGCCCGAATGTGAGAAACAGTTCC
AGCCGTACTTTATTCTATAAAC (SEQ ID NO: 128)

[000254] High affinity CTLA4 (Binding domain: K28H, A29H):

ATGGCCTGCTTGGGCTTCCAAAGGCATAAAGCCCAGCTTAATCTTGCTACTCGCACGTG
GCCCTGCACATTGCTCTTTTTCCTCCTGTTTCATTCCCGTGTTTTGCAAGGCGATGCATGT
GGCACAACCTGCCGTCGTTCTGGCATCATCAAGAGGTATTGCTAGCTTCGTTTGTGAGT
ACGCCTCCCCTGGACATCACACGGAGGTGCGCGTCACTGTATTGCGGCAAGCCGACAG
CCAAGTTACTGAAGTCTGCGCGGCAACGTATATGATGGGCAATGAGCTGACATTCCTT

GACGATTCAATCTGCACGGGAACAAGTAGTGGTAACCAGGTGAATCTCACTATTCAAG
 GTCTGAGAGCCATGGACACCGGCCTCTACATTTGTAAGGTGGAGCTGATGTATCCTCCC
 CCATATTATCTGGGGATCGGAAATGGGACACAGATATATGTTATTGATCCCGAGCCAT
 GTCCCGATAGTGACTTCCTCTTGTGGATACTTGCCGCTGTGAGCAGTGGTTTGTGTTTTTT
 ATTCATTCCTCCTTACGGCAGTATCACTTTCAAAAATGCTCAAGAAGCGAAGTCCTTTG
 ACAACTGGCGTATATGTCAAAATGCCACCAACAGAGCCCGAATGTGAGAAACAGTTCC
 AGCCGTACTTTATTCCTATAAAC (SEQ ID NO: 129)

[000255] Non-limiting exemplary amino acid sequences of CTLA4 are provided:

[000256] Wild-type CTLA4:

MACLGFRHKAQLNLATRTWPCTLLFFLLFIPVFCKAMHVAQPAVVLASSRGIA SFVCEYA
 SPGKATEVRVTVLQRADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRA
 MDTGLYICKVELMYPPPYLIGNGTQIYVIDPEPCPDSDFLLWILAAVSSGLFFYSFLLTAV
 SLSKMLKKRSPLTTGVYVKMPPEPECEKQFQPYFIPIN (SEQ ID NO: 130)

[000257] High affinity CTLA4 (belatacept; Binding domain: A29Y, L104E):

MACLGFRHKAQLNLATRTWPCTLLFFLLFIPVFCKAMHVAQPAVVLASSRGIA SFVCEYA
 SPGKYTEVRVTVLQRADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRA
 MDTGLYICKVELMYPPPYEGIGNGTQIYVIDPEPCPDSDFLLWILAAVSSGLFFYSFLLTAV
 SLSKMLKKRSPLTTGVYVKMPPEPECEKQFQPYFIPIN (SEQ ID NO: 131)

[000258] High affinity CTLA4 (Binding domain: A29H):

MACLGFRHKAQLNLATRTWPCTLLFFLLFIPVFCKAMHVAQPAVVLASSRGIA SFVCEYA
 SPGKHTEVRVTVLQRADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRA
 MDTGLYICKVELMYPPPYLIGNGTQIYVIDPEPCPDSDFLLWILAAVSSGLFFYSFLLTAV
 SLSKMLKKRSPLTTGVYVKMPPEPECEKQFQPYFIPIN (SEQ ID NO: 132)

[000259] High affinity CTLA4 (Binding domain: K28H, A29H):

MACLGFRHKAQLNLATRTWPCTLLFFLLFIPVFCKAMHVAQPAVVLASSRGIA SFVCEYA
 SPGHHTEVRVTVLQRADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRA
 MDTGLYICKVELMYPPPYLIGNGTQIYVIDPEPCPDSDFLLWILAAVSSGLFFYSFLLTAV
 SLSKMLKKRSPLTTGVYVKMPPEPECEKQFQPYFIPIN (SEQ ID NO: 133)

[000260] In some embodiments, the engineered T cells or population of T cells comprising a heterologous sequence encoding a dmTGFB1 under the control of a promoter; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA, and insertion into the cell

of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence, exhibits at least one suppressive activity of a naturally occurring regulatory T cell (nTreg), e.g., suppression of an immune response(s) or biomarker in an in vitro or in vivo assay, e.g., an animal model of GvHD. For example, in a mouse model of GvHD, mice receiving the engineered T cell comprising a heterologous sequence encoding a dmTGFB1 under the control of a promoter; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; and insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence, exhibit improved survival compared to a control, e.g., mice receiving PBMC. In certain embodiments, the regulatory T cell promoting molecule comprises IL10 or CTLA4. In certain embodiments, the regulatory T cell promoting molecule comprises IL10 and CTLA4.

[000261] In certain embodiments, the engineered T cells or population of T cells comprise two or more heterologous coding sequences, e.g., a dmTGFB1 and a regulatory T cell promoting molecule, under the control of a promoter. In certain embodiments, each heterologous coding sequence is under control of a separate promoter. In certain embodiments, two heterologous coding sequences are under control of the same promoter. In certain embodiments, two or more heterologous coding sequences are under control of the same promoter. In certain embodiments, when the engineered T cells or population of T cells comprise three or more heterologous coding sequences, e.g., a dmTGFB1 and a regulatory T cell promoting molecule, under the control of a promoter, each heterologous sequence is each independently under control of a separate promoter or under control of a promoter that controls expression of more than one heterologous coding sequence.

C. T Cell Receptor (TCR)

[000262] In some embodiments, the engineered T cells or population of T cells comprising a modification, e.g., insertion into the cell of heterologous sequence(s) encoding a dmTGFB1 molecule under control of a promoter sequence, further comprising a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s) and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2, further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s).

[000263] In some embodiments, the engineered T cells or population of T cells comprising a modification comprising an insertion into the cell of heterologous sequence(s) encoding a dmTGFB1 molecule under control of a promoter sequence and a modification, e.g., knockdown, of

an endogenous nucleic acid sequence encoding a TGFBR2 further comprising a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and/or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence; further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s).

[000264] In some embodiments, the engineered T cells or population of T cells comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion into the cell of heterologous sequences encoding a dmTGFB1 molecule and a regulatory T cell promoting molecule each under control of a promoter sequence, further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s).

[000265] In some embodiments, the engineered T cells or population of T cells comprising a modification comprising an insertion into the cell of heterologous sequence(s) encoding a dmTGFB1 molecule under control of a promoter sequence further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion into the cell of heterologous sequences encoding a dmTGFB1 molecule and a regulatory T cell promoting molecule each under control of a promoter sequence, insertion into the cell of heterologous sequence(s) encoding a targeting receptor; further comprises a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s).

[000266] Generally, a TCR is a heterodimer receptor molecule that contains two TCR polypeptide chains, α and β . Suitable α and β genomic sequences or loci to target for knockdown are known in the art. In some embodiments, the engineered T cells comprise a modification, e.g., knockdown, of a TCR α -chain gene sequence, e.g., TRAC. *See, e.g.*, NCBI Gene ID: 28755; Ensembl: ENSG00000277734 (T-cell receptor Alpha Constant), US 2018/0362975, and WO2020081613.

[000267] In some embodiments, the engineered T cells or population of T cells comprise a modification comprising insertion of a sequence encoding a dmTGFB1.

[000268] In some embodiments, the engineered T cells or population of T cells comprising an insertion into the cell of heterologous sequence(s) encoding a dmTGFB1 molecule under control of a promoter sequence and a modification, e.g., knockdown, of an endogenous nucleic acid sequence

encoding a TGFBR2 further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion of sequence(s) encoding a regulatory T cell promoting molecule selected from IL10, CTLA4, TIGIT, IDO1, ENTPD1, NT5E, IL22, AREG, IL35, GARP, CD274, FOXP3, IKZF2, EOS, IRF4, LEF1, BACH2, and IL2RA; and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s).

[000269] In some embodiments, the engineered T cells or population of T cells comprising an insertion into the cell of heterologous sequence(s) encoding a dmTGFB1 molecule under control of a promoter sequence and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2 further comprise; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA in combination with modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion of sequence(s) encoding a regulatory T cell promoting molecule selected from IL10, CTLA4, TIGIT, IDO1, ENTPD1, NT5E, IL22, AREG, IL35, GARP, CD274, FOXP3, IKZF2, EOS, IRF4, LEF1, BACH2, and; and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s).

[000270] In some embodiments, the engineered T cells or population of T cells comprising an insertion into the cell of heterologous sequence(s) encoding a dmTGFB1 molecule under control of a promoter sequence and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2 further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion of sequence(s) encoding IL10 or CTLA4, and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s).

[000271] In some embodiments, the engineered T cells or population of T cells comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion of sequence(s) encoding dmTGFB1, IL10, and CTLA4, and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s).

[000272] In some embodiments, the engineered T cells or population of T cells comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion of a sequence encoding a dmTGFB1 molecule, insertion of sequence(s) encoding a

regulatory T cell promoting molecule, and a modification, e.g., knockdown, of an endogenous TCR gene, e.g., a TRAC gene sequence.

[000273] In some embodiments, the engineered T cells or population of T cells comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion of sequence(s) encoding a dmTGFB1 molecule; insertion of sequence(s) encoding a regulatory T cell promoting molecule selected from IL10, CTLA4, TIGIT, IDO1, ENTPD1, NT5E, IL22, AREG, IL35, GARP, CD274, FOXP3, IKZF2, EOS, IRF4, LEF1, BACH2, and IL2RA; and a modification, e.g., knockdown, of an endogenous TCR gene, e.g., a TRAC gene sequence.

[000274] In some embodiments, the engineered T cells or population of T cells comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion of a sequence encoding a dmTGFB1 molecule, insertion of sequence(s) encoding IL10 or CTLA4, and a modification, e.g., knockdown, of a TCR gene, e.g., a TRAC gene sequence.

[000275] In any of these embodiments, the engineered T cells or population of T cells may further comprise insertion of sequence(s) encoding a targeting receptor as described herein, e.g., a CAR, e.g., a CAR targeting MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02).

[000276] In some embodiments, the engineered T cells or population of T cells comprising an insertion into the cell of heterologous sequence(s) encoding a dmTGFB1 molecule under control of a promoter sequence further comprise a modification, e.g., knockdown, of a TRC gene sequence by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 50%, 55%, 60%, 65%, at least 70%, 75%, 80%, 85%, 90%, or 95% of cells comprise an insertion, deletion, or substitution in the endogenous TRC gene sequence. In some embodiments, TRC is decreased by at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the TRC gene has not been modified. Assays for TRC protein and mRNA expression are known in the art.

[000277] In some embodiments, the engineered T cells or population of T cells comprise an insertion of sequence(s) encoding a targeting receptor by gene editing, e.g., as assessed by sequencing, e.g., NGS.

[000278] In some embodiments, a population of T cells comprises T cells that comprise an insertion into the cell of heterologous sequence(s) encoding a dmTGFB1 molecule under control of a promoter sequence are further engineered to comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2. In some embodiments, the population of T cells comprises T cells that further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding insertion of sequence(s) encoding a regulatory T cell promoting molecule, and/or a modification, e.g., knockdown, of at least one TCR gene sequence. In some embodiments, at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the T cells in the population of T cells are engineered to comprise a modification, e.g., knockdown, of at least one TCR gene sequence, e.g., as assessed by sequencing, e.g., NGS.

[000279] In some embodiments, a population of T cells comprises T cells that comprise an insertion into the cell of heterologous sequence(s) encoding a dmTGFB1 molecule under control of a promoter sequence and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2 are further engineered to comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion of sequence(s) encoding a regulatory T cell promoting molecule, and a modification, e.g., knockdown, of at least one TCR gene sequence. In some embodiments, at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the T cells in the population of T cells are engineered to comprise a modification, e.g., knockdown, of at least one TCR gene sequence, e.g., as assessed by sequencing, e.g., NGS.

[000280] In some embodiments, guide RNAs that specifically target sites within the TCR genes, e.g., TRAC gene, are used to provide a modification, e.g., knockdown, of the TCR genes.

[000281] In some embodiments, the TCR gene is modified, e.g., knocked down, in a T cell using a guide RNA with an RNA-guided DNA binding agent. In some embodiments, disclosed herein are T cells engineered by inducing a break (e.g., double-stranded break (DSB) or single-stranded break (nick)) within the TCR genes of a T cell, e.g., using a guide RNA with an RNA-guided DNA-binding agent (e.g., a CRISPR/Cas system). The methods may be used in vitro or ex vivo, e.g., in the manufacture of cell products for suppressing immune response.

[000282] In some embodiments, the guide RNAs mediate a target-specific cutting by an RNA-guided DNA-binding agent (e.g., Cas nuclease) at a site described herein within a TCR gene. It will be appreciated that, in some embodiments, the guide RNAs comprise guide sequences that bind to, or are capable of binding to, said regions.

D. Guide RNA

[000283] In any of the embodiments herein, the guide RNA may further comprise a trRNA. In each composition and method embodiment described herein, the crRNA and trRNA may be associated as a single RNA (sgRNA) or may be on separate RNAs (dgRNA). In the context of sgRNAs, the crRNA and trRNA components may be covalently linked, *e.g.*, via a phosphodiester bond or other covalent bond. In some embodiments, the sgRNA comprises one or more linkages between nucleotides that is not a phosphodiester linkage.

[000284] In each of the composition, use, and method embodiments described herein, the guide RNA may comprise two RNA molecules as a “dual guide RNA” or “dgRNA.” The dgRNA comprises a first RNA molecule comprising a crRNA comprising, *e.g.*, a guide sequence shown herein, and a second RNA molecule comprising a trRNA. The first and second RNA molecules may not be covalently linked, but may form an RNA duplex via the base pairing between portions of the crRNA and the trRNA.

[000285] In each of the composition, use, and method embodiments described herein, the guide RNA may comprise a single RNA molecule as a “single guide RNA” or “sgRNA.” The sgRNA may comprise a crRNA (or a portion thereof) comprising a guide sequence shown herein covalently linked to a trRNA. The sgRNA may comprise 15, 16, 17, 18, 19, or 20 contiguous nucleotides of a guide sequence shown herein. In some embodiments, the crRNA and the trRNA are covalently linked via a linker. In some embodiments, the sgRNA forms a stem-loop structure via the base pairing between portions of the crRNA and the trRNA. In some embodiments, the crRNA and the trRNA are covalently linked via one or more bonds that are not a phosphodiester bond.

[000286] In some embodiments, the trRNA may comprise all or a portion of a trRNA sequence derived from a naturally-occurring CRISPR/Cas system. In some embodiments, the trRNA comprises a truncated or modified wild-type trRNA. The length of the trRNA depends on the CRISPR/Cas system used. In some embodiments, the trRNA comprises or consists of 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more than 100 nucleotides. In some embodiments, the trRNA may comprise certain secondary structures, such as, for example, one or more hairpin or stem-loop structures, or one or more bulge structures.

[000287] In some embodiments, the target sequence or region may be complementary to the guide sequence of the guide RNA. In some embodiments, the degree of complementarity or identity between a guide sequence of a guide RNA and its corresponding target sequence may be 75%, 80%, 85%, 90%, 95%, or 100%. In some embodiments, the target sequence and the guide sequence of the gRNA may be 100% complementary or identical. In other embodiments, the target sequence and the guide sequence of the gRNA may contain one mismatch. For example, the target sequence and the guide sequence of the gRNA may contain 1, 2, 3, 4, or 5 mismatches, where the total length of the guide sequence is about 20, or 20. In some embodiments, the target sequence and the guide

sequence of the gRNA may contain 1-4 mismatches where the guide sequence is about 20, or 20 nucleotides. In some embodiments, the degree of complementarity or identity between a guide sequence and its corresponding target sequence is at least 80%, 85%, 90%, or 95%, for example when, the guide sequence comprises a sequence 24 contiguous nucleotides. In some embodiments, the guide sequence and the target region may be 100% complementary or identical. In other embodiments, the guide sequence and the target region may contain at least one mismatch, i.e., one nucleotide that is not identical or not complementary, depending on the reference sequence. For example, the guide sequence and the target sequence may contain 1-2, e.g., no more than 1 mismatch, where the total length of the target sequence is 19, 20, 21, 22, 23, or 24, nucleotides, or more. In some embodiments, the guide sequence and the target region may contain 1-2 mismatches where the guide sequence comprises at least 24 nucleotides, or more. In some embodiments, the guide sequence and the target region may contain 1-2 mismatches where the guide sequence comprises 24 nucleotides.

[000288] In any of the embodiments herein, each of the guide sequences herein may further comprise additional nucleotides to form a crRNA or guide RNA, *e.g.*, with the following exemplary nucleotide sequence following the guide sequence at its 3' end:

GUUUUAGAGCUAUGCUGUUUUG (SEQ ID NO: 134) in 5' to 3' orientation.

[000289] In the case of a sgRNA, the above guide sequences may further comprise additional nucleotides to form a sgRNA, *e.g.*, with the following exemplary nucleotide sequence following the 3' end of the guide sequence:

GUUUUAGAGCUAGAAAUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAA
AAGUGGCACCGAGUCGGUGCUUUU (SEQ ID NO: 135);

GUUUUAGAGCUAGAAAUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAA
AAGUGGCACCGAGUCGGUGC (SEQ ID NO: 136);

GUUUUAGAGCUAGAAAUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGGCA
CCGAGUCGGUGC (SEQ ID NO: 200);

GUUUUAGAGCUAGAAAUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCACGAAAGGG
CACCGAGUCGGUGC (SEQ ID NO: 201);

GUUGUAGCUCCCUUUCUCAUUUCGGAAACGAAUGAGAACCGUUGCUACAAUAAGG
CCGUCUGAAAAGAUGUGCCGCAACGCUCUGCCCCUAAAGCUUCUGCUUUAAGGGG
CAUCGUUUA (SEQ ID NO: 202);

GUUGUAGCUCCCUUGAAACCGUUGCUACAAUAAGGCCGUCGAAAGAUGUGCCGCAAC
GCUCUGCCUUCUGGCAUCGUU (SEQ ID NO: 203).

[000290] In any embodiments, the guide RNAs disclosed herein bind to a region upstream of a protospacer adjacent motif (PAM). As would be understood by those of skill in the art, the PAM sequence occurs on the strand opposite to the strand that contains the target sequence and varies with the CRISPR/Cas system. That is, the PAM sequence is on the complement strand of the target strand (the strand that contains the target sequence to which the guide RNA binds). In some embodiments, the PAM is selected from NGG, NNGRRT, NNGRR(N), NNAGAAW, NNNNG(A/C)TT, and NNNNRYAC, e.g., when the Cas system includes a SpyCas9. In other embodiments, PAM sequences include NCC, N4GAYW, N4GYTT, N4GTCT, NNNNCC(a), NNNNCAAA (wherein N is defined as any nucleotide, W is defined as either A or T, and R is defined as either A or G; and (a) is a preferred, but not required, A after the second C)), e.g., when the Cas system includes an NmeCas9.

[000291] In some embodiments, the guide RNA sequences provided herein are complementary to a sequence adjacent to a PAM sequence.

[000292] In some embodiments, the guide RNA sequence comprises a sequence that is complementary to a sequence within a genomic region selected from the tables herein according to coordinates in human reference genome hg38. In some embodiments, the guide RNA sequence comprises a sequence that is complementary to a sequence that comprises 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 consecutive nucleotides from within a genomic region selected from the tables herein. In some embodiments, the guide RNA sequence comprises a sequence that is complementary to a sequence that comprises 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 consecutive nucleotides spanning a genomic region selected from the tables herein.

[000293] The guide RNAs disclosed herein mediate a target-specific cutting resulting in a double-stranded break (DSB). The guide RNAs disclosed herein mediate a target-specific cutting resulting in a single-stranded break (SSB or nick).

E. Chemically Modified gRNA

[000294] In some embodiments, the gRNA is chemically modified. A gRNA comprising one or more modified nucleosides or nucleotides is called a “modified” gRNA or “chemically modified” gRNA, to describe the presence of one or more non-naturally or naturally occurring components or configurations that are used instead of or in addition to the canonical A, G, C, and U residues. In some embodiments, a modified gRNA is synthesized with a non-canonical nucleoside or nucleotide, is here called “modified.” Modified nucleosides and nucleotides can include one or more of: (i) alteration, e.g., replacement, of one or both of the non-linking phosphate oxygens or of one or more of the linking phosphate oxygens in the phosphodiester backbone linkage (an exemplary backbone modification); (ii) alteration, e.g., replacement, of a constituent of the ribose sugar, e.g., of the 2'

hydroxyl on the ribose sugar (an exemplary sugar modification); (iii) modification or replacement of a naturally occurring nucleobase, including with a non-canonical nucleobase (an exemplary base modification); and (iv) modification of the 3' end or 5' end of the oligonucleotide to provide exonuclease stability, e.g., with 2' O-me, 2' halide, or 2' deoxy substituted ribose; or inverted abasic terminal nucleotide, or replacement of phosphodiester with phosphothioate.

[000295] Chemical modifications such as those listed above can be combined to provide modified gRNAs comprising nucleosides and nucleotides (collectively “residues”) that can have two, three, four, or more modifications. For example, a modified residue can have a modified sugar and a modified nucleobase. In some embodiments, every base of a gRNA is modified, *e.g.*, all bases have a modified phosphate group, such as a phosphorothioate group. In certain embodiments, all, or substantially all, of the phosphate groups of an gRNA molecule are replaced with phosphorothioate groups. In some embodiments, modified gRNAs comprise at least one modified residue at or near the 5' end of the RNA. In some embodiments, modified gRNAs comprise at least one modified residue at or near the 3' end of the RNA. Certain gRNAs comprise at least one modified residue at or near the 5' end and 3' end of the RNA.

[000296] In some embodiments, the guide RNAs disclosed herein comprise one of the modification patterns disclosed in WO2018107028 the contents of which are hereby incorporated by reference in relevant part. In some embodiments, the guide RNAs disclosed herein comprise one of the structures/modification patterns disclosed in US20170114334, which are hereby incorporated by reference. In some embodiments, the guide RNAs disclosed herein comprise one of the structures/modification patterns disclosed in WO2017136794, WO2017004279, WO2019237069, US2018187186, US2019048338, WO2021119275, or WO2022125968, which are hereby incorporated by reference.

F. mRNAs Encoding RNA-guided DNA-Binding Agents

[000297] In some embodiments, a cell or method comprises an mRNA comprising an open reading frame (ORF) encoding an RNA-guided DNA-binding agent, such as a Cas nuclease as described herein. Cas9 ORFs are provided herein and are known in the art. As one example, the Cas9 ORF can be codon optimized, such that coding sequence includes one or more alternative codons for one or more amino acids. An “alternative codon” as used herein refers to variations in codon usage for a given amino acid, and may or may not be a preferred or optimized codon (codon optimized) for a given expression system. Preferred codon usage, or codons that are well-tolerated in a given system of expression, is known in the art. The Cas9 coding sequences, Cas9 mRNAs, and Cas9 protein sequences of WO2013/176772, WO2014/065596, WO2016/106121, WO2019/067910 and WO2022/125968 are hereby incorporated by reference. In particular, the ORFs and Cas9 amino

acid sequences of the table at paragraph [0449] WO2019/067910, and the Cas9 mRNAs and ORFs of paragraphs [0214] – [0234] of WO2019/067910 are hereby incorporated by reference.

[000298] In some embodiments, the modified ORF may comprise a modified uridine at least at one, a plurality of, or all uridine positions. In some embodiments, the modified uridine is a uridine modified at the 5 position, *e.g.*, with a halogen, methyl, or ethyl. In some embodiments, the modified uridine is a pseudouridine modified at the 1 position, *e.g.*, with a halogen, methyl, or ethyl. The modified uridine can be, for example, pseudouridine, N1-methyl-pseudouridine, 5-methoxyuridine, 5-iodouridine, or a combination thereof. In some embodiments, the modified uridine is 5-methoxyuridine. In some embodiments, the modified uridine is 5-iodouridine. In some embodiments, the modified uridine is pseudouridine. In some embodiments, the modified uridine is N1-methyl-pseudouridine. In some embodiments, the modified uridine is a combination of pseudouridine and N1-methyl-pseudouridine. In some embodiments, the modified uridine is a combination of pseudouridine and 5-methoxyuridine. In some embodiments, the modified uridine is a combination of N1-methyl pseudouridine and 5-methoxyuridine. In some embodiments, the modified uridine is a combination of 5-iodouridine and N1-methyl-pseudouridine. In some embodiments, the modified uridine is a combination of pseudouridine and 5-iodouridine. In some embodiments, the modified uridine is a combination of 5-iodouridine and 5-methoxyuridine.

[000299] In some embodiments, an mRNA disclosed herein comprises a 5' cap, such as a Cap0, Cap1, or Cap2. A 5' cap is generally a 7-methylguanine ribonucleotide (which may be further modified, for example, ARCA (anti-reverse cap analog; Thermo Fisher Scientific Cat. No. AM8045) is a cap analog comprising a 7-methylguanine 3'-methoxy-5'-triphosphate linked to the 5' position of a guanine ribonucleotide) linked through a 5'-triphosphate to the 5' position of the first nucleotide of the 5'-to-3' chain of the mRNA, *i.e.*, the first cap-proximal nucleotide. In Cap0, the riboses of the first and second cap-proximal nucleotides of the mRNA both comprise a 2'-hydroxyl. In Cap1, the riboses of the first and second transcribed nucleotides of the mRNA comprise a 2'-methoxy and a 2'-hydroxyl, respectively. See, *e.g.*, CleanCap™ AG (m7G(5')ppp(5')(2'OMeA)pG; TriLink Biotechnologies Cat. No. N-7113) or CleanCap™ GG (m7G(5')ppp(5')(2'OMeG)pG; TriLink Biotechnologies Cat. No. N-7133). In Cap2, the riboses of the first and second cap-proximal nucleotides of the mRNA both comprise a 2'-methoxy. See, *e.g.*, Katibah et al. (2014) *Proc Natl Acad Sci USA* 111(33):12025-30; Abbas et al. (2017) *Proc Natl Acad Sci USA* 114(11):E2106-E2115.

[000300] In some embodiments, the mRNA further comprises a poly-adenylated (poly-A) tail. In some embodiments, the poly-A tail comprises 20, 30, 40, 50, 60, 70, 80, 90, or 100 adenines (SEQ ID NO: 147), optionally up to 300 adenines (SEQ ID NO: 148). In some embodiments, the poly-A tail comprises 95, 96, 97, 98, 99, or 100 adenine nucleotides (SEQ ID NO: 149).

G. T Cells for Engineering

[000301] The engineered cells provided herein are prepared from a population of cells enriched for T cells. In some embodiments, the T cells are CD4⁺ T cells. The CD4⁺ T cells can be autologous or allogeneic cells. Such cells can be readily obtained from fresh leukopak samples, commercially available from various sources including, e.g., StemCell Technologies. CD4⁺ T cells can be isolated using commercially available kits using routine methods, e.g., by negative selection using the human CD4⁺ T cell isolation kit. However, methods of preparation of CD4⁺ T cells from other sources are also known in the art. For example, 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) can be used to obtain CD4⁺ T cells. Multipotent cells are capable of developing into more than one cell type, but are more limited than pluripotent cells in breadth of differentiation. The multipotent cells may be derived from established cell lines or isolated from human bone marrow or umbilical cords. For 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). Methods to promote differentiation into CD4⁺ T cells are known in the art. T cells engineered from CD4⁺ T cells as described herein can yield, e.g., greater than 500 times, greater than 600 times, greater than 700 times, greater than 800 times, greater than 900 times, or greater than 1000 times more cells as measured, e.g., 8, 9, 10, 11, 12, 13, 14, 15 days post first day of editing, as compared to expanding tTregs according to methods known in the art.

[000302] In some embodiments, the engineered are a population of cells enriched for CD8⁺ T cells. The CD8⁺ T cells can be autologous or allogeneic cells. As with CD4⁺ T cells, the CD8⁺ T cells may be isolated from a sample, e.g., a commercially available sample or a patient sample, or may be prepared from other sources according to methods known in the art. For example, sources may include 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). Methods to promote differentiation into CD8⁺ T cells are known in the art. T cells engineered from CD8⁺ T cells as described herein can yield, e.g., greater than 500 times, greater than 600 times, greater than 700 times, greater than 800 times, greater than 900 times, or greater than 1000 times more cells as measured, e.g., 8, 9, 10, 11, 12, 13, 14, 15 days post first day of editing, as compared to expanding tTregs according to methods known in the art.

III. Method of Delivery

[000303] The guide RNA, RNA-guided DNA binding agents (*e.g.*, Cas nuclease), and nucleic acid sequences disclosed herein can be delivered to a cell or population of cells, *in vitro* or *ex vivo*, for the production of engineered T cells comprising insertion of a sequence encoding a dmTGFB1; optionally further comprising a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TNFA a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding an IFNG or an IL17A, insertion of sequence(s) encoding a regulatory T cell promoting molecule, *e.g.*, IL10, CTLA4; and optionally further comprising insertion of sequence(s) encoding a targeting receptor, *e.g.*, a CAR, and optionally further comprising a modification, *e.g.*, knockdown, of TCR sequence(s), using various known and suitable methods available in the art. The guide RNA, RNA-guided DNA binding agents, and nucleic acid constructs can be delivered individually or together in any combination, using the same or different delivery methods as appropriate.

[000304] Conventional viral and non-viral based gene delivery methods can be used to introduce the guide RNA as well as the RNA-guided DNA-binding agent and donor construct in cells (*e.g.*, mammalian cells) and target tissues. As further provided herein, non-viral vector delivery systems nucleic acids such as non-viral vectors, plasmid vectors, and, *e.g.*, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome, lipid nanoparticle (LNP), or poloxamer. Viral vector delivery systems include DNA and RNA viruses.

[000305] Methods and compositions for non-viral delivery of nucleic acids include electroporation, lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, LNPs, polycation or lipid:nucleic acid conjugates, naked nucleic acid (*e.g.*, naked DNA/RNA), artificial virions, and agent-enhanced uptake of DNA. Sonoporation using, *e.g.*, the Sonitron 2000 system (Rich-Mar) can also be used for delivery of nucleic acids.

[000306] Various delivery systems (*e.g.*, vectors, liposomes, LNPs) containing the guide RNAs, RNA-guided DNA binding agent, and donor construct, singly or in combination, can also be administered to a cell or cell culture *ex vivo*. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood, fluid, or cells including, but not limited to, injection, infusion, topical application and electroporation. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art.

[000307] In certain embodiments, the present disclosure provides DNA or RNA vectors encoding any of the compositions disclosed herein *e.g.*, guide RNAs comprising any one or more of the guide sequences described herein, *e.g.*, for modifying (*e.g.*, knocking down) TGFBR2, IFNG and TNFA or a donor construct comprising a sequence encoding a dmTGFB1 molecule,

sequence(s) encoding a regulatory T cell promoting molecule, e.g., IL10 or CTLA4, or a targeting receptor, e.g., a CAR, e.g., a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), desmoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR. In some embodiments, the vector also comprises a sequence encoding an RNA-guided DNA binding agent. In certain embodiments, the DNA or RNA vectors encode any one or more of the compositions described herein, or in any combination. In some embodiments, the vectors further comprise, *e.g.*, promoters, enhancers, and regulatory sequences. In some embodiments, the vector that comprises a guide RNA comprising any one or more of the guide sequences described herein also comprises one or more nucleotide sequence(s) encoding a crRNA, a trRNA, or a crRNA and trRNA, as disclosed herein.

[000308] In certain embodiments, the present disclosure provides DNA or RNA vectors encoding a regulatory T cell promoting molecules and a targeting receptor. Such vectors allow for selection of cells based on the presence of the receptor for cells that also contain a coding sequence for the regulatory T cell promoting molecule. Positive and negative selection methods based on the presence of cell surface molecules are known in the art.

[000309] In some embodiments, the vector comprises a nucleotide sequence encoding a guide RNA described herein. In some embodiments, the vector comprises one copy of the guide RNA. In other embodiments, the vector comprises more than one copy of the guide RNA. In embodiments with more than one guide RNA, the guide RNAs may be non-identical such that they target different target sequences, or may be identical in that they target the same target sequence. In some embodiments where the vectors comprise more than one guide RNA, each guide RNA may have other different properties, such as activity or stability within a complex with an RNA-guided DNA nuclease, such as a Cas RNP complex. In some embodiments, the nucleotide sequence encoding the guide RNA may be operably linked to at least one transcriptional or translational control sequence, such as a promoter, a 3' UTR, or a 5' UTR. In one embodiment, the promoter may be a tRNA promoter, *e.g.*, tRNA^{Lys3}, or a tRNA chimera. *See* Mefferd et al., *RNA*. 2015 21:1683-9; Scherer et al., *Nucleic Acids Res.* 2007 35: 2620–2628. In some embodiments, the promoter may be recognized by RNA polymerase III (Pol III). Non-limiting examples of Pol III promoters include U6 and H1 promoters. In some embodiments, the nucleotide sequence encoding the guide RNA may be operably linked to a mouse or human U6 promoter. In other embodiments, the nucleotide sequence encoding the guide RNA may be operably linked to a mouse or human H1 promoter. In embodiments with more than one guide RNA, the promoters used to drive expression may be the same or different. In some embodiments, the nucleotide encoding the crRNA of the guide RNA and

the nucleotide encoding the trRNA of the guide RNA may be provided on the same vector. In some embodiments, the nucleotide encoding the crRNA and the nucleotide encoding the trRNA may be driven by the same promoter. In some embodiments, the crRNA and trRNA may be transcribed into a single transcript. For example, the crRNA and trRNA may be processed from the single transcript to form a double-molecule guide RNA. Alternatively, the crRNA and trRNA may be transcribed into a single-molecule guide RNA (sgRNA). In other embodiments, the crRNA and the trRNA may be driven by their corresponding promoters on the same vector. In yet other embodiments, the crRNA and the trRNA may be encoded by different vectors.

[000310] In some embodiments, the nucleotide sequence encoding the guide RNA may be located on the same vector comprising the nucleotide sequence encoding an RNA-guided DNA-binding agent such as a Cas protein. In some embodiments, expression of the guide RNA and of the RNA-guided DNA-binding agent such as a Cas protein may be driven by their own corresponding promoters. In some embodiments, expression of the guide RNA may be driven by the same promoter that drives expression of the RNA-guided DNA-binding agent such as a Cas protein. In some embodiments, the guide RNA and the RNA-guided DNA-binding agent such as a Cas protein transcript may be contained within a single transcript. For example, the guide RNA may be within an untranslated region (UTR) of the RNA-guided DNA-binding agent such as a Cas protein transcript. In some embodiments, the guide RNA may be within the 5' UTR of the transcript. In other embodiments, the guide RNA may be within the 3' UTR of the transcript. In some embodiments, the intracellular half-life of the transcript may be reduced by containing the guide RNA within its 3' UTR and thereby shortening the length of its 3' UTR. In additional embodiments, the guide RNA may be within an intron of the transcript. In some embodiments, suitable splice sites may be added at the intron within which the guide RNA is located such that the guide RNA is properly spliced out of the transcript. In some embodiments, expression of the RNA-guided DNA-binding agent such as a Cas protein and the guide RNA from the same vector in close temporal proximity may facilitate more efficient formation of the CRISPR RNP complex.

[000311] In some embodiments, the nucleotide sequence encoding the guide RNA or RNA-guided DNA-binding agent may be located on the same vector comprising the construct that comprises the sequence encoding the dmTGFB1 molecule, the regulatory T cell promoting molecule, e.g., IL10, CTLA4; or targeting receptor, e.g., a CAR, e.g., a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demogelin 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR. In some embodiments, proximity of the construct comprising the sequence encoding the dmTGFB1 molecule, the regulatory T cell promoting molecule, e.g., IL10,

CTLA4; or targeting receptor, e.g., a CAR, e.g., a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demogelin 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR and the guide RNA (or the RNA-guided DNA binding agent) on the same vector may facilitate more efficient insertion of the construct into a site of insertion created by the guide RNA/RNA-guided DNA binding agent.

[000312] In certain embodiments, DNA and RNA vectors can include more than one open reading frame for expression under a single promoter, either present in the vector or at the genomic insertion site. In such embodiments, a coding sequence for a self-cleaving peptide can be included between the open reading frames. The self-cleaving peptide may be, for example, a 2A peptide, for example, a P2A peptide, an E2A peptide, a F2A peptide, or a T2A peptide.

[000313] In some embodiments, the vector comprises one or more nucleotide sequence(s) encoding a sgRNA and an mRNA encoding an RNA-guided DNA binding agent, which can be a Cas protein, such as Cas9 or Cpf1. In some embodiments, the vector comprises one or more nucleotide sequence(s) encoding a crRNA, a trRNA, and an mRNA encoding an RNA-guided DNA binding agent, which can be a Cas protein, such as, Cas9 or Cpf1. In one embodiment, the Cas9 is from *Streptococcus pyogenes* (i.e., Spy Cas9). In some embodiments, the Cas nuclease is the Cas9 nuclease from *Neisseria meningitidis* (i.e., Nme Cas9, e.g., Nme1, Nme2, or Nme3 Cas9). In some embodiments, the nucleotide sequence encoding the crRNA, trRNA, or crRNA and trRNA (which may be a sgRNA) comprises or consists of a guide sequence flanked by all or a portion of a repeat sequence from a naturally-occurring CRISPR/Cas system. The nucleic acid comprising or consisting of the crRNA, trRNA, or crRNA and trRNA may further comprise a vector sequence wherein the vector sequence comprises or consists of nucleic acids that are not naturally found together with the crRNA, trRNA, or crRNA and trRNA.

[000314] In some embodiments, the crRNA and the trRNA are encoded by non-contiguous nucleic acids within one vector. In other embodiments, the crRNA and the trRNA may be encoded by a contiguous nucleic acid. In some embodiments, the crRNA and the trRNA are encoded by opposite strands of a single nucleic acid. In other embodiments, the crRNA and the trRNA are encoded by the same strand of a single nucleic acid.

[000315] In some embodiments, the vector comprises a donor construct comprising a sequence that encodes the dmTGFB1 molecule, the regulatory T cell promoting molecule, e.g., IL10 or CTLA4, or targeting receptor, e.g., a CAR, e.g., MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein),

proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR, as disclosed herein. In some embodiments, in addition to the donor construct disclosed herein, the vector may further comprise nucleic acids that encode the guide RNAs described herein or nucleic acid encoding an RNA-guided DNA-binding agent (*e.g.*, a Cas nuclease such as Cas9). In some embodiments, a nucleic acid encoding an RNA-guided DNA-binding agent are each or both on a separate vector from a vector that comprises the donor construct disclosed herein. In any of the embodiments, the vector may include other sequences that include, but are not limited to, promoters, enhancers, regulatory sequences, as described herein. In some embodiments, the promoter does not drive the expression of the regulatory T cell promoting molecule, *e.g.*, IL10 or CTLA4, or targeting receptor, *e.g.*, a CAR, *e.g.*, MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR, of the donor construct. In some embodiments, the vector comprises one or more nucleotide sequence(s) encoding a crRNA, a trRNA, or a crRNA and trRNA. In some embodiments, the vector comprises one or more nucleotide sequence(s) encoding a sgRNA and an mRNA encoding an RNA-guided DNA nuclease, which can be a Cas nuclease (*e.g.*, Cas9). In some embodiments, the vector comprises one or more nucleotide sequence(s) encoding a crRNA, a trRNA, and an mRNA encoding an RNA-guided DNA nuclease, which can be a Cas nuclease, such as, Cas9. In some embodiments, the Cas9 is from *Streptococcus pyogenes* (*i.e.*, Spy Cas9). In some embodiments, the Cas nuclease is the Cas9 nuclease from *Neisseria meningitidis*. In some embodiments, the nucleotide sequence encoding the crRNA, trRNA, or crRNA and trRNA (which may be a sgRNA) comprises or consists of a guide sequence flanked by all or a portion of a repeat sequence from a naturally occurring CRISPR/Cas system. The nucleic acid comprising or consisting of the crRNA, trRNA, or crRNA and trRNA may further comprise a vector sequence wherein the vector sequence comprises or consists of nucleic acids that are not naturally found together with the crRNA, trRNA, or crRNA and trRNA.

[000316] In some embodiments, the vector may be circular. In other embodiments, the vector may be linear. In some embodiments, the vector may be enclosed in a lipid nanoparticle, liposome, non-lipid nanoparticle, or viral capsid. Non-limiting exemplary vectors include plasmids, phagemids, cosmids, artificial chromosomes, minichromosomes, transposons, viral vectors, and expression vectors.

[000317] In some embodiments, the vector may be a viral vector. In some embodiments, the viral vector may be genetically modified from its wild-type counterpart. For example, the viral vector may comprise an insertion, deletion, or substitution of one or more nucleotides to facilitate cloning or such that one or more properties of the vector is changed. Such properties may include packaging capacity, transduction efficiency, immunogenicity, genome integration, replication, transcription, and translation. In some embodiments, a portion of the viral genome may be deleted such that the virus is capable of packaging exogenous sequences having a larger size. In some embodiments, the viral vector may have an enhanced transduction efficiency. In some embodiments, the immune response induced by the virus in a may be reduced. In some embodiments, viral genes (such as, *e.g.*, integrase) that promote integration of the viral sequence into a genome may be mutated such that the virus becomes non-integrating. In some embodiments, the viral vector may be replication defective. In some embodiments, the viral vector may comprise exogenous transcriptional or translational control sequences to drive expression of coding sequences on the vector. In some embodiments, the virus may be helper-dependent. For example, the virus may need one or more helper virus to supply viral components (such as, *e.g.*, viral proteins) required to amplify and package the vectors into viral particles. In such a case, one or more helper components, including one or more vectors encoding the viral components, may be introduced into a cell or population of cells along with the vector system described herein. In other embodiments, the virus may be helper-free. For example, the virus may be capable of amplifying and packaging the vectors without a helper virus. In some embodiments, the vector system described herein may also encode the viral components required for virus amplification and packaging.

[000318] Non-limiting exemplary viral vectors include adeno-associated virus (AAV) vector, lentivirus vectors, adenovirus vectors, helper dependent adenoviral vectors (HDAd), herpes simplex virus (HSV-1) vectors, bacteriophage T4, baculovirus vectors, and retrovirus vectors. In some embodiments, the viral vector may be an AAV vector. In other embodiments, the viral vector may be a lentivirus vector.

[000319] In some embodiments, “AAV” refers all serotypes, subtypes, and naturally occurring AAV as well as recombinant AAV. “AAV” may be used to refer to the virus itself or a derivative thereof. The term “AAV” includes AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV6.2, AAV7, AAVrh.64R1, AAVhu.37, AAVrh.8, AAVrh.32.33, AAV8, AAV9, AAV-DJ, AAV2/8, AAVrh10, AAVLK03, AV10, AAV11, AAV12, rh10, and hybrids thereof, avian AAV, bovine AAV, canine AAV, equine AAV, primate AAV, nonprimate AAV, and ovine AAV. In certain embodiments, the AAV is acceptable for use in ex vivo applications for human cells. In certain embodiments, the AAV is AAV6. The genomic sequences of various serotypes of AAV, as well as the sequences of the native terminal repeats (TRs), Rep proteins, and capsid subunits are known in the art. Such sequences may be found in the literature or in public databases such as

GenBank. A “AAV vector” as used herein refers to an AAV vector comprising a heterologous sequence not of AAV origin (*i.e.*, a nucleic acid sequence heterologous to AAV), typically comprising a sequence encoding a heterologous polypeptide of interest. The construct may comprise an AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV6.2, AAV7, AAVrh.64R1, AAVhu.37, AAVrh.8, AAVrh.32.33, AAV8, AAV9, AAV-DJ, AAV2/8, AAVrh10, AAVLK03, AV10, AAV11, AAV12, rh10, and hybrids thereof, avian AAV, bovine AAV, canine AAV, equine AAV, primate AAV, nonprimate AAV, and ovine AAV capsid sequence. In general, the heterologous nucleic acid sequence (the transgene) is flanked by at least one, and generally by two, AAV inverted terminal repeat sequences (ITRs). An AAV vector may either be single-stranded (ssAAV) or self-complementary (scAAV).

[000320] In some embodiments, the lentivirus may be integrating. In some embodiments, the lentivirus may be non-integrating. In some embodiments, the viral vector may be an adenovirus vector. In some embodiments, the adenovirus may be a high-cloning capacity or “gutless” adenovirus, where all coding viral regions apart from the 5' and 3' inverted terminal repeats (ITRs) and the packaging signal ('I') are deleted from the virus to increase its packaging capacity. In yet other embodiments, the viral vector may be an HSV-1 vector. In some embodiments, the HSV-1-based vector is helper dependent, and in other embodiments it is helper independent. For example, an amplicon vector that retains only the packaging sequence requires a helper virus with structural components for packaging, while a 30kb-deleted HSV-1 vector that removes non-essential viral functions does not require helper virus. In additional embodiments, the viral vector may be bacteriophage T4. In some embodiments, the bacteriophage T4 may be able to package any linear or circular DNA or RNA molecules when the head of the virus is emptied. In further embodiments, the viral vector may be a baculovirus vector. In yet further embodiments, the viral vector may be a retrovirus vector. In embodiments using AAV or other vectors, which have smaller cloning capacity, more than one vector may be used to deliver all the components of a vector system as disclosed herein. For example, one AAV vector may contain sequences encoding an RNA-guided DNA-binding agent such as a Cas protein (*e.g.*, Cas9), while a second AAV vector may contain one or more guide sequences.

[000321] In some embodiments, the vector system may be capable of driving expression of one or more nuclease components in a cell. In some embodiments, the vector does not comprise a promoter that drives expression of one or more coding sequences once it is integrated in a cell (*e.g.*, uses the cell's endogenous promoter such as when inserted at specific genomic loci of the cell, as exemplified herein). Suitable promoters to drive expression in different types of cells, *e.g.*, CD4+ T cells, are known in the art. In some embodiments, the promoter may be wild-type. In other embodiments, the promoter may be modified for more efficient or efficacious expression. In yet other embodiments, the promoter may be truncated yet retain its function. For example, the

promoter may have a normal size or a reduced size that is suitable for proper packaging of the vector into a virus.

[000322] In some embodiments, the vector may comprise a nucleotide sequence encoding an RNA-guided DNA-binding agent such as a Cas protein (*e.g.*, Cas9) described herein. In some embodiments, the nuclease encoded by the vector may be a Cas protein. In some embodiments, the vector system may comprise one copy of the nucleotide sequence encoding the nuclease. In other embodiments, the vector system may comprise more than one copy of the nucleotide sequence encoding the nuclease. In some embodiments, the nucleotide sequence encoding the nuclease may be operably linked to at least one transcriptional or translational control sequence. In some embodiments, the nucleotide sequence encoding the nuclease may be operably linked to at least one promoter.

[000323] In some embodiments, the vector may comprise any one or more of the constructs comprising a sequence encoding the dmTGFB1 molecule; the regulatory T cell promoting molecule, *e.g.*, IL10, CTLA4; or targeting receptor, *e.g.*, a CAR, *e.g.*, a MAdCAM-1 CAR, as described herein. In some embodiments, the sequence of the dmTGFB1 molecule, the regulatory T cell promoting molecule, *e.g.*, IL10, CTLA4; or targeting receptor, *e.g.*, a CAR, *e.g.*, a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demogelin 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR, may be operably linked to at least one transcriptional or translational control sequence. In some embodiments, the sequence of the dmTGFB1 molecule, the regulatory T cell promoting molecule, *e.g.*, IL10, CTLA4; or targeting receptor, *e.g.*, a CAR, *e.g.*, a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demogelin 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR may be operably linked to at least one promoter. In some embodiments, the sequence of the dmTGFB1 molecule, the regulatory T cell promoting molecule, *e.g.*, IL10, CTLA4; or targeting receptor, *e.g.*, a CAR, *e.g.*, a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demogelin 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR, is not linked to a promoter that drives the expression of the heterologous gene.

[000324] In some embodiments, the promoter may be constitutive, inducible, or tissue-specific. In some embodiments, the promoter may be a constitutive promoter. Non-limiting exemplary constitutive promoters include cytomegalovirus immediate early promoter (CMV), simian virus (SV40) promoter, adenovirus major late (MLP) promoter, Rous sarcoma virus (RSV) promoter, mouse mammary tumor virus (MMTV) promoter, phosphoglycerate kinase (PGK) promoter, elongation factor- α (EF1 α) promoter, ubiquitin promoters, actin promoters, tubulin promoters, immunoglobulin promoters, a functional fragment thereof, or a combination of any of the foregoing. In some embodiments, the promoter may be a CMV promoter. In some embodiments, the promoter may be a truncated CMV promoter. In other embodiments, the promoter may be an EF1 α promoter. In some embodiments, the promoter may be an inducible promoter. Non-limiting exemplary inducible promoters include those inducible by heat shock, light, chemicals, peptides, metals, steroids, antibiotics, or alcohol. In some embodiments, the inducible promoter may be one that has a low basal (non-induced) expression level, such as, *e.g.*, the Tet-On[®] promoter (Clontech).

[000325] In certain embodiments, the promoter is acceptable for use in *ex vivo* applications in human cells.

[000326] In some embodiments, the promoter may be a tissue-specific promoter, *e.g.*, a promoter specific for expression in a T cell.

[000327] In some embodiments, the compositions comprise a vector system. In some embodiments, the vector system may comprise one single vector. In other embodiments, the vector system may comprise two vectors. In additional embodiments, the vector system may comprise three vectors. When different guide RNAs are used for multiplexing, or when multiple copies of the guide RNA are used, the vector system may comprise more than three vectors.

[000328] In some embodiments, the vector system may comprise inducible promoters to start expression only after it is delivered to a target cell. Non-limiting exemplary inducible promoters include those inducible by heat shock, light, chemicals, peptides, metals, steroids, antibiotics, or alcohol. In some embodiments, the inducible promoter may be one that has a low basal (non-induced) expression level, such as, *e.g.*, the Tet-On[®] promoter (Clontech).

[000329] In additional embodiments, the vector system may comprise tissue-specific promoters.

[000330] Non-limiting exemplary viral vector sequences are provided below:

[000331] CTLA4 insert (nucleotide sequence)

ATGGCCTGCTTGGGCTTCCAAAGGCATAAAGCCCAGCTTAATCTTGCTACTCGCACGTG
GCCCTGCACATTGCTCTTTTTCCTCCTGTTTCATTCCCGTGTTTTGCAAGGCGATGCATGT

GGCACAACCTGCCGTCGTTCTGGCATCATCAAGAGGTATTGCTAGCTTCGTTTGTGAGT
ACGCCTCCCCTGGAAAAGCGACGGAGGTGCGCGTCACTGTATTGCGGCAAGCCGACAG
CCAAGTTACTGAAGTCTGCGCGGCAACGTATATGATGGGCAATGAGCTGACATTCTT
GACGATTCAATCTGCACGGGAACAAGTAGTGGTAACCAGGTGAATCTCACTATTCAAG
GTCTGAGAGCCATGGACACCGGCCTCTACATTTGTAAGGTGGAGCTGATGTATCCTCCC
CCATATTATCTGGGGATCGGAAATGGGACACAGATATATGTTATTGATCCCGAGCCAT
GTCCCGATAGTGACTTCCTCTTGTGGATACTTGCCGCTGTGAGCAGTGGTTTGTTTTTT
ATTCAATCCTCCTTACGGCAGTATCACTTTCAAAAATGCTCAAGAAGCGAAGTCCTTG
ACAACCTGGCGTATATGTCAAAAATGCCACCAACAGAGCCCGAATGTGAGAAACAGTTCC
AGCCGTACTTTATTCTATAAACTGA (SEQ ID NO: 137)

[000332] CTLA4 insert (amino acid sequence)

MACLGFRHKAQLNLATRTWPCTLLFLLFIPVFCKAMHVAQPAVVLASSRGIA SFVCEYA
SPGKATEVRVTVLRRQADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVNLTIQGLRA
MDTGLYICKVELMYPPPYLIGINGTQIYVIDPEPCPDSDFLWILAAVSSGLFFYSFLLTAV
SLSKMLKKRSPLTTGVYVKMPPEPECEKQFQPYFIPIN (SEQ ID NO: 130)

[000333] IL10 insert (nucleotide sequence)

ATGCACAGCTCAGCACTGCTCTGTTGCCTGGTCCTCTGACTGGGGTGAGGGCCAGCCC
AGGCCAGGGCACCCAGTCTGAGAACAGCTGCACCCACTTCCCAGGCAACCTGCCTAAC
ATGCTTCGAGATCTCCGAGATGCCTTCAGCAGAGTGAAGACTTTCTTTCAAATGAAGGA
TCAGCTGGACAACTTGTGTAAAGGAGTCCTTGCTGGAGGACTTTAAGGGTTACCTGG
GTTGCCAAGCCTTGCTGAGATGATCCAGTTTTACCTGGAGGAGGTGATGCCCCAAGCT
GAGAACCAAGACCCAGACATCAAGGCGCATGTGAACTCCCTGGGGGAGAACCTGAAG
ACCTCAGGCTGAGGCTACGGCGCTGTCATCGATTTCTTCCCTGTGAAAACAAGAGCA
AGGCCGTGGAGCAGGTGAAGAATGCCTTTAATAAGCTCCAAGAGAAAGGCATCTACAA
AGCCATGAGTGAGTTTGACATCTTCATCAACTACATAGAAGCCTACATGACAATGAAG
ATACGAAACTGA (SEQ ID NO: 138)

[000334] IL10 insert (amino acid sequence)

MHSSALLCCLVLLTGVRASPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFQMKDQ
LDNLLLKESLLEDFKGYLGQCALSEMIQFYLEEVMPPQAENQDPDIKAHVNSLGENLKTTRL
RLRRCHRFPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN (SEQ
ID NO: 124)

[000335] Empty lentivector

ACGCGTGTAGTCTTATGCAATACTCTTGTAGTCTTGCAACATGGTAACGATGAGTTAGC
AACATGCCTTACAAGGAGAGAAAAAGCACCGTGCATGCCGATTGGTGGAAGTAAGGT

GGTACGATCGTGCCTTATTAGGAAGGCAACAGACGGGTCTGACATGGATTGGACGAAC
CACTGAATTGCCGCATTGCAGAGATATTGTATTTAAGTGCCTAGCTCGATACATAAACG
GGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCA
CTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCAAGTAGTGTGTGCCCCGTCTGTT
GTGTGACTCTGGTAACTAGAGATCCCCTCAGACCCCTTTTAGTCAGTGTGAAAAATCTCTA
GCAGTGGCGCCCGAACAGGGACTTGAAAGCGAAAAGGGAACCAGAGGAGCTCTCTCG
ACGCAGGACTCGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGT
GAGTACGCCAAAAATTTTACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAG
CGTCAGTATTAAGCGGGGGAGAATTAGATCGCGATGGGAAAAAATTCGGTTAAGGCCA
GGGGGAAAGAAAAAATATAAATTAACATATAGTATGGGCAAGCAGGGAGCTAGAA
CGATTGCGAGTTAATCCTGGCCTGTTAGAAACATCAGAAGGCTGTAGACAAATACTGG
GACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATATAATAC
AGTAGCAACCTCTATTGTGTGCATCAAAGGATAGAGATAAAAGACACCAAGGAAGCT
TTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGACCACCGCACAGCAAGCGGCC
ACTGATCTTCAGACCTGGAGGAGGAGATATGAGGGACAATTGGAGAAGTGAATTATAT
AAATATAAAGTAGTAAAAATTGAACCATTAGGAGTAGCACCCACCAAGGCAAAGAGA
AGAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAATAGGAGCTTTGTTCTTGGGTTCT
TGGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATGACGCTGACGGTACAGGCCA
GACAATTATTGTCTGGTATAGTGCAGCAGCAGAACAATTTGCTGAGGGCTATTGAGGC
GCAACAGCATCTGTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAGAATC
CTGGCTGTGGAAAGATACCTAAAGGATCAACAGCTCCTGGGGATTTGGGGTTGCTCTG
GAAAACCTCATTTGCACCACTGCTGTGCCTTGAATGCTAGTTGGAGTAATAAATCTCTG
GAACAGATTTGGAATCACACGACCTGGATGGAGTGGGACAGAGAAATTAACAATTACA
CAAGCTTAATACACTCCTTAATTGAAGAATCGCAAAACCAGCAAGAAAAGAATGAACA
AGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTTTAACATAACAAAT
TGGCTGTGGTATATAAAATTATTACATAATGATAGTAGGAGGCTTGGTAGGTTTAAGAAT
AGTTTTTGCTGTACTTTCTATAGTGAATAGAGTTAGGCAGGGATATTCACCATTATCGT
TTCAGACCCACCTCCCAACCCCGAGGGGACCCGACAGGCCCGAAGGAATAGAAGAAG
AAGGTGGAGAGAGAGACAGAGACAGATCCATTTCGATTAGTGAACGGATCTCGACGGT
ATCGATGGCCGCCCCCTTCACCGAGGGCCTATTTCCCATGATTCTTCATATTTGCATAT
ACGATACAAGGCTGTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATA
TTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTAA
ATTATGTTTTAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTTCGATTTCT
GGCTTTATATATCTTGTGGAAAGGACGAAACACCGGAGTCTTCTTTTTTGAAGACACTT
CGGACTGTAGAACTCTGAACCTCGAGCAATTTAAAAGAAAAGGGGGGATTGGGGGGT
ACAGTGCAGGGGAAAGAATAGTAGACATAATAGCAACAGACATACAACTAAAGAAT
TACAAAAACAAATTACAAAAATTCAAAATTTCTGCGTTGTTGTCGGTGCTCGTTCTCTG

CTCTTCACGCTACTGAATTCATCACCGGTTCTTCGAAGGCCTCCGCGCCGGGTTTTGGC
GCCTCCCGCGGGCGCCCCCTCCTCACGGCGAGCGCTGCCACGTCAGACGAAGGGCGC
AGCGAGCGTCTGATCCTTCCGCCCGGACGCTCAGGACAGCGGCCCCGCTGCTCATAAG
ACTCGGCCTTAGAACCCCAGTATCAGCAGAAGGACATTTTAGGACGGGACTTGGGTGA
CTCTAGGGCACTGGTTTTCTTTCCAGAGAGCGGAACAGGCGAGGAAAAGTAGTCCCTT
CTCGGCGATTCTGCGGAGGGATCTCCGTGGGGCGGTGAACGCCGATGATTATATAAGG
ACGCGCCGGGTGTGGCACAGCTAGTTCCGTGCGAGCCGGGATTTGGGTGCGGGTTCTTG
TTTGTGGATCGCTGTGATCGTCACTTGGTCTAGACGCCACCATGAGCGGGGGCGAGGA
GCTGTTCCGCCGGCATCGTGCCCGTGCTGATCGAGCTGGACGGCGACGTGCACGGCCAC
AAGTTCAGCGTGCGCGGCGAGGGCGAGGGCGACGCCGACTACGGCAAGCTGGAGATC
AAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGGCCCACCCTGGTGACCACCCT
CTGCTACGGCATCCAGTGCTTCGCCCCGCTACCCCGAGCACATGAAGATGAACGACTTCT
TCAAGAGCGCCATGCCCCGAGGGCTACATCCAGGAGCGCACCATCCAGTTCCAGGACGA
CGGCAAGTACAAGACCCGCGGCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCG
CATCGAGCTGAAGGGCAAGGACTTCAAGGAGGACGGCAACATCCTGGGCCACAAGCT
GGAGTACAGCTTCAACAGCCACAACGTGTACATCCGCCCCGACAAGGCCAACAACGGC
CTGGAGGCTAACTTCAAGACCCGCCACAACATCGAGGGCGGCGGCGTGCAGCTGGCCG
ACCACTACCAGACCAACGTGCCCCCTGGGGCGACGGCCCCGTGCTGATCCCCATCAACCA
CTACCTGAGCACTCAGACCAAGATCAGCAAGGACCGCAACGAGGCCCGCGACCACATG
GTGCTCCTGGAGTCCTTCAGCGCCTGCTGCCACACCCACGGCATGGACGAGCTGTACA
GGGGATCCGAGGGCAGAGGAAGCCTTCTAACATGCGGTGACGTGGAGGAGAATCCCG
GCCCTTCCGGGATGACCGAGTACAAGCCCACGGTGCGCCTCGCCACCCGCGACGACGT
CCCCAGGGCCGTACGCACCTCGCCGCCGCGTTCGCCGACTACCCCGCCACGCGCCAC
ACCGTCGATCCGGACCGCCACATCGAGCGGGTCAACGAGCTGCAAGAACTCTTCCTCA
CGCGCGTCGGGCTCGACATCGGCAAGGTGTGGGTGCGGGACGACGGCGCCGCGGTGGC
GGTCTGGACCACGCCGGAGAGCGTGAAGCGGGGGCGGTGTTCCGCCGAGATCGGCCCG
CGCATGGCCGAGTTGAGCGGTTCCCGGCTGGCCGCGCAGCAACAGATGGAAGGCCTCC
TGGCGCCGCACCGGCCCAAGGAGCCCGGTGGTTCTTGGCCACCGTCGGCGTCTCGCC
CGACCACCAGGGCAAGGGTCTGGGCAGCGCCGTCGTGCTCCCCGGAGTGGAGGCGGCC
GAGCGCGCCGGGTGCCCCCTTCTTGAGACCTCCGCGCCCCGCAACCTCCCCTTCTA
CGAGCGGCTCGGCTTACCGTCAACGCCGACGTGAGGTGCCCCGAAGGACCGCGCACC
TGGTGCATGACCCGCAAGCCCGGTGCCTGAATCTAGGTGACAATCAACCTCTGGATT
ACAAAATTTGTGAAAGATTGACTGGTATTCTTAACTATGTTGCTCCTTTTACGCTATGTG
GATACGCTGCTTTAATGCCTTTGTATCATGCTATTGCTTCCCGTATGGCTTTTCATTTTCT
CCTCCTTGTATAAATCCTGGTTGCTGTCTCTTTATGAGGAGTTGTGGCCCGTTGTCAGGC
AACGTGGCGTGGTGTGCACTGTGTTTGCTGACGCAACCCCCACTGGTTGGGGCATTGCC
ACCACCTGTCAGCTCCTTTCCGGGACTTTTCGCTTTCCCCCTCCCTATTGCCACGGCGGAA

CTCATCGCCGCCTGCCTTGCCCGCTGCTGGACAGGGGCTCGGCTGTTGGGCACTGACAA
TTCCGTGGTGTGTGTCGGGAAATCATCGTCCTTTTCCTTGGCTGCTCGCCTGTGTTGCCAC
CTGGATTCTGCGCGGGACGTCTTCTGCTACGTCCCTTCGGCCCTCAATCCAGCGGACC
TTCCTTCCCGCGGCCTGCTGCCGGCTCTGCGGCCTCTTCCGCGTCTCCGCCTTCGCCCTC
AGACGAGTCGGATCTCTCTTTGGGCCGCCTCCCCGCCTGGTACCTTTAAGACCAATGAC
TTACAAGGCAGCTGTAGATCTTAGCCACTTTTTTAAAAGAAAAGGGGGGACTGGAAGGG
CTAATTCACCTCCCAACGAAGATAAGATCTGCTTTTTGCTTGTACTGGGTCTCTCTGGTTA
GACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTC
AATAAAGCTTGCCCTGAGTGCTTCAAGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGT
AACTAGAGATCCCTCAGACCTTTTAGTCAGTGTGGAATACTCTAGCAGTAGTAGTTC
ATGTCATCTTATTATTAGTATTTATAACTTGCAAAGAAATGAATATCAGAGAGTGAGA
GGAACCTGTTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTC
ACAAATAAAGCATTTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTA
TCTTATCATGTCTGGCTCTAGCTATCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTC
CGCCAGTTCGCCCCATTCTCCGCCCATGGCTGACTAATTTTTTTTTATTTATGCAGAGG
CCGAGGCCGCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGG
CCTAGACTTTTGCAGAGACCAAATTCGTAATCATGTCATAGCTGTTTCCTGTGTGAAAT
TGTTATCCGCTCACAATCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCT
GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGTCACTGCCCCGTTTC
CAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAG
GCGGTTTGCATTGCGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTGCG
TTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGA
ATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAA
CCGTAAAAAGGCCGCGTTGCTGGCGTTTTTTCATAGGCTCCGCCCCCTGACGAGCATC
ACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACC
AGGCGTTTCCCCCTGGAAGCTCCCTCGTGCCTCTCCTGTTCCGACCCTGCCGCTTACC
GGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGCGCTTTCTCATAGCTCACGCTG
TAGGTATCTCAGTTCGGTGTAGGTCGTTGCTCCAAGCTGGGCTGTGTGCACGAACCCC
CCGTTACAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGTA
AGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT
ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAG
AACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTA
GCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAG
CAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGT
CTGACGCTCAGTGGAACGAAAACCTCACGTTAAGGGATTTTGGTCATGAGATTATCAAA
AAGGATCTTCACCTAGATCCTTTTAAATTAATAAATGAAGTTTTAAATCAATCTAAAGTA
TATATGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCA

GCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACG
ATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCT
CACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAA
GTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGAAGCTAGA
GTAAGTAGTTCCGCCAGTTAATAGTTTTCGCGCAACGTTGTTGCCATTGCTACAGGCATCGT
GGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCTCAACGATCAAGGC
GAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCTCTCCGATC
GTTGTGAGAAGTAAGTTGGCCGAGTGTTATCACTCATGGTTATGGCAGCACTGCATAA
TTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAA
GTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGG
ATAATACCGCGCCACATAGCAGAACTTTAAAAAGTGCTCATCATTGGAAAACGTTCTTCG
GGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCG
TGACCCCAACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAA
CAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATAC
TCATACTCTTCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCG
GATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCC
CGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAA
ATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTC
TGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCA
GACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGCGGGCTGGCTTAACTA
TGCGGCATCAGAGCAGATTGTAAGTGCAGAGTGACCATATGCGGTGTGAAATACCGCAC
AGATGCGTAAGGAGAAAAATACCGCATCAGGCGCCATTTCGCCATTACAGGCTGCGCAACT
GTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG
ATGTGCTGCAAGGCGATTAAAGTTGGGTAACGCCAGGGTTTTTCCCAGTCACGACGTTGTA
AAACGACGGCCAGTGCCAAGCTG (SEQ ID NO: 141)

[000336] Empty lentivector

GCGATCGCAGTAATCAATTACGGGGTTCATTAGTTCATAGCCCATATATGGAGTTCCGCG
TTACATAACTTACGGTAAATGGCCCCGCTGGCTGACCGCCCAACGACCCCCGCCATTG
ACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA
ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGC
CAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCCGCTGGCATTATGCCCA
GTACATGACCTTATGGGACTTTCTACTTGGCAGTACATCTACGTATTAGTCATCGCTA
TTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCA
CGGGGATTTCCAAGTCTCCACCCCATGACGTCAATGGGAGTTTGTGTTTGGCACCAAAA
TCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAAATGGGCGGT
AGGCGTGACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGGGGTCTCT

CTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTA
AGCCTCAATAAAGCTTGCCTTGAGTGCTTCAAGTAGTGTGTGCCCCGTCTGTTGTGTGAC
TCTGGTAACTAGAGATCCCTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTGG
CGCCCGAACAGGGACCTGAAAGCGAAAGGGAAACCAGAGCTCTCTCGACGCAGGACT
CGGCTTGCTGAAGCGCGCACGGCAAGAGGGCGAGGGGCGGGCGACTGGTGAGTACGCCA
AAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTATT
AAGCGGGGGAGAATTAGATCGCGATGGGAAAAAATTCGGTTAAGGCCAGGGGGAAAG
AAAAAATATAAATTAACATATAGTATGGGCAAGCAGGGAGCTAGAACGATTTCGCA
GTTAATCCTGGCCTGTTAGAAACATCAGAAGGCTGTAGACAAATACTGGGACAGCTAC
AACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATATAATACAGTAGCAAC
CCTCTATTGTGTGCATCAAAGGATAGAGATAAAAGACACCAAGGAAGCTTTAGACAAG
ATAGAGGAAGAGCAAAACAAAAGTAAGACCACCGCACAGCAAGCGGCCGCTGATCTT
CAGACCTGGAGGAGGAGATATGAGGGACAATTGGAGAAGTGAATTATATAAATATAA
AGTAGTAAAAATTGAACCATTAGGAGTAGCACCCACCAAGGCAAAGAGAAGAGTGGT
GCAGAGAGAAAAAAGAGCAGTGGGAATAGGAGCTTTGTTCCCTTGGGTTCTTGGGAGCA
GCAGGAAGCACTATGGGCGCAGCCTCAATGACGCTGACGGTACAGGCCAGACAATTAT
TGTCTGGTATAGTGCAGCAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAGCA
TCTGTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAGAATCCTGGCTGTG
GAAAGATACCTAAAGGATCAACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAACTCA
TTTGCACCACTGCTGTGCCTTGGAATGCTAGTTGGAGTAATAAATCTCTGGAACAGATT
TGGAATCACACGACCTGGATGGAGTGGGACAGAGAAATTAACAATTACACAAGCTTAA
TACACTCCTTAATTGAAGAATCGCAAAACCAGCAAGAAAAGAATGAACAAGAATTATT
GGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTTTAACATAACAAATTGGCTGTGG
TATATAAAATTATTACATAATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGC
TGTACTTTCTATAGTGAATAGAGTTAGGCAGGGATATTCACCATTATCGTTTCAGACCC
ACCTCCCAACCCCGAGGGGACCCGACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAG
AGAGAGACAGAGACAGATCCATTCGATTAGTGAACGGATCTCGACGGTATCGGTTAAC
TTTTAAAAGAAAAGGGGGGATTGGGGGGTACAGTGCAGGGGAAAGAATAGTAGACAT
AATAGCAACAGACATACAACTAAAGAATTACAAAAACAAATTACAAAAATTCAAAA
TTTTGGCTCCCGATCGTTGCGTTACACACACAATTACTGCTGATCGAGTGTAGCCTTCG
AATGAAAGACCCACCTGTAGGTTTGGCAAGATAGCTGCAGTAACGCCATTTTGCAAG
GCATGGAAAAATACCAACCAAGAATAGAGAAGTTCAGATCAAGGGCGGGTACATGA
AAATAGCTAACGTTGGGCCAAACAGGATATCTGCGGTGAGCAGTTTCGGCCCCGGCCC
GGGGCCAAGAACAGATGGTCACCGCAGTTTCGGCCCCGGCCGAGGCCAAGAACAGA
TGGTCCCCAGATATGGCCCAACCCTCAGCAGTTTCTTAAGACCCATCAGATGTTTCCAG
GCTCCCCCAAGGACCTGAAATGACCCTGCGCCTTATTTGAATTAACCAATCAGCCTGCT
TCTCGCTTCTGTTTCGCGCGCTTCTGCTTCCCGAGCTCTATAAAAGAGCTCACAAACCCTC

ACTCGGCGCGCCAGTCCTCCGATTGACTGAGTCGCCCTGATCATTGTGTCGATCCTACCAT
CCACTCGACACACCCGCCAGGGCCCTGCCAAGCTTCCGAGCTCTCGATATCAAAGGAG
GTACCCAACATGGTCAGCAAGGGCGAGGAACTGTTACCGGGGTGGTGCCCATCCTGG
TCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGG
GCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGTACCACCGGCAAGCTGCC
CGTGCCCTGGCCACCCCTCGTGACCACCCTGACCTACGGCGTGCAATGCTTCAGCCGCT
ACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCCAAGGCTACGT
CCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTG
AAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGG
AGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACACTACAACAGCCACAACGTCTA
TATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAAC
ATCGAGGACGGCAGCGTGCAACTCGCCGACCACTACCAGCAGAACACCCCCATCGGCG
ACGGCCCCGTGCTGCTGCCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAA
AGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGG
ATCACTCTCGGCATGGACGAGCTGTACAAGTAGAAGTTGTCTCCTCCTGCACTGACTGA
CTGATACAATCGATTTCTGGATCCGCAGGCCTCTGCTAGAAGTTGTCTCCTCCTGCACT
GACTGACTGATACAATCGATTTCTGGATCCGCAGGCCTCTGCTAGCTTGACTGACTGAG
TCGACAATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTTAACTAT
GTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTATTGCT
TCCCGTATGGCTTTTCATTTTCTCCTCCTTGTATAAATCCTGGTTGCTGTCTCTTTATGAG
GAGTTGTGGCCCGTTGTCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGCTGACGCAAC
CCCCACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTTTTCGCTTTCC
CCTCCCTATTGCCACGGCGGAACCTCATCGCCGCCTGCCTTGCCCCGTGCTGGACAGGGG
CTCGGCTGTTGGGCACTGACAATTCCGTGGTGTGTCGGGGAAGCTGACGTCTTTCCA
TGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGCGCGGGACGTCCTTCTGCTACGTCCCT
TCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCGGCTCTGCGGCCTCT
TCCGCGTCTTCGCCTTCGCCCTCAGACGAGTCGGATCTCCCTTTGGGCCGCCTCCCCGC
CTGGAATTCGAGCTCGGTACCTTTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTA
GCCACTTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATTCCTCCCAACGAAGACA
AGATCTGCTTTTTGCTTGTACTGGGTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGC
TCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCCTTGAGTGCTT
CAAGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCCCTCAGACCCTT
TTAGTCAGTGTGGAATACTCTAGCAGTCCTGGCCAACGTGAGCACCGTGCTGACCTCC
AAATATCGTTAAGCTGGAGCCTGGGAGCCGGCCTGGCCCTCCGCCCCCCCCACCCCCG
CAGCCCACCCCTGGTCTTTGAATAAAGTCTGAGTGAGTGGCCGACAGTGCCCGTGAGG
TTCTCGTGACCTGAGGTGCAGGGCCGGCGCTAGGGACACGTCCGTGCACGTGCCGAGG
CCCCCTGTGCAGCTGCAAGGGACAGGCCTAGCCCTGCAGGCCTAACTCCGCCCATCCC

GCCCCTAACTCCGCCAGTTCCGCCATTCTCCGCCTCATGGCTGACTAATTTTTTTTAT
TTATGCAGAGGCCGAGGCCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGACGC
TTTTTTGGAGGCCGAGGCTTTTGCAAAGATCGAACAAGAGACAGGACCTGCAGGTAA
TTAAATTTAAATCATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGC
CGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGAC
GCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTTCCCC
TGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCG
CCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTT
CGGTGTAGGTGCTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTACAGCCCGAC
CGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATC
GCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCT
ACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTA
TCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGC
AAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCA
GAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTG
GAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACC
TAGATCCTTTTAAATTAATAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAAC
TTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTAT
TTCGTTTATCCATAGTTGCATTTAAATGGCCGGCCTGGCGCGCCGTTTAAACCTAGATA
TTGATAGTCTGATCGGTCAACGTATAATCGAGTCCTAGCTTTTGCAACATCTATCAAG
AGACAGGATCAGCAGGAGGCTTTTCGCATGAGTATTCAACATTTCCGTGTGCGCCCTTATT
CCCTTTTTTTCGCGCATTTTGCCTTCCTGTTTTTTGCTCACCCAGAAACGCTGGTGAAAGTA
AAAGATGCTGAAGATCAGTTGGGTGCGCGAGTGGGTTACATCGAACTGGATCTCAACA
GCGGTAAGATCCTTGAGAGTTTTTCGCCCCGAAGAACGCTTTCCAATGATGAGCACTTTT
AAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCG
GTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTATTCACCAGTCACAGAAAAG
CATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTG
ATAACACTGCGGCCAACTTACTTCTGACAACGATTGGAGGACCGAAGGAGCTAACCGC
TTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGGGAACCGGAGCTGA
ATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAAC
CTTGCGTAAACTATTAATACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAGTTGATAG
ACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGG
CTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAG
CACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCA
GGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAG
CATTGGTAACCGATTCTAGGTGCATTGGCGCAGAAAAAAATGCCTGATGCGACGCTGC
GCGTCTTATACTCCACATATGCCAGATTCAGCAACGGATACGGCTTCCCCAACTTGCC

CACTTCCATACGTGTCTCCTTACCAGAAATTTATCCTTAAGATCCCGAATCGTTTAAAC (SEQ ID NO: 142)

[000337] Other sequences are provided throughout the specifications and in the table below; and the Sequence Listing filed herewith which forms part of the specifications.

Table of Sequences

[000338] In the following table, the terms “mA,” “mC,” “mU,” or “mG” are used to denote a nucleotide that has been modified with 2’-O-Me.

[000339] In the following table, each “N” is used to independently denote any nucleotide (e.g., A, U, T, C, G). In certain embodiments, the nucleotide is an unmodified RNA nucleotide residue, i.e., a ribose sugar and a phosphodiester backbone.

[000340] In the following table, a “*” is used to denote a PS modification. In this application, the terms A*, C*, U*, or G* may be used to denote a nucleotide that is linked to the next (e.g., 3’) nucleotide with a PS bond.

[000341] It is understood that if a DNA sequence (comprising Ts) is referenced with respect to an RNA, then Ts should be replaced with Us (which may be modified or unmodified depending on the context), and vice versa.

[000342] In the following table, single amino acid letter code is used to provide peptide sequences.

Table 4: Table of Sequences

SEQ ID	Description	Sequences
204	Nucleic acid sequence encoding TGFB1	ATGCCGCCCTCCGGGCTGCGGCTGCTGCTGCTGCTGCTACC GCTGCTGTGGCTACTGGTGCTGACGCCTGGCCGGCCGGCC GCGGGACTATCCACCTGCAAGACTATCGACATGGAGCTGG TGAAGCGGAAGCGCATCGAGGCCATCCGCGGCCAGATCCT GTCCAAGCTGCGGCTCGCCAGCCCCCGAGCCAGGGGGAG GTGCCGCCCGGCCGCTGCCCCGAGGCCGTGCTCGCCCTGTA CAACAGCACCCGCGACCGGGTGCCGGGGAGAGTGCAGA ACCGGAGCCCGAGCCTGAGGCCGACTACTACGCCAAGGAG GTCACCCGCGTGCTAATGGTGGAACCCACAACGAAATCT ATGACAAGTTCAAGCAGAGTACACACAGCATATATATGTT CTTCAACACATCAGAGCTCCGAGAAGCGGTACCTGAACCC GTGTTGCTCTCCCGGGCAGAGCTGCGTCTGCTGAGGCTCAA GTTAAAAGTGGAGCAGCACGTGGAGCTGTACCAGAAATAC AGCAACAATTCTGGCGATACCTCAGCAACCGGCTGCTGG CACCCAGCGACTCGCCAGAGTGTTATCTTTTGATGTCACC GGAGTTGTGCGGCAGTGGTTGAGCCGTGGAGGGGAAATTG AGGGCTTTCGCCTTAGCGCCCACTGCTCCTGTGACAGCAGG GATAACACACTGCAAGTGGACATCAACGGGTTCACTACCG GCCGCCGAGGTGACCTGGCCACCATTCATGGCATGAACCG GCCTTTCCTGCTTCTCATGGCCACCCCGCTGGAGAGGGCCC

<u>SEQ ID</u>	<u>Description</u>	<u>Sequences</u>
		AGCATCTGCAAAGCTCCCGGCACCGCCGAGCCCTGGACAC CAACTATTGCTTCAGCTCCACGGAGAAGAACTGCTGCGTG CGGCAGCTGTACATTGACTTCCGCAAGGACCTCGGCTGGA AGTGGATCCACGAGCCCAAGGGCTACCATGCCAACTTCTG CCTCGGGCCCTGCCCCTACATTTGGAGCCTGGACACGCAGT ACAGCAAGGTCCTGGCCCTGTACAACCAGCATAACCCGGG CGCCTCGGCGGGCGCCGTGCTGCGTGCCGCAGGCGCTGGAG CCGCTGCCCATCGTGTAACGTGGGCCGCAAGCCCAAGG TGGAGCAGCTGTCCAACATGATCGTGCGCTCCTGCAAGTG CAGC
205	Nucleic acid sequence encoding TGFB1 R218H	ATGCCGCCCTCCGGGCTGCGGCTGCTGCTGCTGCTACC GCTGCTGTGGCTACTGGTGCTGACGCCTGGCCGGCCGGCC GCGGGACTATCCACCTGCAAGACTATCGACATGGAGCTGG TGAAGCGGAAGCGCATCGAGGCCATCCGCGGCCAGATCCT GTCCAAGCTGCGGCTCGCCAGCCCCCGAGCCAGGGGGAG GTGCCGCCCGGGCCGCTGCCGAGGCCGTGCTCGCCCTGTA CAACAGCACCCGCGACCGGGTGCCGGGGAGAGTGCAGA ACCGGAGCCCGAGCCTGAGGCCGACTACTACGCCAAGGAG GTCACCCGCGTGCTAATGGTGGAACCCACAACGAAATCT ATGACAAGTTCAAGCAGAGTACACACAGCATATATATGTT CTTCAACACATCAGAGCTCCGAGAAGCGGTACCTGAACCC GTGTTGCTCTCCCGGGCAGAGCTGCGTCTGCTGAGGCTCAA GTTAAAAGTGGAGCAGCACGTGGAGCTGTACCAGAAATAC AGCAACAATTCTGGCGATACCTCAGCAACCGGCTGCTGG CACCCAGCGACTCGCCAGAGTGGTTATCTTTTGATGTCACC GGAGTTGTGCGGCAGTGGTTGAGCCGTGGAGGGGAAATTG AGGGCTTTCACCTTAGCGCCCACTGCTCCTGTGACAGCAGG GATAACACACTGCAAGTGGACATCAACGGGTTCACTACCG GCCGCCGAGGTGACCTGGCCACCATTATGGCATGAACCG GCCTTTCTGCTTCTCATGGCCACCCCGCTGGAGAGGGCCC AGCATCTGCAAAGCTCCCGGCACCGCCGAGCCCTGGACAC CAACTATTGCTTCAGCTCCACGGAGAAGAACTGCTGCGTG CGGCAGCTGTACATTGACTTCCGCAAGGACCTCGGCTGGA AGTGGATCCACGAGCCCAAGGGCTACCATGCCAACTTCTG CCTCGGGCCCTGCCCCTACATTTGGAGCCTGGACACGCAGT ACAGCAAGGTCCTGGCCCTGTACAACCAGCATAACCCGGG CGCCTCGGCGGGCGCCGTGCTGCGTGCCGCAGGCGCTGGAG CCGCTGCCCATCGTGTAACGTGGGCCGCAAGCCCAAGG TGGAGCAGCTGTCCAACATGATCGTGCGCTCCTGCAAGTG CAGC
206	Nucleic acid sequence encoding TGFB1 R218C	ATGCCGCCCTCCGGGCTGCGGCTGCTGCTGCTGCTACC GCTGCTGTGGCTACTGGTGCTGACGCCTGGCCGGCCGGCC GCGGGACTATCCACCTGCAAGACTATCGACATGGAGCTGG TGAAGCGGAAGCGCATCGAGGCCATCCGCGGCCAGATCCT GTCCAAGCTGCGGCTCGCCAGCCCCCGAGCCAGGGGGAG GTGCCGCCCGGGCCGCTGCCGAGGCCGTGCTCGCCCTGTA CAACAGCACCCGCGACCGGGTGCCGGGGAGAGTGCAGA ACCGGAGCCCGAGCCTGAGGCCGACTACTACGCCAAGGAG GTCACCCGCGTGCTAATGGTGGAACCCACAACGAAATCT ATGACAAGTTCAAGCAGAGTACACACAGCATATATATGTT CTTCAACACATCAGAGCTCCGAGAAGCGGTACCTGAACCC GTGTTGCTCTCCCGGGCAGAGCTGCGTCTGCTGAGGCTCAA GTTAAAAGTGGAGCAGCACGTGGAGCTGTACCAGAAATAC

SEQ ID	Description	Sequences
		AGCAACAATTCTGGCGATACCTCAGCAACCGGCTGCTGG CACCCAGCGACTCGCCAGAGTGGTTATCTTTTGATGTCACC GGAGTTGTGCGGCAGTGGTTGAGCCGTGGAGGGGAAATTG AGGGCTTTTGCCTTAGCGCCCACTGCTCCTGTGACAGCAGG GATAACACACTGCAAGTGGACATCAACGGGTTCACTACCG GCCGCCGAGGTGACCTGGCCACCATTTCATGGCATGAACCG GCCTTTCTGCTTCTCATGGCCACCCCGCTGGAGAGGGCCC AGCATCTGCAAAGCTCCCGGCACCGCCGAGCCCTGGACAC CAACTATTGCTTCAGCTCCACGGAGAAGAACTGCTGCGTG CGGCAGCTGTACATTGACTTCCGCAAGGACCTCGGCTGGA AGTGGATCCACGAGCCCAAGGGCTACCATGCCAACTTCTG CCTCGGGCCCCTGCCCCTACATTTGGAGCCTGGACACGCAGT ACAGCAAGGTCTGCGCCCTGTACAACCAGCATAACCCGGG CGCCTCGGCGGCGCCGTGCTGCGTGCCGCAGGCGCTGGAG CCGCTGCCCATCGTGTACTACGTGGGCGCAAGCCCAAGG TGGAGCAGCTGTCCAACATGATCGTGCGCTCCTGCAAGTG CAGC
207	Nucleic acid sequence encoding TGFB1 C225R	ATGCCGCCCTCCGGGCTGCGGCTGCTGCTGCTGCTGCTACC GCTGCTGTGGCTACTGGTGCTGACGCCTGGCCGGCCGGCC GCGGGACTATCCACCTGCAAGACTATCGACATGGAGCTGG TGAAGCGGAAGCGCATCGAGGCCATCCGCGGCCAGATCCT GTCCAAGCTGCGGCTCGCCAGCCCCCGAGCCAGGGGGAG GTGCCGCCCGGCCCGCTGCCCAGGCGCGTGCTCGCCCTGTA CAACAGCACCCGCGACCGGGTGCCCGGGGAGAGTGCAGA ACCGGAGCCCGAGCCTGAGGCCGACTACTACGCCAAGGAG GTCACCCGCGTGCTAATGGTGGAACCCACAACGAAATCT ATGACAAGTTCAAGCAGAGTACACACAGCATATATATGTT CTTCAACACATCAGAGCTCCGAGAAGCGGTACCTGAACCC GTGTTGCTCTCCCGGGCAGAGCTGCGTCTGCTGAGGCTCAA GTTAAAAGTGGAGCAGCACGTGGAGCTGTACCAGAAATAC AGCAACAATTCTGGCGATACCTCAGCAACCGGCTGCTGG CACCCAGCGACTCGCCAGAGTGGTTATCTTTTGATGTCACC GGAGTTGTGCGGCAGTGGTTGAGCCGTGGAGGGGAAATTG AGGGCTTTTGCCTTAGCGCCCACTGCTCCAGAGACAGCAG GGATAACACACTGCAAGTGGACATCAACGGGTTCACTACC GGCCGCCGAGGTGACCTGGCCACCATTTCATGGCATGAACC GGCCTTTCTGCTTCTCATGGCCACCCCGCTGGAGAGGGCC CAGCATCTGCAAAGCTCCCGGCACCGCCGAGCCCTGGACA CCAACTATTGCTTCAGCTCCACGGAGAAGAACTGCTGCGT GCGGCAGCTGTACATTGACTTCCGCAAGGACCTCGGCTGG AAGTGGATCCACGAGCCCAAGGGCTACCATGCCAACTTCT GCCTCGGGCCCCTGCCCCTACATTTGGAGCCTGGACACGCA GTACAGCAAGGTCTGCGCCCTGTACAACCAGCATAACCCG GGCGCTCGGCGGCGCCGTGCTGCGTGCCGCAGGCGCTGG AGCCGCTGCCCATCGTGTACTACGTGGGCGCAAGCCCAA GGTGGAGCAGCTGTCCAACATGATCGTGCGCTCCTGCAAG TGCAGC
208	Nucleic acid sequence encoding TGFB1 R218H C225R	ATGCCGCCCTCCGGGCTGCGGCTGCTGCTGCTGCTGCTACC GCTGCTGTGGCTACTGGTGCTGACGCCTGGCCGGCCGGCC GCGGGACTATCCACCTGCAAGACTATCGACATGGAGCTGG TGAAGCGGAAGCGCATCGAGGCCATCCGCGGCCAGATCCT GTCCAAGCTGCGGCTCGCCAGCCCCCGAGCCAGGGGGAG GTGCCGCCCGGCCCGCTGCCCAGGCGCGTGCTCGCCCTGTA

<u>SEQ ID</u>	<u>Description</u>	<u>Sequences</u>
		CAACAGCACCCGCGACCGGGTGGCCGGGGAGAGTGCAGA ACCGGAGCCCCGAGCCTGAGGCCGACTACTACGCCAAGGAG GTCACCCGCGTGCTAATGGTGGAACCCACAACGAAATCT ATGACAAGTTCAAGCAGAGTACACACAGCATATATATGTT CTTCAACACATCAGAGCTCCGAGAAGCGGTACCTGAACCC GTGTTGCTCTCCCGGGCAGAGCTGCGTCTGCTGAGGCTCAA GTTAAAAGTGGAGCAGCACGTGGAGCTGTACCAGAAATAC AGCAACAATTCTGGCGATACCTCAGCAACCGGCTGCTGG CACCCAGCGACTCGCCAGAGTGGTTATCTTTTGATGTCACC GGAGTTGTGCGGCAGTGGTTGAGCCGTGGAGGGGAAATTG AGGGCTTTCACCTTAGCGCCCACTGCTCCAGAGACAGCAG GGATAACACACTGCAAGTGGACATCAACGGGTTCACTACC GGCCGCCGAGGTGACCTGGCCACCATTGATGGCATGAACC GGCCTTTCCTGCTTCTCATGGCCACCCCGCTGGAGAGGGCC CAGCATCTGCAAAGCTCCCGGCACCGCCGAGCCCTGGACA CCAATAATTGCTTCAGCTCCACGGAGAAGAAGTGTGCGT GCGGCAGCTGTACATTGACTTCCGCAAGGACCTCGGCTGG AAGTGGATCCACGAGCCCAAGGGCTACCATGCCAATTCT GCCTCGGGCCCTGCCCTACATTTGGAGCCTGGACACGCA GTACAGCAAGGTCCTGGCCCTGTACAACCAGCATAACCCG GGCGCCTCGGCGGCGCCGTGCTGCGTGCCGCAGGCGCTGG AGCCGCTGCCCATCGTGTACTACGTGGGCGCAAGCCCAA GGTGGAGCAGCTGTCCAACATGATCGTGCGCTCCTGCAAG TGCAGC
209	Nucleic acid sequence encoding GFP	ATGTGGAAGGGAGAAGAAGTGTTCACAGGAGTCGTCCCGA TCCTGGTTCGAAGTGGACGGAGACGTCAACGGACACAAGTT CTCGGTCAGAGGAGAAGGAGAAGGAGACGCAACAAACGG AAAGCTGACACTGAAGTTCATCTGCACAACAGGAAAGCTG CCGGTCCCGTGGCCGACACTGGTCACAACACTGACATAACG GAGTCCAGTGCTTTTCGAGATACCCGACCACTGAAAGAG ACACGACTTCTTCAAGTCGGCAATGCCGGAAGGATACGTC CAGGAAAGAACAATCTCGTTCAAGGACGACGGAACATACA AGACAAGAGCAGAAGTCAAGTTCGAAGGAGACACACTGG TCAACAGAATCGAACTGAAGGGAATCGACTTCAAGGAAGA CGGAAACATCCTGGGACACAAGCTGGAATACAATTCAAC TCGCACAACGTCTACATCACAGCAGACAAGCAGAAGAACG GAATCAAGGCAAACTTCAAGATCAGACACAACGTGGAAGA CGGATCGGTCCAGCTGGCAGACCACTACCAGCAGAACACA CCGATCGGAGACGGACCGGTCTGCTGCCGACAACTACT ACCTGTCGACACAGTCGGTCTGTCGAAGGACCCGAACGA AAAGAGAGACCACATGGTCTGCTGGAATTCGTACAGCA GCAGGAATCACACACGGAATGGACGAACTGTACAAGT
210	Amino acid sequence for TGFB1	MPPSGLRLLLLLLPLLWLLVLTTPGRPAAGLSTCKTIDMELVKR KRIEAIRGQILSKRLASPPSQGEVPPGPLPEAVLALYNSTRDR VAGESAEPEPEPEADYYAKEVTRVLMVETHNEIYDKFKQSTH SIYMFNTSELREAVPEPVLLSRAELRLRLKLKVEQHVELYQ KYSNNSWRYLSNRLLAPSDSPEWLSFDVTGVVRQWLSRGGEI EGFRLSAHSCDSRDNTLQVDINGFTTGRRGDLATIHGMNRP FLLLMTPLERAQHLQSSRHRRALDTNYCFSSTEKNCCVRQL YIDFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVL ALYNQHNP GASAAPCCVPQALEPLPIVYVGRKPKVEQLSN MIVRSCKCS

SEQ ID	Description	Sequences
211	Amino acid sequence for TGFB1 R218H	MPPSGLRLLLLLLPLLWLLVLTTPGRPAAGLSTCKTIDMELVKR KRIEAI RGQILSKLRLASPPSQGEVPPGPLPEAVLALYNSTRDR VAGESAEPEPEPEADYYAKEVTRVLMVETHNEIYDKFKQSTH SIYMFNTSELREAVPEPVLLSRAELRLLRLKLVKEQHVELYQ KYSNNSWRYLSNRLLAPSDSPEWLSFDVTGVVRQWLSRGGEI EGFHLSAHCSDSRDNTLQVDINGFTTGRRGDLATIHGMNRP FLLMATPLERAQHLQSSRHRRALDTNYCFSSTEKNCCVRQL YIDFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVL ALYNQHNP GASAAPCCVPQALEPLPIVYYVGRKPKVEQLSN MIVRSCKCS
212	Amino acid sequence for TGFB1 R218C	MPPSGLRLLLLLLPLLWLLVLTTPGRPAAGLSTCKTIDMELVKR KRIEAI RGQILSKLRLASPPSQGEVPPGPLPEAVLALYNSTRDR VAGESAEPEPEPEADYYAKEVTRVLMVETHNEIYDKFKQSTH SIYMFNTSELREAVPEPVLLSRAELRLLRLKLVKEQHVELYQ KYSNNSWRYLSNRLLAPSDSPEWLSFDVTGVVRQWLSRGGEI EGFCLSAHCSDSRDNTLQVDINGFTTGRRGDLATIHGMNRP FLLMATPLERAQHLQSSRHRRALDTNYCFSSTEKNCCVRQL YIDFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVL ALYNQHNP GASAAPCCVPQALEPLPIVYYVGRKPKVEQLSN MIVRSCKCS
213	Amino acid sequence for TGFB1 C225R	MPPSGLRLLLLLLPLLWLLVLTTPGRPAAGLSTCKTIDMELVKR KRIEAI RGQILSKLRLASPPSQGEVPPGPLPEAVLALYNSTRDR VAGESAEPEPEPEADYYAKEVTRVLMVETHNEIYDKFKQSTH SIYMFNTSELREAVPEPVLLSRAELRLLRLKLVKEQHVELYQ KYSNNSWRYLSNRLLAPSDSPEWLSFDVTGVVRQWLSRGGEI EGFRLSAHCSDSRDNTLQVDINGFTTGRRGDLATIHGMNRP FLLMATPLERAQHLQSSRHRRALDTNYCFSSTEKNCCVRQL YIDFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVL ALYNQHNP GASAAPCCVPQALEPLPIVYYVGRKPKVEQLSN MIVRSCKCS
214	Amino acid sequence for TGFB1 R218H C225R	MPPSGLRLLLLLLPLLWLLVLTTPGRPAAGLSTCKTIDMELVKR KRIEAI RGQILSKLRLASPPSQGEVPPGPLPEAVLALYNSTRDR VAGESAEPEPEPEADYYAKEVTRVLMVETHNEIYDKFKQSTH SIYMFNTSELREAVPEPVLLSRAELRLLRLKLVKEQHVELYQ KYSNNSWRYLSNRLLAPSDSPEWLSFDVTGVVRQWLSRGGEI EGFHLSAHCSDSRDNTLQVDINGFTTGRRGDLATIHGMNRP FLLMATPLERAQHLQSSRHRRALDTNYCFSSTEKNCCVRQL YIDFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVL ALYNQHNP GASAAPCCVPQALEPLPIVYYVGRKPKVEQLSN MIVRSCKCS
215	Amino acid sequence for GFP	MSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGK LTLKFICTTGKLPVPWPTLVTTLTYGVCFSRYPDHMKRHDF FKSAMPEGYVQERTISFKDDGTYKTRA EVKFEGD TLVNRIEL KGIDFKEDGNILGHKLEYNFN SHNVYITADKQKNGIKANFKIR HNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSLTSQSVLSKD PNEKRDH MVLLLEFVTAAGITHGMDELYK
216	Lentivirus vector	TATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAG TGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTTCATCCA TAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGG GAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGC GAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAA CCAGCCAGCCGGAAGGGCCGAGCGCAGAAAGTGGTCTCTGCA ACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGA

SEQ ID	Description	Sequences
		AGCTAGAGTAAGTAGTTTCGCCAGTTAATAGTTTGC GCAAC GTTGTTGCCATTGCTACAGGCATCGTGGTGTCAACGTCGTC GTTTGGTATGGCTTCATTCAGCTCCGTTCCCAACGATCAA GGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGT TAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAAGTAAGTTGG CCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAAT TCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACT GGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGC GGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAA TACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTG GAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACC GCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCA ACTGATCTTCAGCATCTTTTACTTTACCAGCGTTTCTGGGT GAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAA TAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTT TTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCAT GAGCGGATACATATTGAATGTATTTAGAAAAATAAACAA ATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTG ACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAA AATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGG TGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAG ACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGAC AAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCG GGGCTGGCTTAAGTATGCGGCATCAGAGCAGATTGTACTG AGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCG TAAGGAGAAAAATACCGCATCAGGCGCCATTCCGCCATTAG GCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCT TCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAA GGCGATTAAAGTTGGGTAAACGCCAGGGTTTCCAGTACAG ACGTTGTAAACGACGCGCCAGTGCCAAGCTGACGCGTGTA GTCTTATGCAATACTCTTGTAAGTCTTGCAACATGGTAACGA TGAGTTAGCAACATGCCTTACAAGGAGAGAAAAAGCACCG TGCATGCCGATTGGTGGAAGTAAGGTGGTACGATCGTGCC TTATTAGGAAGGCAACAGACGGGTCTGACATGGATTGGAC GAACCACTGAATTGCCGCATTGCAGAGATATTGTATTAAAG TGCCTAGCTCGATACATAAACGGGTCTCTCTGGTTAGACCA GATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCA CTGCTTAAGCCTCAATAAAGCTTGCTTGAGTGCTTCAAGT AGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGAT CCCTCAGACCCTTTTAGTCAGTGTGAAAAATCTCTAGCAGT GGCGCCCGAACAGGGACTTGAAAGCGAAAGGGAAACAG AGGAGCTCTCTCGACGCAGGACTCGGCTTGCTGAAGCGCG CACGGCAAGAGGCGAGGGGCGGCGACTGGTGAGTACGCC AAAAATTTTGAAGTACGGAGGCTAGAAGGAGAGAGATGG GTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATCG CGATGGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAA AAATATAAATTAACACATATAGTATGGGCAAGCAGGGAGC TAGAACGATTTCAGTAAATCCTGGCCTGTTAGAAACATCA GAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCC TTCAGACAGGATCAGAAGAACTTAGATCATTATATAATAC AGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATA AAAGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAG CAAAACAAAAGTAAGACCACCGCACAGCAAGCGGCCACT

SEQ ID	Description	Sequences
		GATCTTCAGACCTGGAGGAGGAGATATGAGGGACAATTGG AGAAGTGAATTATATAAATATAAAGTAGTAAAAATTGAAC CATTAGGAGTAGCACCCACCAAGGCAAAGAGAAGAGTGGT GCAGAGAGAAAAAAGAGCAGTGGGAATAGGAGCTTTGTTC CTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAG CGTCAATGACGCTGACGGTACAGGCCAGACAATTATTGTC TGGTATAGTGCAGCAGCAGAACAATTTGCTGAGGGCTATT GAGGCGCAACAGCATCTGTTGCAACTCACAGTCTGGGGCA TCAAGCAGCTCCAGGCAAGAATCCTGGCTGTGGAAAGATA CCTAAAGGATCAACAGCTCCTGGGGATTTGGGGTTGCTCTG GAAAACTCATTTGCACCACTGCTGTGCCTTGGAATGCTAGT TGGAGTAATAAATCTCTGGAACAGATTTGGAATCACACGA CCTGGATGGAGTGGGACAGAGAAATTAACAATTACACAAG CTTAATACACTCCTTAATTGAAGAATCGCAAACCAGCAA GAAAAGAATGAACAAGAATTATTGGAATTAGATAAATGGG CAAGTTTGTGGAATTGGTTTAACATAACAAATTGGCTGGG TATATAAAATTATTCATAATGATAGTAGGAGGCTTGGTAG GTTTAAGAATAGTTTTTGTCTGTACTTTCTATAGTGAATAGA GTTAGGCAGGGATATTCACCATTATCGTTTCAGACCCACCT CCCAACCCCGAGGGGACCCGACAGGCCCGAAGGAATAGA AGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTCTG ATTAGTGAACGGATCTCGACGGTATCGATTTAAAGAAAA GGGGGGATTGGGGGGTACAGTGCAGGGGAAAGAATAGTA GACATAATAGCAACAGACATACAACTAAAGAATTACAAA AACAAATTACAAAAAATTCAAAATTTTCGGGTTTATTACAGG GACAGCAGAGATCCAGTTTGCTAGACCATAGAGCCCACCG CATCCCCAGCATGCCTGCTATTGTCTTCCCAATCCTCCCCCT TGCTGTCTCTGCCCCACCCACCCCGCAGAATAGAATGACAC CTACTCAGACAATGCGATGCAATTTCTCTATTATTAGGA AAGGACAGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGC ACGGGGGAGGGGCAACAACAGATGGCTGGCAACTAGAA GGCACAGGCTAGCTAGGTGGATCCGAATAAGGCCTGCAAC GACACACACACGAACAAGCAGAGCCGCTGGACGCCGACTG CGGGACGAAAGGCCCGGAGATGAGGAAGAGGAGAACAGC GCGGCAGACGTGCGCTTTTGAAGCGTGCAGAATGCCGGGC CTCCGGAGGACCTTCGGGCGCCCGCCCCGCCCCCTGAGCCC GCCCCTGAGCCCGCCCCCGGACCCACCCCTTCCAGCCTCT GAGCCCAGAAAGCGAAGGAGCAAAGCTGCTATTGGCCGCT GCCCCAAAGGCCTACCCGCTTCCATTGCTCAGCGGTGCTGT CCATCTGCACGAGACTAGTGAGACGTGCTACTTCCATTTGT CACGTCCTGCACGACGCGAGCTGCGGGGCGGGGGGAACT TCCTGACTAGGGGAGGAGTAGAAGGTGGCGCGAAGGGGC CACCAAAGAACGGAGCCGGTTGGCGCCTACCGGTGGATGT GGAATGTGTGCGAGGCCAGAGGCCACTTGTGTAGCGCAA GTGCCCAGCGGGGCTGCTAAAGCGCATGCTCCAGACTGCC TTGGGAAAAGCGCCTCCCCTACCCATCGATGGCTCCGGTGC CCGTCAGTGGGCAGAGCGCACATCGCCACAGTCCCCGAG AAGTTGGGGGGAGGGGTTCGGCAATTGAACCGGTGCCTAGA GAAGGTGGCGCGGGGTAACTGGGAAAGTGATGTCTGTGTA CTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATA TAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTCGCAACGGG TTTGCCGCCAGAACACAGGTAAGTGCCGTGTGTGGTTCCCG CGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTGCCTT

SEQ ID	Description	Sequences
		GAATTACTTCCACCTGGCTGCAGTACGTGATTCTTGATCCC GAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGAGGCCCTT GCGCTTAAGGAGCCCCCTTCGCCTCGTGCTTGAGTTGAGGCC TGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGC ACCTTCGCGCCTGTCTCGCTGCTTTTCGATAAGTCTCTAGCC ATTTAAAAATTTTTGATGACCTGCTGCGACGCTTTTTTTCTGG CAAGATAGTCTTGTAATGCGGGCCAAGATCTGCACACTG GTATTTGCGTTTTTTGGGGCCGCCGGGCGGCGACGGGGCCCCG TGCCTCCAGCGCACATGTTTCGGCGAGGCGGGGCGCTGCGA GCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTG GCCGGCCTGCTCTGGTGCCTGGTCTCGCGCCGCCGTGTATC GCCCCGCCCTGGGCGGCAAGGCTGGCCCCGGTCGGCACCAG TTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTGC AGGGAGCTCAAAATGGAGGACGACGCGCTCGGGAGAGCG GGCGGGTGAGTCACCCACACAAAGGAAAAGGGCCTTTCCG TCCTCAGCCGTCGCTTCATGTGACTCCACGAGTACCGGGC GCCGTCCAGGCACCTCGATTAGTTCTCGAGCTTTTGAGTA CGTCGTCTTTAGGTTGGGGGGAGGGGTTTTATGCGATGGA GTTTCCCCACACTGAGTGGGTGGAGACTGAAGTTAGGCCA GCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCCTTTTT GAGTTTGGATCTTGGTTCAATTCTCAAGCCTCAGACAGTGGT TCAAAGTTTTTTTCTTCCATTTACAGGTGTCGTGATCTAGATG CCACCGATGCATGTCGACAATCAACCTCTGGATTACAAAA TTTGTGAAAGATTGACTGGTATTCTTAACCTATGTTGCTCCTT TTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCAT GCTATTGCTTCCCGTATGGCTTTCATTTTCTCCTCCTTGAT AAATCCTGGTTGCTGTCTCTTTATGAGGAGTTGTGGCCCCGT TGTCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGCTGACG CAACCCCCACTGGTTGGGGCATTGCCACCACCTGTACAGCTC CTTTCCGGGACTTTTCGCTTTCCCCCTCCCTATTGCCACGGCG GAACTCATCGCCGCTGCCTTGCCCGCTGCTGGACAGGGG CTCGGCTGTTGGGCACTGACAATTCCGTGGTGTGTGCGGGG AAATCATCGTCCTTTCTTGGCTGCTCGCCTGTGTTGCCAC CTGGATTCTGCGCGGGACGTCCTTCTGCTACGTCCTTCGG CCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCG GCTCTGCGGCCTCTTCCGCGTCTCCGCCTTCGCCCTCAGAC GAGTCGGATCTCTCTTTGGGCCGCTCCCCGCCTGGTACCT TTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCC ACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATTCA CTCCAACGAAGATAAGATCTGCTTTTTGCTTGTACTGGGT CTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGC TAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCCTTGC CTTGAGTGCTTCAAGTAGTGTGTGCCCCGTGTTGTGTGAC TCTGGTAACTAGAGATCCCTCAGACCCCTTTTAGTCAGTGTG GAAAATCTCTAGCAGTAGTAGTTTCATGTCATCTTATTATTC AGTATTTATAACTTGCAAAGAAATGAATATCAGAGAGTGA GAGGAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAA GCAATAGCATCACAAATTTACAAATAAAGCATTTTTTTTCA CTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATC TTATCATGTCTGGCTCTAGCTATCCCGCCCCCTAACTCCGCC CATCCCCCCCCCTAACTCCGCCAGTTCCGCCATTCTCCGC CCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAG GCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAG

SEQ ID	Description	Sequences
		GCTTTTTTGGAGGCCTAGACTTTTTGCAGAGACCAAATTCGT AATCATGTGCATAGCTGTTTCTGTGTGAAATTGTTATCCGC TCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTG TAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTA ATTGCGTTGCGCTCACTGCCCCGCTTTCAGTCGGGAAACCT GTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGG AGAGGCGGTTTTCGTATTGGGCGCTCTTCCGCTTCTCGCT CACTGACTCGCTGCGCTCGGTCGTTTCGGCTGCGGCGAGCG GTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAG AATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAG GCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCT GGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACA AAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGG ACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCG TGCGCTCTCTGTTCGACCCCTGCCGCTTACCGGATACCTG TCCGCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAG CTCACGCTGTAGGTATCTCAGTTCGGTGATAGGTCGTTTCGCT CCAAGCTGGGCTGTGTGCACGAACCCCCCGTTTCAGCCCGA CCGCTGCGCCTTATCCGGTAACATCGTCTTGAGTCCAACC CGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGG TAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACA GAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAA GAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACC TTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAA CCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAG ATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGA TCTTTTCTACGGGGTCTGACGCTCAGTGAACGAAAACCTCA CGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTT CACCTAGATCCTTTTAAATTAATAAAGTGTAAATCAA TCTAAAGTATA
119	G019753	mC*mC*mA*GAGCAUCCAAAAGAGUGGUUUUAGAmGmCm UmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGU CCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmG mCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*m U
120	G019757	mA*mG*mA*GCUCUUACCUACAACAUGUUUUAGAmGmCm UmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGU CCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmG mCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*m U
217	G027259	mU*mC*mA*CAGAGGGAUAUCUCUCAGUUUUAGAmGmCm UmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGU CCGUUAUCAmAmCmUmUmGmAmAmAmAmGmUmGmG mCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*m U
218	Open reading frame for SpCas9	AUGGACAAGAAGUACUCCAUCGGCCUGGACAUCGGCACC AACUCCGUGGGCUGGGCCGUGAUCACCGACGAGUACAAG GUGCCCUCCAAGAAGUUAAGGUGCUGGGCAACACCGAC CGGCACUCCAUAAGAAGAACCUGAUCGGCGCCUGCUG UUCGACUCCGGCGAGACCGCCGAGGCCACCCGGCUGAAG CGGACCGCCCGGCGGCGGUACACCCGGCGGAAGAACCGG AUCUGCUACCUGCAGGAGAUUCUCCAACGAGAUGGCC AAGGUGGACGACUCCUUCUCCACCGGCUGGAGGAGUCC

SEQ ID	Description	Sequences
		UUCUUGGUGGAGGAGGACAAGAAGCACGAGCGGCACCCC AUCUUCGGCAACAUCGUGGACGAGGUGGCCUACCACGAG AAGUACCCCAACUACUACCACCGCGGAAGAAGCUGGUG GACUCCACCGACAAGGCCGACCUGCGGCUGAUUACCUG GCCCUGGCCACAUGAUCAAGUUCGGGGCCACUUCUG AUCGAGGGCGACCUGAACCCCGACAACUCCGACGUGGAC AAGCUGUUAUCCAGCUGGUGCAGACCUACAACCAGCUG UUCGAGGAGAACCCCAUCAACGCCUCCGGCGUGGACGCC AAGGCCAUCCUGUCCGGCCCGCUGUCCAAGUCCCGGCGG CUGGAGAACCUGAUCGCCAGCUGCCCGGCGAGAAGAAG AACGGCCUGUUCGGCAACCUGAUCGCCUGUCCUGGGC CUGACCCCAACUUAAGUCCAACUUCGACCUGGCCGAG GACGCCAAGCUGCAGCUGUCCAAGGACACCUACGACGAC GACCUGGACAACCUGCUGGCCCAGAUCCGGCGACCAGUAC GCCGACCUGUCCUGGCCGCCAAGAACCUGUCCGACGCC AUCCUGCUGUCCGACAUCUCCUGCGGGUGAACACGAGAU ACCAAGGCCCCCGUGUCCGCCUCCAUGAUCAAGCGGUAC GACGAGCACCAACGAGGACCUGACCCUGCUGAAGGCCUG GUGCGGCAGCAGCUGCCCGAGAAGUACAAGGAGAUUUC UUCGACCAGUCCAAGAACGGCUACGCCGGCUACAUCGAC GGCGGCGCCUCCAGGAGGAGUUCUACAAGUUAUCAAG CCCAUCCUGGAGAAGAUGGACGGCACCGAGGAGCUGCUG GUGAAGCUGAACCAGGAGGACCUGCUGCGGAAGCAGCGG ACCUUCGACAACGGCUCCAUCCCCCACCAGAUCCACCUGG GCGAGCUGCACGCCAUCCUGCGGCGGCAGGAGGACUUCU ACCCCUUCCUGAAGGACAACCGGGAGAAGAUCGAGAAGA UCCUGACCUUCCGGAUCCCCUACUACGUGGGCCCCCUGGC CCGGGGCAACUCCCGGUUCGCCUGGAUGACCCGGAAGUC CGAGGAGACCAUACACCCCGGAACUUCGAGGAGGUGGU GGACAAGGGCGCCUCCGCCAGUCCUUAUCGAGCGGAU GACCAACUUCGACAAGAACCUGCCCAACGAGAAGGUGCU GCCCAAGCACUCCUGCUGUACGAGUACUUCACCGUGUA CAACGAGCUGACCAAGGUGAAGUACGUGACCGAGGGCAU GCGGAAGCCCGCCUCCUGUCCGGCGAGCAGAAGAAGGC CAUCGUGGACCUGCUGUUAAGACCAACCGGAAGGUGAC CGUGAAGCAGCUGAAGGAGGACUACUUAAGAAGAUCGA GUGCUUCGACUCCUGGAGAUUCCGGCGUGGAGGACCG GUUCAACGCCUCCUGGGCACCACCGACCGUGUGAA GAUCAUAAGGACAAGGACUUCUGGACAACGAGGAGAA CGAGGACAUCUGGAGGACAUCGUGCUGACCCUGACCCU GUUCGAGGACCGGGAGAUGAUCGAGGAGCGGCUGAAGAC CUACGCCCACCUGUUCGACGACAAGGUGAUGAAGCAGCU GAAGCGGCGGCGGUACACCGGCUGGGGCGGCUGUCCCG GAAGCUGAUCAACGGCAUCCGGGACAAGCAGUCCGGCAA GACCAUCCUGGACUUCUGAAGUCCGACGGCUUCGCCAA CCGGAACUUAUGCAGCUGAUCCACGACGACUCCUGAC CUUCAAGGAGGACAUCAGAAAGGCCAGGUGUCCGGCCA GGGCGACUCCUGCACGAGCACAUCGCCAACCGGCCGG CUCCCCGCCAUAAGAAGGGCAUCCUGCAGACCGUGAA GGUGGUGGACGAGCUGGUGAAGGUGAUGGGCCGGCACA GCCCCGAGAACAUCGUGAUCGAGAUGGCCCGGGAGAACCA GACCACCCAGAAGGGCCAGAAGAACUCCCGGGAGCGGAU GAAGCGGAUCGAGGAGGGCAUCAAGGAGCUGGGCUCCCA

<u>SEQ ID</u>	<u>Description</u>	<u>Sequences</u>
		GAUCCUGAAGGAGCACCCCGUGGAGAACACCCAGCUGCA GAACGAGAAGCUGUACCUGUACUACCUGCAGAACGGCCG GGACAUGUACGUGGACCAGGAGCUGGACAUAACCGGCU GUCCGACUACGACGUGGACCACAUCGUGCCCCAGUCCUU CCUGAAGGACGACUCCAUCGACAACAAGGUGCUGACCCG GUCCGACAAGAACCGGGGCAAGUCCGACAACGUGCCCUC CGAGGAGGUGGUGAAGAAGAUGAAGAACUACUGGCGGCA GCUGCUGAACGCCAAGCUGAUCACCCAGCGGAAGUUCGA CAACCUGACCAAGGCCGAGCGGGGCGGCCUGUCCGAGCU GGACAAGGCCGGCUUCAUCAAGCGGCAGCUGGUGGAGAC CCGGCAGAUACCAAGCACGUGGCCCAGAUCCUGGACUC CCGGAUGAACACCAAGUACGACGAGAACGACAAGCUGAU CCGGGAGGUGAAGGUGAUCACCCUGAAGUCCAAGCUGGU GUCCGACUUCGGGAAGGACUUCAGUUCUACAAGGUGCG GGAGAUCAACAACUACCACCACGCCACGACGCCUACCU GAACGCCGUGGUGGGCACCGCCUGAUCAGAAGUACCC CAAGCUGGAGUCCGAGUUCGUGUACGGCGACUACAAGGU GUACGACGUGCGGAAGAUGAUCGCCAAGUCCGAGCAGGA GAUCGGCAAGGCCACCGCCAAGUACUUCUUCUACUCCAA CAUCAUGAACUUCUUAAGACCGAGAUACCCUGGCCAA CGGCGAGAUCCGGAAGCGGCCCCUGAUCGAGACCAACGG CGAGACCGGCGAGAUUCGUGUGGGACAAGGGCCGGGACUU CGCCACCGUGCGGAAGGUGCUGUCCAUGCCCCAGGUGAA CAUCGUGAAGAAGACCGAGGUGCAGACCGGCGGCUUCUC CAAGGAGUCCAUCCUGCCCAAGCGGAACUCCGACAAGCU GAUCGCCCAGGAAGAAGGACUGGGACCCCAAGAAGUACGG CGGCUUCGACUCCCCACCGUGGCCUACUCCGUGCUGGU GGUGGCCAAGGUGGAGAAGGGCAAGUCCAAGAAGCUGAA GUCCGUGAAGGAGCUGCUGGGCAUACCAUACUGGAGCG GUCCUCCUUCGAGAAGAACCCCAUCGACUUCUGGAGGC CAAGGGCUACAAGGAGGUGAAGAAGGACCUGAUCAUCAA GCUGCCCAAGUACUCCUGUUCGAGCUGGAGAACGGCCG GAAGCGGAUGCUGGCCUCCGCCGGCGAGCUGCAGAAGGG CAACGAGCUGGCCUCCUCCUCCAAGUACGUGAACUUCU GUACCUGGCCUCCACUACGAGAAGCUGAAGGGCUCCCC CGAGGACAACGAGCAGAAGCAGCUGUUCGUGGAGCAGCA CAAGCACUACCUGGACGAGAUCAUCGAGCAGAUUCCGA GUUCUCCAAGCGGGUGAUCCUGGCCGACGCCAACCUGGA CAAGGUGCUGUCCGCCUACAACAAGCACCGGGACAAGCC CAUCCGGGAGCAGGCCGAGAACAUAUCCACCUGUUCAC CCUGACCAACCUGGGCGCCCCCGCCGCCUUAAGUACUUC GACACCACCAUCGACCGGAAGCGGUACACCUCCACCAAG GAGGUGCUGGACGCCACCCUGAUCCACCAGUCCAUCACC GGCCUGUACGAGACCCGGAUCGACCUGUCCAGCUGGGC GGCGACGGCGGGCGGCUCCCCCAAGAAGAAGCGGAAGGUG UGA
219	Amino acid sequence for SpCas9	MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHS IKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIF SNEMAKVDDSFHRLVESFLVEEDKKHERHPIFGNIVDEVAY HEKYPTIYHLRKKLVSTDKADLRILIYLAHMIKFRGHFLIE GDLNPDNSDVKLFIQLVQTYNQLFEENPINASGVDAKAILS RLSKSRLENLIAQLPGEKKNGLFGNLIASLGLTPNFKSNFDL AEDAKLQLSKDQYDDDLNLLAQIGDQYADLFLAAKNLSDA

SEQ ID	Description	Sequences
		ILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQ LPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDG TEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQED FYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEE TITPWNFEVVVDKGASAQSFIERMTNFDKNLPNEKVLPHKSL YEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTN RKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLL KIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHL FDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLK SDGFANRNFMLIHDDSLTFKEDIQKAQVSGQGDSLHEHIAN LAGSPAIIKKGILQTVKVVDLVKVMGRHKPENIVIEMARENQ TTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKL YLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSID NKVLTRSDKNRGKSDNPSEEVVKMKMKNYWRQLLNAKLITQ RKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDS RMNTKYDENDKLIREVKVITLKSCLVSDFRKDFQFYKVIN NYHHAHDAYLNAVVGTAIIKKYPKLESEFVYGDYKVVDVR KMIKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLI ETNGETGEIVWDKGRDFATVRKVLSPQVNVKKTETVQTGG FSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVV AKVEKGKSKKLKSVKELLGITIMERSSEKPNIDFLEAKGYKE VKKDLIIKLPKYSLELENGRKRMLASAGELQKGNELALPSK YVNFLYLASHYEKLKGSPEDEQKQLFVEQHKHYLDEIIEQIS EFSKRVLADANLDKVL SAYNKHDKPIREQAENIIHLFTLTN LGAPAAFKYFDTTIDRKRYTSTKEVLDTLIHQSIITGLYETRID LSQLGGDGGGSPKKKRKV
228	G021925	mU*mU*mC*UUUGUAGAACUUGAAGUGUUUUAGAmGmCm UmAmGmAmAmAmUmAmGmCAAGUUAUUAAAGGCUAGU CCGUUAUCAmAmCmUmUmGmAmAmAmAmAmUmGmGm mCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*m U
229	Amino acid sequence for IL10 D25/E96A	MHSSALLCCLVLLTGVRASPGQGTQSENSCTHFPGNLPNMLR ALRDAFSRVKTFQMKDQLDNLKESLLEDFKGYLGCCQALS EMIQFYLEEVMPPQAENQDPDIKAHVNSLGANLKTLLRLRRC HRFLPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIE AYMTMKIRN
230	Nucleic acid sequence for IL10 D25/E96A	ATGCACAGCTCAGCACTGCTCTGTTGCCTGGTCCTCCTGAC TGGGGTGAGGGCCAGCCCAGGCCAGGGCACCCAGTCTGAG AACAGCTGCACCCACTTCCCAGGCAACCTGCCTAACATGCT TCGAGCTCTCCGAGATGCCTTCAGCAGAGTGAAGACTTTCT TTcaaatgaaggatcagctggacaACTTGTTGTTAAAGGAGTCCTTGCT GGAGGACTTTAAGGGTTACCTGGGTTGCCAAGCCTTGCTG AGATGATCCAGTTTTACCTGGAGGAGGTGATGCCCCAAGC TGAGAACCAAGACCCAGACATCAAGGCGCATGTGAATCC CTGGGGGCTAACCTGAAGACCCCTCAGGCTGAGGCTACGGC GCTGTCATCGATTTCTTCCCTGTGAAAACAAGAGCAAGGCC GTGGAGCAGGTGAAGAATGCCTTTAATAAGCTCCAAGAGA AAGGCATCTACAAAGCCATGAGTGAGTTTGACATCTTCATC AACTACATAGAAGCCTACATGACAATGAAGATACGAACT GA

<u>SEQ ID</u>	<u>Description</u>	<u>Sequences</u>
351	G029528	mU*mC*mG*CUUUGCUGAGGUCUAUAGUUUUAGAmGmCm UmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGU CCGUUAUCACGAAAGGGCACCGAGUCGmU*mG*mC*mU

[000343] The vector comprising: a guide RNA, RNA-binding DNA binding agent, or donor construct comprising a sequence encoding the dmTGFB1 molecule, the regulatory T cell promoting molecule, e.g., IL10, CTLA4; or targeting receptor, e.g., a CAR, individually or in any combination, may be delivered by liposome, a nanoparticle, an exosome, or a microvesicle. The vector may also be delivered by a lipid nanoparticle (LNP). One or more guide RNA, RNA-binding DNA binding agent (*e.g.*, mRNA), or donor construct comprising a sequence encoding a heterologous protein, individually or in any combination, may be delivered by LNP, liposome, a nanoparticle, an exosome, or a microvesicle. One or more guide RNA, RNA-binding DNA binding agent (*e.g.*, mRNA), or donor construct comprising a sequence encoding a heterologous protein, individually or in any combination, may be delivered by LNP. In some embodiments, one or more guide RNA and an RNA-guided DNA-binding agent (*e.g.*, mRNA) are delivered by LNP. A donor construct may be delivered by viral vector.

[000344] Lipid nanoparticles (LNPs) are a well-known means for delivery of nucleotide and protein cargo, and may be used for delivery of any of the guide RNAs, RNA-guided DNA binding agent, or donor construct disclosed herein.

[000345] As used herein, lipid nanoparticle (LNP) refers to a particle that comprises a plurality of (*i.e.*, more than one) lipid molecules physically associated with each other by intermolecular forces. The LNPs may be, *e.g.*, microspheres (including unilamellar and multilamellar vesicles, *e.g.*, “liposomes”—lamellar phase lipid bilayers that, in some embodiments, are substantially spherical and, in more particular embodiments, can comprise an aqueous core, *e.g.*, comprising a substantial portion of RNA molecules), a dispersed phase in an emulsion, micelles, or an internal phase in a suspension (see, *e.g.*, WO2017173054, the contents of which are hereby incorporated by reference in their entirety). Any LNP known to those of skill in the art to be capable of delivering nucleotides to subjects may be utilized. Exemplary LNP formulations and methods for delivery of agents to T cell for modification are provided, for example, in WO2021222287.

[000346] In some embodiments, provided herein is a method for delivering any of the guide RNAs described herein or donor construct disclosed herein, alone or in combination, to a cell or a population of cells or a subject, wherein any one or more of the components is associated with an LNP. In some embodiments, the method further comprises an RNA-guided DNA-binding agent (*e.g.*, Cas9 or a sequence encoding Cas9).

[000347] In some embodiments, provided herein is a composition comprising any of the guide RNAs described herein or donor construct disclosed herein, alone or in combination, with an LNP. In some embodiments, the composition further comprises an RNA-guided DNA-binding agent (*e.g.*, Cas9 or a sequence encoding Cas9).

[000348] In some embodiments, the LNPs comprise cationic lipids. In some embodiments, the LNPs comprise (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-(((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-(((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate) or another ionizable lipid. See, *e.g.*, lipids of WO2019067992, WO2017173054, WO2015095340, and WO2014136086, as well as references provided therein. In some embodiments, the LNPs comprise molar ratios of a cationic lipid amine to RNA phosphate (N:P) of about 4.5, 5.0, 5.5, 6.0, or 6.5. In some embodiments, the term cationic and ionizable in the context of LNP lipids is interchangeable, *e.g.*, wherein ionizable lipids are cationic depending on the pH.

[000349] In some embodiments, LNPs associated with the construct disclosed herein are for use in preparing a cell-based medicament for suppressing immune response. Methods for preparation of cell-based therapeutics and reagents for use in cell based therapeutics are known in the art.

[000350] In some embodiments, any of the guide RNAs described herein, RNA-guided DNA binding agents, or donor construct disclosed herein, alone or in combination, whether naked or as part of a vector, is formulated in or administered via a lipid nanoparticle; see *e.g.*, WO2019067992, WO2017173054, or WO2021222287 the contents of which are hereby incorporated by reference in their entirety.

[000351] In some embodiments, an LNP composition comprising: an RNA component and a lipid component, wherein the lipid component comprises an amine lipid such as a biodegradable, ionizable lipid. In some instances, the lipid component comprises biodegradable, ionizable lipid, cholesterol, DSPC (distearoylphosphatidylcholine), and PEG-DMG (1,2-dimyristoyl-rac-glycero-3-methylpolyoxyethylene glycol 2000 (PEG2k-DMG)). In certain embodiments, the lipid nucleic acid assemblies contained ionizable Lipid A ((9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-(((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-(((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate), cholesterol, DSPC (distearoylphosphatidylcholine), and PEG2k-DMG (1,2-dimyristoyl-rac-glycero-3-methylpolyoxyethylene glycol 2000 (PEG2k-DMG)). In certain embodiments, the components are present in a 50 Lipid A:38 cholesterol:9 DSPC:3 PEG-DMG molar ratio, respectively. In certain embodiments, the components are present in a 35 Lipid A:47.5

cholesterol:15 DSPC:2.5 PEG-DMG molar ratio, respectively. The lipid nucleic acid assemblies may be formulated with a lipid amine to RNA phosphate (N:P) molar ratio of about 6, and a ratio of gRNA to mRNA of 2:1, 1:1, or 1:2 by weight.

[000352] It will be apparent that a guide RNA, an RNA-guided DNA-binding agent (*e.g.*, Cas nuclease or a nucleic acid encoding a Cas nuclease), and a donor construct comprising a sequence encoding the dmTGFB1 molecule; the regulatory T cell promoting molecule, *e.g.*, IL10, or targeting receptor, *e.g.*, a CAR can be delivered using the same or different systems. For example, the guide RNA, Cas nuclease, and construct can be carried by the same vector (*e.g.*, AAV). Alternatively, the Cas nuclease (as a protein or mRNA) or gRNA can be carried by a plasmid or LNP, while the donor construct can be carried by a vector such as AAV.

[000353] The different delivery systems can be delivered simultaneously or in any sequential order. In some embodiments, the donor construct, guide RNA, and Cas nuclease can be delivered simultaneously, *e.g.*, in one vector, two vectors, individual vectors, one LNP, two LNPs, individual LNPs, or a combination thereof. In some embodiments, the donor construct can be delivered as a vector or associated with a LNP, prior to (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or more days) delivering the guide RNA or Cas nuclease, as a vector or associated with a LNP singly or together or as a ribonucleoprotein (RNP). As a further example, the guide RNA and Cas nuclease, as a vector or associated with a LNP singly or together or as a ribonucleoprotein (RNP), can be delivered prior to (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or more days) delivering the construct, as a vector or associated with a LNP.

IV. Method of Engineering T Cells

[000354] The disclosure provides methods of engineering T cells to comprise a modification by insertion into the cell of heterologous sequence encoding dmTGFB1 under control of a promoter sequence and a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TGFB2. The disclosure provides methods of engineering T cells further comprising a modification *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and/or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence. The disclosure provides methods of engineering T cells to comprise a modification by insertion into the cell of heterologous sequence encoding dmTGFB1 under control of a promoter sequence and a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TGFB2. The disclosure provides methods of engineering T cells further comprising a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and/or insertion into the cell of heterologous sequence(s) encoding IL10 under control of a

promoter sequence. The disclosure provides methods of engineering T cells to comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG and/or; and insertion into the cell of heterologous sequence(s) encoding CTLA4 under control of a promoter sequence. The disclosure provides methods of engineering T cells to comprise a modification by insertion into the cell of heterologous sequence encoding dmTGFB1 under control of a promoter sequence and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2. The disclosure provides methods of engineering T cells further comprising a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and/or insertion into the cell of heterologous sequences encoding IL10 and CTLA4, each under control of a promoter sequence.

[000355] In some embodiments, the methods comprise engineering T cells to comprise a modification by insertion into the cell of heterologous sequence encoding dmTGFB1 under control of a promoter sequence and e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2 and, optionally, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and/or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule, e.g., IL10 or CTLA4, and further comprise a modification, e.g., knockdown, of TCR sequence(s).

[000356] In some embodiments, the methods comprise engineering T cells to comprise a modification by insertion into the cell of heterologous sequence encoding dmTGFB1 under control of a promoter sequence, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule, e.g., IL10 or CTLA4, and further comprise insertion into the cell of heterologous sequence(s) encoding a targeting receptor, e.g., a CAR.

[000357] In some embodiments, the methods comprise engineering T cells to comprise a modification by insertion into the cell of heterologous sequence encoding dmTGFB1 under control of a promoter sequence, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion into the cell of heterologous sequence(s) encoding a regulatory T cell

promoting molecule, e.g., IL10 or CTLA4, a modification, e.g., knockdown, of TCR sequence(s), and insertion into the cell of heterologous sequence(s) encoding a targeting receptor, e.g., a CAR.

[000358] In some embodiments, the methods comprise modification by insertion into the cell of heterologous sequence encoding dmTGFB1 under control of a promoter sequence, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; the modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule, optional knockdown of a TCR gene and optional insertion into the cell of a targeting receptor, e.g., a CAR, are engineered using the CRISPR/Cas system and the guide RNAs disclosed herein.

[000359] In these embodiments, the regulatory T cell promoting molecule to be inserted may be provided via a donor construct. The regulatory T cell promoting molecule provided via a donor construct may be selected from IL10, CTLA4, TIGIT, IDO1, ENTPD1, NT5E, IL22, AREG, IL35, GARP, CD274, FOXP3, IKZF2, EOS, IRF4, LEF1, BACH2, and IL2RA; and a modification, e.g., knockdown, of TCR gene sequence(s).

In these embodiments, the targeting receptor to be inserted may be provided via a donor construct. In some embodiments, the targeting receptor may be a chimeric antigen receptor (CAR), a T-cell receptor (TCR), or a receptor for a cell surface molecule operably linked through at least a transmembrane domain in an internal signaling domain capable of being activated upon binding of the extracellular receptor portion to a target. In some embodiments, the targeting receptor may be a receptor present on the surface of an engineered cell, e.g., a T cell, to permit binding of the cell to a target site, e.g., a specific cell or tissue in an organism. In some embodiments, the targeting receptor may permit binding of an engineered T cell to a specific target cell, wherein the engineered T cell homes to a site of disease (e.g., mucosal cells at a site of inflammation) and acts directly on the target cell (e.g., kills or suppresses the target cell); and/or the targeting receptor may permit binding of an engineered T cell to a specific target cell that is not specific to a site of disease (e.g., activated immune cells of the lymph system), and acts directly on the target cell (e.g., kills or suppresses the target cell).

[000360] In some of these embodiments, the targeting receptor is a CAR capable of targeting MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02).

[000361] Suitable gene editing systems for engineering the T cells to comprise insertions and modifications, e.g., knockdowns, are disclosed herein and known in the art. In some embodiments, the gene editing systems include but are not limited to the CRISPR/Cas system; zinc finger nuclease (ZFN) system; transcription activator-like effector nuclease (TALEN) system. Generally, the gene editing systems involve the use of engineered cleavage systems to induce a double strand break (DSB) or a nick (e.g., a single strand break, or SSB) in a target DNA sequence. Cleavage or nicking can occur through the use of specific nucleases such as engineered ZFN, TALENs, or using the CRISPR/Cas system with an engineered guide RNA to guide specific cleavage or nicking of a target DNA sequence, such as a CRISPR/Cas9 system. Further, targeted nucleases are being developed based on the Argonaute system (e.g., from *T. thermophilus*, known as 'TtAgo', see Swarts et al (2014) *Nature* 507(7491): 258-261), which also may have the potential for uses in genome editing and gene therapy.

[000362] Transcription activator-like effector nucleases (TALEN) are restriction enzymes that can be engineered to cut specific sequences of DNA. They are made by fusing a TAL effector DNA-binding domain to a DNA cleavage domain (a nuclease which cuts DNA strands). Transcription activator-like effectors (TALEs) can be engineered to bind to a desired DNA sequence, to promote DNA cleavage at specific locations (see, e.g., Boch, TALEs of genome targeting *Nature Biotech.* 29:135–136 (2011)). The restriction enzymes can be introduced into cells, for use in gene editing or for genome editing in situ, a technique known as genome editing with engineered nucleases. Such methods and compositions for use therein are known in the art. See, e.g., WO2019147805, WO2014040370, WO2018073393, the contents of which are hereby incorporated in their entireties.

[000363] Zinc-finger nucleases (ZFNs) are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. Zinc finger domains can be engineered to target specific desired DNA sequences to enables zinc-finger nucleases to target unique sequences within complex genomes. The non-specific cleavage domain from the type II restriction endonuclease FokI is typically used as the cleavage domain in ZFNs. Cleavage is repaired by endogenous DNA repair machinery, allowing ZFN to precisely alter the genomes of higher organisms. Such methods and compositions for use therein are known in the art. See, e.g., WO2011091324, the contents of which are hereby incorporated in their entireties.

[000364] RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression or translation, by neutralizing targeted mRNA molecules. Small interfering RNAs (siRNAs) are central to RNA interference. RNAs are the direct products of genes, and these small RNAs (typically each strand being 19-23 nucleotides in length forming a duplex of 19-21 nucleotides) can direct the RNA induced silencing (RISC) complex to degrade messenger RNA (mRNA) molecules and thus decrease their activity by preventing translation, via post-

transcriptional gene silencing. Short hairpin RNAs (shRNAs) are siRNAs that are a single RNA strand wherein the strands forming the duplex region have a hairpin structure, often generated by transcription from an expression vector. RNAi can also be accomplished by longer RNA duplex structures referred to as Dicer substrate molecules, which are cleaved by the enzyme Dicer before being loaded into RISC to promote target mRNA cleavage. Such methods and compositions for use are known in the art. In the compositions and methods provided herein, it is preferred that the RNA molecule to promote RNA interference is provided as an expression vector for durability, see, e.g., WO2018208837, the contents of which are hereby incorporated in their entireties. In some embodiments, RNAi is used with an expression vector.

[000365] It will be appreciated that the present disclosure contemplates methods of insertion performed with or without the guide RNAs disclosed herein (*e.g.*, using a ZFN system to cause a break in a target DNA sequence, creating a site for insertion of the construct). For methods that use guide RNAs disclosed herein, the methods include the use of the CRISPR/Cas system to modify, *e.g.*, knockdown, a nucleic acid sequence encoding TGFBR2, TNFA, IFNG, or TCR. It will also be appreciated that the present disclosure contemplates methods of modifying, *e.g.*, knocking down, TGFBR2, TNFA, IFNG, or TCR, which can be performed without the guide RNAs disclosed herein (*e.g.*, using a ZFN system to cause a break in a target DNA sequence, creating a site for insertion of the construct).

[000366] In some embodiments, the donor construct comprising the sequence for insertion, *e.g.*, a sequence encoding a dmTGFB1 or a regulatory T cell promoting molecule, *e.g.*, IL10, or CTLA4, is inserted at a genomic locus for a sequence that is targeted for modification, *e.g.*, knockdown, *e.g.*, a TCR gene.

[000367] In some embodiments, a CRISPR/Cas system (*e.g.*, a guide RNA and RNA-guided DNA binding agent) can be used to create a site of insertion at a desired locus within a genome, at which site a donor construct comprising a sequence encoding dmTGFB1; a regulatory T cell promoting molecule, *e.g.*, IL10, CTLA4; or targeting receptor, *e.g.*, a CAR, *e.g.*, a MAdCAM-1 CAR, disclosed herein can be inserted to express dmTGFB1; a regulatory T cell promoting molecule, *e.g.*, IL10, CTLA4, or a CAR, *e.g.*, a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demogelin 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR. The targeting receptor, *e.g.*, a CAR, *e.g.*, a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2

(SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR; or inserted sequence, dmTGFB1 or a regulatory T cell promoting molecule, e.g., IL10, or CTLA4, may be heterologous with respect to its insertion site or locus, for example a safe harbor locus or a TCR locus from which dmTGFB1; a regulatory T cell promoting molecule, e.g., IL10, CTLA4; or targeting receptor, e.g., a CAR, e.g., MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR, is not normally expressed, as described herein. In some embodiments, a guide RNA described herein can be used according to the present methods with an RNA-guided DNA-binding agent (e.g., Cas nuclease) to create a site of insertion, at which site a donor construct comprising a sequence encoding dmTGFB1; a regulatory T cell promoting molecule, e.g., IL10, CTLA4; or targeting receptor, e.g., a CAR, e.g., a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR can be inserted to express dmTGFB1; a regulatory T cell promoting molecule, e.g., IL10, CTLA4; or a CAR, e.g., a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR. The guide RNAs for insertion of dmTGFB1; a regulatory T cell promoting molecule, e.g., IL10, CTLA4; or targeting receptor, e.g., a CAR, e.g., a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR, into specific genomic loci, are exemplified and described herein. In alternative embodiments, the targeting receptor is a TNFA targeting receptor.

[000368] In some embodiments, CD4⁺ T cells are engineered by transduction (e.g., using viral or non-viral delivery) with a gRNA (e.g., gRNA targeting TGFBR2, IFNG, TNFA, or TCR for knockdown), an RNA-guided DNA-binding agent (e.g., Cas nuclease), a donor construct. In some embodiments, the engineered T cells are: 1) transduced with a gRNA targeting a nucleic acid

sequence encoding TGFBR2 and, optionally, a pro-inflammatory cytokine, e.g., IFNG or TNFA, an RNA guided DNA binding agent (e.g., Cas nuclease), and 2) transduced with a donor construct comprising nucleic acid sequence(s) encoding dmTGFB1 and, optionally, a regulatory T cell promoting molecule, e.g., IL10 or CTLA4; and a targeting receptor, e.g., a CAR, e.g., a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR; or a TNFA targeting receptor. In certain embodiments, the engineered cells are selected for expression of the targeting receptor.

[000369] In some embodiments, CD4⁺ T cells are engineered by transduction with a gRNA (e.g., gRNA targeting TGFBR2, IFNG, TNFA, or TCR for knockdown), an RNA-guided DNA-binding agent (e.g., Cas nuclease) and a donor construct. In some embodiments, the engineered T cells are: 1) transduced with a donor construct comprising nucleic acid sequence(s) encoding dmTGFB1 and, optionally, a regulatory T cell promoting molecule, e.g., IL10 or CTLA4, and/or a targeting receptor, e.g., a CAR, e.g., a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR, and 2) transduced with a gRNA targeting a nucleic acid sequence encoding TGFBR2 and, optionally, a pro-inflammatory cytokine, e.g., IFNG or TNFA, an RNA guided DNA binding agent (e.g., Cas nuclease). In certain embodiments, the engineered cells are selected for expression of the targeting receptor.

[000370] In some embodiments, CD4⁺ T cells are engineered by transduction with a gRNA (e.g., gRNA targeting TGFBR2, IFNG, TNFA, or TCR for knockdown), an RNA-guided DNA-binding agent (e.g., Cas nuclease), a donor construct. In some embodiments, the engineered T cells are: 1) transduced with a donor construct comprising nucleic acid sequence(s) encoding dmTGFB1 and, optionally, a regulatory T cell promoting molecule, e.g., IL10 or CTLA4, and/or a targeting receptor, e.g., a CAR, e.g., a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR, and 2) transduced with a gRNA targeting a nucleic acid sequence encoding TGFBR2 and, optionally, a

pro-inflammatory cytokine, e.g., IFNG or TNFA; and an RNA guided DNA binding agent (e.g., Cas nuclease).

[000371] As described herein, the donor construct comprising a sequence encoding dmTGFB1; a regulatory T cell promoting molecule, e.g., IL10, CTLA4; or a targeting receptor e.g., a CAR, guide RNA (e.g., gRNA targeting TGFBR2, IFNG, TNFA, or TCR for knockdown), and RNA-guided DNA-binding agent can be delivered using any suitable delivery system and method known in the art. In some embodiments, the guide RNA and Cas nuclease are associated with an LNP and delivered to the cell or the population of cells prior to delivering the donor construction comprising a sequence encoding dmTGFB1; a regulatory T cell promoting molecule, e.g., IL10, CTLA4; or a targeting receptor, e.g., a CAR. In some embodiments, the guide RNA and Cas nuclease are associated with an LNP and delivered to the cell or the population of cells after delivering the donor construction comprising a sequence encoding dmTGFB1; a regulatory T cell promoting molecule, e.g., IL10, CTLA4, or a targeting receptor, e.g., a CAR.

[000372] In some embodiments, administration of the gRNAs, donor construct, and RNA-guided DNA binding agents described herein to a naturally occurring T cell is capable of converting the naturally occurring T cell, e.g., a CD4⁺ T cell, to a cell that exhibits the characteristics, e.g., immune response suppressive characteristics, of a regulatory T cell.

[000373] gRNAs, donor constructs, and RNA-guided DNA binding agents for modifying, e.g., knocking down, TGFBR2, IFNG, TNFA, or TCR gene expression or inserting a sequence encoding dmTGFB1; a regulatory T cell promoting molecule, e.g., IL10, CTLA4, or a targeting receptor, e.g., a CAR, e.g., a MAdCAM-1, TNFA, CD70, CEACAM6, VCAM-1, citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNFSF7, TNFRSF17, dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), demoglein 3 (DSG3), or MHC class I HLA-A (HLA-A*02) CAR, may be introduced to a conventional T cell or population of conventional T cells to generate the engineered T cells or population of T cells described herein.

[000374] Methods of using various RNA-guided DNA-binding agents, e.g., a nuclease, such as a Cas nuclease, e.g., Cas9, are also well known in the art. While the use of a CRISPR/Cas system is exemplified herein, it will be appreciated that suitable variations to the system can also be used. It will be appreciated that, depending on the context, the RNA-guided DNA-binding agent can be provided as a nucleic acid (e.g., DNA or mRNA), such as the mRNAs encoding an RNA-guided DNA-binding agent provided above, or as a protein. In some embodiments, the present method can be practiced in a cell that already comprises or expresses an RNA-guided DNA-binding agent.

[000375] In some embodiments, the RNA-guided DNA-binding agent, such as a Cas9 nuclease, has cleavage activity, which can also be referred to as double-strand endonuclease activity. In some embodiments, the RNA-guided DNA-binding agent, such as a Cas9 nuclease, has nickase activity, which can also be referred to as single-strand endonuclease activity. In some embodiments, the RNA-guided DNA-binding agent comprises a Cas nuclease. Examples of Cas nucleases include those of the type II CRISPR systems of *S. pyogenes*, *S. aureus*, *Neisseria meningitidis*, and other prokaryotes (see, e.g., the list in the next paragraph), and variant or mutant (e.g., engineered, non-naturally occurring, naturally occurring, or other variant) versions thereof. See, e.g., US20160312198; US 20160312199.

[000376] Non-limiting exemplary species that the Cas nuclease can be derived from include *Streptococcus pyogenes*, *Streptococcus thermophilus*, *Streptococcus sp.*, *Staphylococcus aureus*, *Listeria innocua*, *Lactobacillus gasseri*, *Francisella novicida*, *Wolinella succinogenes*, *Sutterella wadsworthensis*, *Gammaproteobacterium*, *Neisseria meningitidis*, *Campylobacter jejuni*, *Pasteurella multocida*, *Fibrobacter succinogene*, *Rhodospirillum rubrum*, *Nocardiopsis dassonvillei*, *Streptomyces pristinaespiralis*, *Streptomyces viridochromogenes*, *Streptomyces viridochromogenes*, *Streptosporangium roseum*, *Streptosporangium roseum*, *Alicyclobacillus acidocaldarius*, *Bacillus pseudomycoides*, *Bacillus selenitireducens*, *Exiguobacterium sibiricum*, *Lactobacillus delbrueckii*, *Lactobacillus salivarius*, *Lactobacillus buchneri*, *Treponema denticola*, *Microscilla marina*, *Burkholderiales bacterium*, *Polaromonas naphthalenivorans*, *Polaromonas sp.*, *Crocospaera watsonii*, *Cyanothece sp.*, *Microcystis aeruginosa*, *Synechococcus sp.*, *Acetohalobium arabaticum*, *Ammonifex degensii*, *Caldicelulosiruptor beccii*, *Candidatus Desulforudis*, *Clostridium botulinum*, *Clostridium difficile*, *Finogoldia magna*, *Natranaerobius thermophilus*, *Pelotomaculum thermopropionicum*, *Acidithiobacillus caldus*, *Acidithiobacillus ferrooxidans*, *Allochrochromatium vinosum*, *Marinobacter sp.*, *Nitrosococcus halophilus*, *Nitrosococcus watsoni*, *Pseudoalteromonas haloplanktis*, *Ktedonobacter racemifer*, *Methanohalobium evestigatum*, *Anabaena variabilis*, *Nodularia spumigena*, *Nostoc sp.*, *Arthrospira maxima*, *Arthrospira platensis*, *Arthrospira sp.*, *Lyngbya sp.*, *Microcoleus chthonoplastes*, *Oscillatoria sp.*, *Petrotoga mobilis*, *Thermosipho africanus*, *Streptococcus pasteurianus*, *Neisseria cinerea*, *Campylobacter lari*, *Parvibaculum lavamentivorans*, *Corynebacterium diphtheria*, *Acidaminococcus sp.*, *Lachnospiraceae bacterium ND2006*, and *Acaryochloris marina*.

[000377] In some embodiments, the Cas nuclease is the Cas9 nuclease from *Streptococcus pyogenes*. In some embodiments, the Cas nuclease is the Cas9 nuclease from *Streptococcus thermophilus*. In some embodiments, the Cas nuclease is the Cas9 nuclease from *Neisseria meningitidis*. In some embodiments, the Cas nuclease is the Cas9 nuclease is from *Staphylococcus aureus*. In some embodiments, the Cas nuclease is the Cpf1 nuclease from *Francisella novicida*. In some embodiments, the Cas nuclease is the Cpf1 nuclease from *Acidaminococcus sp.* In some

embodiments, the Cas nuclease is the Cpf1 nuclease from *Lachnospiraceae* bacterium ND2006. In further embodiments, the Cas nuclease is the Cpf1 nuclease from *Francisella tularensis*, *Lachnospiraceae* bacterium, *Butyrivibrio proteoclasticus*, *Peregrinibacteria bacterium*, *Parcubacteria bacterium*, *Smithella*, *Acidaminococcus*, *Candidatus Methanoplasma termitum*, *Eubacterium eligens*, *Moraxella bovoculi*, *Leptospira inadai*, *Porphyromonas crevioricanis*, *Prevotella disiens*, or *Porphyromonas macacae*. In certain embodiments, the Cas nuclease is a Cpf1 nuclease from an *Acidaminococcus* or *Lachnospiraceae*.

[000378] In some embodiments, the gRNA together with an RNA-guided DNA-binding agent is called a ribonucleoprotein complex (RNP). In some embodiments, the RNA-guided DNA-binding agent is a Cas nuclease. In some embodiments, the gRNA together with a Cas nuclease is called a Cas RNP. In some embodiments, the RNP comprises Type-I, Type-II, or Type-III components. In some embodiments, the Cas nuclease is the Cas9 protein from the Type-II CRISPR/Cas system. In some embodiment, the gRNA together with Cas9 is called a Cas9 RNP.

[000379] Wild-type Cas9 has two nuclease domains: RuvC and HNH. The RuvC domain cleaves the non-target DNA strand, and the HNH domain cleaves the target strand of DNA. In some embodiments, the Cas9 protein comprises more than one RuvC domain or more than one HNH domain. In some embodiments, the Cas9 protein is a wild-type Cas9. In each of the composition, use, and method embodiments, the Cas induces a double strand break in target DNA.

[000380] In some embodiments, chimeric Cas nucleases are used, where one domain or region of the protein is replaced by a portion of a different protein. In some embodiments, a Cas nuclease domain may be replaced with a domain from a different nuclease such as FokI. In some embodiments, a Cas nuclease may be a modified nuclease.

[000381] In other embodiments, the Cas nuclease may be from a Type-I CRISPR/Cas system. In some embodiments, the Cas nuclease may be a component of the Cascade complex of a Type-I CRISPR/Cas system. In some embodiments, the Cas nuclease may be a Cas3 protein. In some embodiments, the Cas nuclease may be from a Type-III CRISPR/Cas system. In some embodiments, the Cas nuclease may have an RNA cleavage activity.

[000382] In some embodiments, the RNA-guided DNA-binding agent has single-strand nickase activity, *i.e.*, can cut one DNA strand to produce a single-strand break, also known as a “nick.” In some embodiments, the RNA-guided DNA-binding agent comprises a Cas nickase. A nickase is an enzyme that creates a nick in dsDNA, *i.e.*, cuts one strand but not the other of the DNA double helix. In some embodiments, a Cas nickase is a version of a Cas nuclease (*e.g.*, a Cas nuclease discussed above) in which an endonucleolytic active site is inactivated, *e.g.*, by one or more alterations (*e.g.*, point mutations) in a catalytic domain. See, *e.g.*, US Pat. No. 8889356 for

discussion of Cas nickases and exemplary catalytic domain alterations. In some embodiments, a Cas nickase such as a Cas9 nickase has an inactivated RuvC or HNH domain.

[000383] In some embodiments, the RNA-guided DNA-binding agent is modified to contain only one functional nuclease domain. For example, the agent protein may be modified such that one of the nuclease domains is mutated or fully or partially deleted to reduce its nucleic acid cleavage activity. In some embodiments, a nickase is used having a RuvC domain with reduced activity. In some embodiments, a nickase is used having an inactive RuvC domain. In some embodiments, a nickase is used having an HNH domain with reduced activity. In some embodiments, a nickase is used having an inactive HNH domain.

[000384] In some embodiments, a conserved amino acid within a Cas protein nuclease domain is substituted to reduce or alter nuclease activity. In some embodiments, a Cas nuclease may comprise an amino acid substitution in the RuvC or RuvC-like nuclease domain. Exemplary amino acid substitutions in the RuvC or RuvC-like nuclease domain include D10A (based on the *S. pyogenes* Cas9 protein). *See, e.g.*, Zetsche et al. (2015) *Cell* Oct 22:163(3): 759-771. In some embodiments, the Cas nuclease may comprise an amino acid substitution in the HNH or HNH-like nuclease domain. Exemplary amino acid substitutions in the HNH or HNH-like nuclease domain include E762A, H840A, N863A, H983A, and D986A (based on the *S. pyogenes* Cas9 protein). *See, e.g.*, Zetsche et al. (2015). Further exemplary amino acid substitutions include D917A, E1006A, and D1255A (based on the *Francisella novicida* U112 Cpf1 (FnCpf1) sequence (UniProtKB - A0Q7Q2 (CPF1_FRATN))). Exemplary amino acid substitutions in the HNH or HNH-like nuclease domain or RuvC or RuvC-like domains for *N. meningitidis* include Nme2Cas9D16A (HNH nickase) and Nme2Cas9H588A (RuvC nickase).

[000385] In some embodiments, a nickase is provided in combination with a pair of guide RNAs that are complementary to the sense and antisense strands of the target sequence, respectively. In this embodiment, the guide RNAs direct the nickase to a target sequence and introduce a DSB by generating a nick on opposite strands of the target sequence (*i.e.*, double nicking). In some embodiments, a nickase is used together with two separate guide RNAs targeting opposite strands of DNA to produce a double nick in the target DNA. In some embodiments, a nickase is used together with two separate guide RNAs that are selected to be in close proximity to produce a double nick in the target DNA.

[000386] In some embodiments, the RNA-guided DNA-binding agent comprises one or more heterologous functional domains (*e.g.*, is or comprises a fusion polypeptide).

[000387] In some embodiments, the heterologous functional domain may facilitate transport of the RNA-guided DNA-binding agent into the nucleus of a cell. For example, the heterologous functional domain may be a nuclear localization signal (NLS). In some embodiments, the RNA-

guided DNA-binding agent may be fused with 1-5 NLS(s). In some embodiments, the RNA-guided DNA-binding agent may be fused with 2, 3, or 4 NLS(s). In some embodiments, the RNA-guided DNA-binding agent may be fused with two NLS(s). In some embodiments, the RNA-guided DNA-binding agent may be fused with one NLS. Where one NLS is used, the NLS may be linked at the N-terminus or the C-terminus of the RNA-guided DNA-binding agent sequence. In some embodiments, the NLS is not linked to the C-terminus. It may also be inserted within the RNA-guided DNA-binding agent sequence. In other embodiments, the RNA-guided DNA-binding agent may be fused with more than one NLS. In certain circumstances, at least the two NLSs are the same (*e.g.*, two SV40 NLSs). In certain embodiments, at least two different NLSs are present the RNA-guided DNA binding agent. In some embodiments, the RNA-guided DNA-binding agent is fused to two SV40 NLS sequences linked at the carboxy terminus. In some embodiments, the RNA-guided DNA-binding agent may be fused with two NLSs, one linked at the N-terminus and one at the C-terminus. In some embodiments, the RNA-guided DNA-binding agent may be fused with 3 NLSs. In some embodiments, the RNA-guided DNA-binding agent may be fused with no NLS. In some embodiments, the NLS may be a monopartite sequence, such as, *e.g.*, the SV40 NLS, PKKKRKV (SEQ ID NO: 143) or PKKKRRV (SEQ ID NO: 144). In some embodiments, the NLS may be a bipartite sequence, such as the NLS of nucleoplasmin, KRPAATKKAGQAKKKK (SEQ ID NO: 145). In a specific embodiment, a single PKKKRKV (SEQ ID NO: 143) NLS may be linked at the C-terminus of the RNA-guided DNA-binding agent. One or more linkers are optionally included at the fusion site.

V. Method of Treatment

[000388] The disclosure provides methods for suppressing an immune response in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding a dmTGFB1 under control of a promoter sequence and a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TGFB2. The engineered T cells may, optionally, further comprise a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding an IFNG; or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence. The disclosure provides methods for suppressing an immune response in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding a dmTGFB1 under control of a promoter sequence; a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, *e.g.*, knockdown, of an endogenous nucleic acid sequence encoding a TNFA; and insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence. The disclosure provides methods for suppressing an immune response in a subject, comprising

administering engineered T cells comprising a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion into the cell of a heterologous sequence encoding IL10 under control of a promoter sequence. The disclosure provides methods for suppressing an immune response in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding a dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion into the cell of a heterologous sequence encoding CTLA4 under control of a promoter sequence. The disclosure provides methods for suppressing an immune response in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding a dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion into the cell of heterologous sequences encoding IL10 and CTLA4, each under control of a promoter sequence.

[000389] The disclosure provides methods for treating an autoimmune disorder in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding a dmTGFB1 under control of a promoter sequence and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2. The engineered T cells may, optionally, further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence. The disclosure provides methods for treating an autoimmune disorder in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFBR2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA, and insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence. The disclosure provides methods for treating an autoimmune disorder in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence

encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA, and insertion into the cell of heterologous sequence encoding IL10 under control of a promoter sequence. The disclosure provides methods for treating an autoimmune disorder in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA, and insertion into the cell of heterologous sequence(s) encoding CTLA4 under control of a promoter sequence. The disclosure provides methods for treating an autoimmune disorder in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG, a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA, and insertion into the cell of heterologous sequences encoding IL10 and CTLA4, each under control of a promoter sequence.

[000390] The disclosure provides methods for treating GvHD in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding a dmTGFB1 under control of a promoter sequence and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2. The engineered T cells may, optionally, further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence. The disclosure provides methods for treating GvHD in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence. The disclosure provides methods for treating GvHD in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter

sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG and insertion into the cell of heterologous sequence(s) encoding IL10 under control of a promoter sequence. The disclosure provides methods for treating GvHD in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion into the cell of heterologous sequence(s) encoding CTLA4 under control of a promoter sequence. The disclosure provides methods for treating GvHD in a subject, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion into the cell of heterologous sequences encoding IL10 and CTLA4, each under control of a promoter sequence.

[000391] The disclosure provides methods for treating a subject receiving a transplant or transfusion, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding a dmTGFB1 under control of a promoter sequence and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2 – optionally, before prior to, concurrently with, or after having received the transplant or transfusion. The engineered T cells may, optionally, further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence. The disclosure provides methods for treating a subject receiving a transplant or transfusion, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence – optionally, before prior to, concurrently with, or after having received the transplant or transfusion.. The disclosure provides methods for treating a subject receiving a

transplant or transfusion, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG and insertion into the cell of heterologous sequence(s) encoding IL10 under control of a promoter sequence – optionally, before prior to, concurrently with, or after having received the transplant or transfusion. The disclosure provides methods for treating a subject receiving a transplant or transfusion, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion into the cell of heterologous sequence(s) encoding CTLA4 under control of a promoter sequence – optionally, before prior to, concurrently with, or after having received the transplant or transfusion.. The disclosure provides methods for treating a subject receiving a transplant or transfusion, comprising administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding dmTGFB1 under control of a promoter sequence; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; and insertion into the cell of heterologous sequences encoding IL10 and CTLA4, each under control of a promoter sequence – optionally, before prior to, concurrently with, or after having received the transplant or transfusion..

[000392] In some embodiments, the methods comprise administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding a dmTGFB1 under control of a promoter sequence and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2, and further comprising insertion of sequence(s) encoding a targeting receptor, e.g., a CAR. The engineered T cells may, optionally, further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence. In some embodiments, the methods comprise administering engineered T cells comprising a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an

IFNG; insertion of sequence(s) encoding a regulatory T cell promoting molecule, and further comprising insertion of sequence(s) encoding a targeting receptor, e.g., a CAR.

[000393] In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to the gastrointestinal system, e.g., the targeting receptor is a CAR targeting MAdCAM-1, e.g., for suppressing immune responses, including inflammation, in disorders such as inflammatory bowel disease, ulcerative colitis, or Crohn's disease. In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to tissues comprising endothelial cells, e.g., the targeting receptor is a CAR targeting VCAM-1, e.g., for suppressing immune responses, including inflammation, in disorders such as Crohn's disease and multiple sclerosis. In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to endothelial cells, e.g., the targeting receptor is a CAR targeting CEACAM6, e.g., for suppressing immune responses in disorders such as Crohn's disease. In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to B cells, e.g., the targeting receptor is a CAR targeting CD19, e.g., for suppressing immune responses in disorders such as in multiple sclerosis and systemic lupus erythematosus. In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to B cells, e.g., the targeting receptor is a CAR targeting CD20, e.g., for suppressing immune responses in disorders such as in multiple sclerosis and systemic lupus erythematosus. In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to an inflammatory tissue, e.g., the targeting receptor is a CAR targeting TNFA, e.g., for suppressing immune responses in disorders such as inflammatory bowel disease, ulcerative colitis, or Crohn's disease. In some embodiments, the targeting receptor, e.g., a CAR is capable of targeting engineered T cells to a neurological tissue, e.g., the targeting receptor is a CAR targeting MBP, MOG, or PLP, e.g., for suppressing immune responses in disorders such as multiple sclerosis. In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to immune cells expressing TNFSF7 including but not limited to activated T lymphocytes, B lymphocytes, dendritic cells, and/or NK cells, e.g., the CAR targets TNFSF7, e.g., for suppressing immune responses in disorders such as Crohn's disease, ulcerative colitis, systemic lupus erythematosus, or multiple sclerosis. In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to tissues comprising mature B lymphocytes, e.g., the targeting receptor is a CAR targeting TNFRSF17, e.g., for suppressing immune responses in disorders such as systemic lupus erythematosus. In some embodiments, the targeting receptor, e.g., a CAR, is capable of targeting engineered T cells to synovial tissue, e.g., the targeting receptor is a CAR targeting citrullinated vimentin e.g., for suppressing immune responses in disorders such as rheumatoid arthritis.

[000394] In some embodiments, the targeting receptor is a CAR targeting CD70, e.g., for suppressing immune responses in disorders such as GvHD. CD70 is a transmembrane protein that is

transiently expressed on the surface of CD4+ and CD8+ T-cells, regulatory T-cells (Tregs), B cells, antigen-presenting cells such as dendritic cells, and natural killer (NK) cells in response to immune activation. Without being bound by theory, CD70 may be elevated in immune cells such as T cells of patients with autoimmune diseases. In some embodiments, the engineered T cells as described herein may suppress activity of CD70-expressing immune cells and/or kill these cells.

[000395] In some embodiments, the targeting receptor is a CAR targeting DPP6, SCL2A2, glutamate decarboxylase (GAD2), demoglein 3 (DSG3), and MHC class I HLA-A (HLA-A*02).

[000396] In some embodiments, the methods comprise administering engineered T cells comprising an insertion into the cell of a heterologous sequence encoding a dmTGFB1 under control of a promoter sequence and a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2, and further comprising a modification, e.g., knockdown, of TCR sequence(s). The engineered T cells may, optionally, further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; or insertion into the cell of heterologous sequence(s) encoding a regulatory T cell promoting molecule under control of a promoter sequence. In some embodiments, the methods comprise administering engineered T cells comprising a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TGFB2; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding a TNFA; a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding an IFNG; insertion of sequence(s) encoding a regulatory T cell promoting molecule, and further comprising a modification, e.g., knockdown, of TCR sequence(s).

[000397] In some embodiments, the sequence(s) to be inserted are inserted into the sequence(s) to be modified, e.g., knocked down, e.g., a CAR sequence is inserted into a TNFA genomic sequence, thereby modifying, e.g., knocking down, the TNFA sequence.

[000398] In some embodiments, the methods comprise administering a population of T cells comprising T cells that are engineered as described above. In some embodiments, at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the T cells in the population of T cells are engineered, e.g., as assessed by sequencing, e.g., NGS.

[000399] In some embodiments, the autoimmune disorder is selected from ulcerative colitis, Crohn's disease, rheumatoid arthritis, psoriasis, multiple sclerosis, systemic lupus erythematosus, type 1 diabetes, and graft versus host disease (GvHD). In some embodiments, the engineered T cells have autologous or allogenic use.

[000400] In some embodiments, the effectiveness of treatment using the engineered T cell described above can be assessed in an animal model, e.g., mouse model, of graft versus host disease by measuring the animal's weight or survival (wherein the animals are sacrificed after loss of a

substantial portion of body weight, e.g., 20% of starting body weight) following administration of the engineered T cell. In some embodiments, effective treatment results in a statistically significant increase in survival rate as compared to a suitable control, e.g., an animal treated with PBMC.

EXAMPLES

[000401] The following examples are provided to illustrate certain disclosed embodiments and are not to be construed as limiting the scope of this disclosure in any way.

Example 1. General Methods

Example 1.1 Preparation of lipid nanoparticles

[000402] In general, the lipid components were dissolved in 100% ethanol at various molar ratios. The RNA cargos (*e.g.*, Cas9 mRNA and sgRNA) were dissolved in 25 mM citrate buffer, 100 mM NaCl, pH 5.0, resulting in a concentration of RNA cargo of approximately 0.45 mg/mL.

[000403] Unless otherwise specified, the lipid nucleic acid assemblies contained ionizable Lipid A ((9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-(((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-(((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate), cholesterol, DSPC, and PEG2k-DMG in a 35 Lipid A:47.5 cholesterol:15 DSPC:2.5 PEG-DMG molar ratio, respectively. The lipid nucleic acid assemblies were formulated with a lipid amine to RNA phosphate (N:P) molar ratio of about 6, and a ratio of gRNA to mRNA of 1:1 by weight.

[000404] LNPs were prepared using a cross-flow technique utilizing impinging jet mixing of the lipid in ethanol with two volumes of RNA solutions and one volume of water. First, the lipids in ethanol were mixed through a mixing cross with the two volumes of RNA solution. Then, a fourth stream of water was mixed with the outlet stream of the cross through an inline tee (*See* WO2016010840 Fig. 2.). The LNPs were held for 1 hour at room temperature, and further diluted with water (approximately 1:1 v/v). LNPs were concentrated using tangential flow filtration on a flat sheet cartridge (Sartorius, 100kD MWCO) and buffer exchanged using PD-10 desalting columns (GE) into 50 mM Tris, 45 mM NaCl, 5% (w/v) sucrose, pH 7.5 (TSS). Alternatively, the LNPs were optionally concentrated using 100 kDa Amicon spin filter and buffer exchanged using PD-10 desalting columns (GE) into TSS. The resulting mixture was then filtered using a 0.2 µm sterile filter. The final LNP was stored at 4°C or -80°C until further use.

Example 1.2. In vitro transcription (“IVT”) of mRNA

[000405] Capped and polyadenylated mRNA containing N1-methyl pseudo-U was generated by in vitro transcription using a linearized plasmid DNA template and T7 RNA polymerase. The linearized plasmid DNA containing a T7 promoter, and a sequence for transcription was linearized by restriction endonuclease digestion followed by heat inactivation of the reaction mixture and purified from enzyme and buffer salts. Messenger RNA was synthesized and purified using standard techniques known in the art.

[000406] Messenger RNA was generated from plasmid DNA encoding an open reading frame according to SEQ ID NO: 218 in Table 4. When the sequences cited in this paragraph are referred to below with respect to RNAs, it is understood that Ts should be replaced with Us (which can be modified nucleosides as described above). Messenger RNAs used in the Examples include a 5' cap and a 3' polyadenylation sequence e.g., up to 100 nts. Guide RNAs were chemically synthesized by commercial vendors or using standard in vitro synthesis techniques with modified nucleotides.

Example 2. Suppressive activity of engineered T cells overexpressing Treg-associated factors

Example 2.1. T cell preparation

[000407] Human PBMCs were prepared from a fresh leukopak (AllCells,) and cryopreserved for future use by methods known in the art. CD4⁺ T cells were isolated from PBMCs by negative selection using the human CD4⁺ T cell isolation kit (Miltenyi; Cat no. 130-096-533) following the manufacturer's protocol. The CD4⁺ T cells were activated by plating at a density of 5×10^6 cells/mL in OpTmizer base media including CTS OpTmizer T Cell Expansion SFM (Gibco, Cat. A3705001), 1% Penicillin-Streptomycin, 1X Glutamax, 10 mM HEPES, 2.5% human AB serum (Gemini, Cat. 100-512) containing 200U/mL IL-2, 5ng/mL IL-7, 5ng/mL IL-15 and 25uL/mL ImmunoCult Human CD3/CD28 T cell Activator (Stemcell Technologies, Cat. 10991) and cultured at 37°C for 24 hours prior to lentiviral transduction.

[000408] Natural regulatory T cells (nTregs), also referred to herein as thymic Tregs (tTregs) were prepared using methods known in the art. CD4⁺ CD25⁺ T cell populations are known to be enriched for natural Tregs (nTregs). CD25⁺ cells were isolated from CD4⁺ T cells using Human CD25 Microbeads II (Miltenyi; Cat No. 130-092-983) according to the manufacturer's instructions. Isolated CD25⁺ CD4⁺ T cells were plated at a density of 5×10^6 cells per well in a 6-well Grex containing 30ml of OpTmizer base media including CTS OpTmizer T Cell Expansion SFM (Gibco, Cat. A3705001), 1% Penicillin-Streptomycin, 1X Glutamax, 10 mM HEPES, 2.5% human AB serum (Gemini, Cat. 100-512) and supplemented every other day with 1000 U/mL of recombinant human interleukin-2 (Miltenyi Biotec; Cat No. 130-097-746). Cells were activated by addition of 25 uL/mL ImmunoCult Human CD3/CD28 T cell Activator (Stemcell Technologies, Cat. 10991) and cultured at 37°C for 2 days until lentiviral transduction. After additional 3 days, activated CD25⁺ T cells were harvested and labelled with 50 ug/mL biotinylated anti-LAP antibody

(Miltenyi; Cat. 130-095-213) for 15 min at 4°C. Latency-associated peptide (LAP) is a marker of activated Treg cells. The labelled cells were washed twice and further labelled with anti-biotin microbeads (Miltenyi; Cat. 130-090-485) according to manufacturer's instructions. The LAP⁺ cells were isolated using LS columns (Miltenyi; Cat. 130-042-401). The isolated natural Tregs were cultured in 6-well Grex plates with 30 mL OpTmizer base media including CTS OpTmizer T Cell Expansion SFM (Gibco, Cat. A3705001), 1% Penicillin-Streptomycin, 1X Glutamax, 10 mM HEPES, 2.5% human AB serum (Gemini, Cat. 100-512) supplemented with 1000 U/mL IL-2 for expansion every other day until day of injection.

Example 2.2. T cell transduction and cell sorting

[000409] To engineer suppressive T cells, activated CD4⁺ T cells were serially transduced with lentiviral constructs one day apart as described in Table 5. One lentiviral construct encoded both IL10DE (SEQ ID NO: 229) and CTLA4 (SEQ ID NO: 130) as well as GFP and mScarlet as markers. The second lentiviral construct encoded constitutively active dual mutant TGFB1 (caTGFB1; R218H C225R; SEQ ID NO: 214). CD25⁺ T cells were transduced with a lentiviral construct encoding GFP. For transduction, concentrated viral supernatant was added to T cells and samples were centrifuged 60 mins at 37°C. T cells were resuspended in the cell/viral supernatant mixture and incubated in OpTmizer media. Transduced CD4⁺ cell samples were supplemented with 200 U/mL IL-2, 5 ng/mL IL-7 and 5 ng/mL IL-15 and transduced CD25⁺ samples were supplemented with 1000 U/mL IL-2. Cells were split, and media or cytokines replenished regularly.

Table 5. T cell editing treatment groups. “LV” is for lentivirus, “DKI” is for double knock-in, “DKO” is for double knock-out, “TKI” is for triple knock-in & “TKO” is for triple knock-out.

Group	Starting Cell	LV GFP	LV CTLA4 & IL-10DE	LV caTGFB1	LNP IFN γ	LNP TNF α	LNP TGFB2
nTreg	CD4 ⁺ CD25 ⁺	+	-	-	-	-	-
DKI DKI	CD4 ⁺	-	+	-	+	+	-
TKI DKI	CD4 ⁺	-	+	+	+	+	-
TKI TKO	CD4 ⁺	-	+	+	+	+	+

[000410] T cells were edited according to Table 5 with LNPs. Each LNP species was coformulated with mRNA encoding Cas9 and guide at the following dose: INF-gamma (G019753) at 0.67 ug/ml total RNA cargo, TNF-alpha (G019757) at 0.67 ug/ml total RNA cargo, TGFB2 (G029528) at 1 ug/ml total RNA cargo. LNPs were generated as described in Example 1 using a molar ratio of 35 Lipid A/47.5 cholesterol/15 DSPC/2.5 PEG2k-DMG. The LNPs were formulated with a lipid amine to RNA phosphate (N:P) molar ratio of about 6. LNPs were prepared with a ratio of 1:1 by weight of gRNA to Cas9 mRNA cargo.

[000411] Briefly, T cells were resuspended in serum free media at a concentration of 1×10^6 cells/mL supplemented with 1000 U/mL IL-2, 10 ng/mL IL-7 and 10 ng/mL IL-15 after which cells were transferred to T-75 flasks and incubated at 37°C. Each LNP preparation was incubated in T cell base media supplemented with 5 ug/ml recombinant human ApoE3 (Peprotech, Cat. 350-02). LNP mix was incubated for 15 minutes at 37°C. Equal volume of pre-incubated LNP mix was added to each T cell sample, leading to double the original volume.

[000412] Twenty-four hours after LNP treatment, T cells were collected, washed, and suspended in OpTmizer base media supplemented with 500 U/mL recombinant human IL-2, 5 ng/ml recombinant human IL-7, and 5 ng/ml recombinant human IL-15. C Cells were expanded in 6 well Grex plates with cytokines were replenished every other day.

Example 2.3. Confirmation of cell engineering

[000413] Engineered samples were sorted by flow cytometry to obtain enriched cell populations.

[000414] Engineered T cells were assessed by flow cytometry to determine phenotype, gene insertions and disruptions. Cells were washed and stained in FACS buffer (PBS + 2% FBS + 2 mM EDTA). Engineered T cells were incubated with antibodies targeting CD4 (Biolegend) or TGFB2 (Miltenyi). T cells were subsequently washed and analyzed on a Cytoflex instrument (Beckman Coulter). Data analysis was performed using FlowJo software package. T cells were gated based on size, single cells, CD4 expression, and expression of markers. Target expression of TGFB2 verified by flow cytometry is shown in **Table 6**. Values shown correspond to the mean percentage of cells positive for TGFB2 expression, and the TGFB2 expression level as measured by median fluorescence for each cell population.

Table 6. Mean fluorescent intensity of TGFB2 expression in engineered CD4+ T cells

Group	TGFB2		n
	Mean % of cells TGFB2+	Mean TGFB2 expression (MFI)	
Unstained CD4+	0.5	108	2
Unedited CD4+	84.4	1401	2
TKI DKO	74.8	1206	1
TKI TKO	2.8	217	2

Example 2.4. Suppressive capacity of engineered T cells

[000415] A mixed lymphocyte reaction (MLR) assay was used to assay the suppressive function of sorted transduced engineered T cells. MLR is an inflammatory reaction caused by T cells recognizing leukocytes of another (allogenic leukocytes) as foreign. Tregs suppress the MLR inflammatory reaction by suppressing proliferation of inflammatory T cells. Cell proliferation is

measured by labeling the initial population with an intracellular fluorescent dye that becomes diluted as it is passed as part of the cell contents to each successive generation.

[000416] The MLR assay was conducted in a 96-well U-bottom plate using OpTmizer base media including CTS OpTmizer T Cell Expansion SFM (Gibco, Cat. A3705001), 1% Penicillin-Streptomycin, 1X Glutamax, 10 mM HEPES and 2.5% human AB serum (Gemini, Cat. 100-512). Untransduced CD3+CD4+ T cells from the same donor as the transduced T cells (autologous T cells) were used as the responding cells. These untransduced cells were labelled with CellTrace Violet (CTV) (ThermoFisher Scientific; Cat No. C34557) according to manufacturer's instructions. CD3-depleted PBMC from an allogenic donor relative to the transduced T cells were used to stimulate the responding, untransduced T cells. Cultures were prepared by combining 50,000 CTV-labelled autologous T cells, 50,000 CD3-depleted allogenic PBMC, and approximately 50,000 (1 to 1), 16,666 (3 to 1) or 5,555 (9 to 1) sorted transduced CD3+CD4+ T cells per well. Following 5 days of culture at 37°C, the culture plate was centrifuged. The cell pellet was resuspended in FACS buffer containing APC/Fire 750 anti-CD4 and placed at 4°C for 30 mins. The cells were subsequently washed, processed on a CytoFlex flow cytometer (Beckman Coulter), and analyzed using the FlowJo software package. Cells were first gated by positive CD4 expression, followed by CTV signal and finally on the undiluted CTV population. Suppression of CTV-dilution was calculated using the following formula:

$$\frac{\log_2(y) \text{ of CTV T cells} - \log_2(y) \text{ of CTV T cells with Treg}}{\log_2(y) \text{ of CTV T cells}} * 100$$

[000417] Where y = mean fluorescent intensity of the entire CTV-labelled population / mean fluorescent intensity of the undiluted portion of the CTV-labelled population. Data are shown in **Figure 1** and **Table 7**.

Table 7. Calculated mean percent suppression of autologous T cell proliferation by engineered T cells

Sample	1:1		1:3		1:9		1:27	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Untransduced T cells (Tconv)	2.74	2.20	3.86	3.86	1.92	1.92	6.09	0.69
tTregs	82.10	0.97	79.05	3.01	70.12	3.31	51.23	5.81
TKI DKO	80.41	2.02	75.64	0.57	46.80	7.56	39.34	3.16
TKI TKO	69.79	0.49	70.71	5.57	64.68	3.61	55.94	1.47

Example 3. In vivo functional characterization of engineered suppressive T cells**Example 3.1. In vivo assessment of inflammatory response in a GvHD model**

[000418] The in vivo suppressive function of the engineered T cells was assessed using a graft versus host disease (GvHD) mouse model.

[000419] Sorted T cells for in vivo injections were harvested and resuspended in CTS OpTmizer T cell Expansion Media (Gibco, Cat. A1048501) at 4×10^6 cells/mL. PBMCs autologous to the engineered T cells were thawed and cell density adjusted to 8×10^6 cells/mL. PBMCs were added to each assay population at a 0.5:1 ratio and cells resuspended in RPMI supplemented with 10mM HEPES and 2% FBS to 6×10^6 cells/100 μ L. The PBMC only group was resuspended at 4×10^6 cells/100 μ L.

[000420] Female NOG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac; Taconic, Cat No. NOG-F) were conditioned for cellular transplant by sublethal irradiation (150 rads) using X-rays (RS-2000 irradiator; Rad Source Technologies) one day before injection. Cohorts of irradiated NOG mice were injected intravenously with 100 μ L of each test cell population described above. Eight irradiated mice injected with injection media were used as the irradiation only control (Vehicle). Body weight was monitored daily. Upon 20% weight loss, mice were sacrificed, and the cellular composition of their spleens was assessed. Survival data is plotted in **Table 8** and **Figure 2**.

Table 8. Percent survival of mice in each cohort surviving at each timepoint after injection.

N	8	7	8	8	8	8
Days	Vehicle	PBMC only	tTreg	DKI DKO	TKI DKO	TKI TKO
12	100	85.7	100	100	100	100
13	100	71.4	100	100	100	100
16	100	57.1	100	100	87.5	100
17	87.5	42.9	100	100	87.5	100
19	87.5	28.6	100	100	87.5	100
22	87.5	14.3	100	100	87.5	100
23	87.5	0.0	100	100	87.5	100
24	87.5	0.0	100	87.5	62.5	100
26	87.5	0.0	100	87.5	50	100
28	87.5	0.0	100	75	50	100
28	87.5	0.0	100	75	37.5	100
35	87.5	0.0	100	75	37.5	87.5
44	87.5	0.0	100	62.5	37.5	87.5
49	87.5	0.0	100	62.5	25	87.5
52	87.5	0.0	100	50	25	87.5
55	87.5	0.0	75	37.5	12.5	75
62	87.5	0.0	62.5	37.5	12.5	75

63	87.5	0.0	62.5	37.5	12.5	75
----	------	-----	------	------	------	----

Example 3.2. Persistence ability of engineered T cells

[000421] To confirm persistence of the engineered T cells, the splenic composition was assessed. Treg persistence is evaluated by assessing the relative cell counts of Tregs and total splenocytes in the spleen post-euthanasia. At the time of euthanasia, each animal's spleen was collected in a 15mL conical tube containing complete Optimizer media. The spleens were dissociated manually using the rubber side of a 1ml syringe plunger. The cell suspension was filtered through a 70-micron cell strainer (Corning, Cat. 08-771-2) and cells were counted using the Cellometer K2 (Nexcelom Bioscience). The splenocytes were cryopreserved in CryoStor CS10 media (Stemcell Technologies; Cat# 07930). Following the completion of the study, all the splenocytes were thawed and counted using the Cellometer K2 (Nexcelom Bioscience). Approximately one million viable splenocytes were resuspended in Optimizer media and 6×10^5 cells were stained for 30 min at 4°C with panel of antibodies including, APC anti-human CD45 (BD Pharmingen, Cat. 561864) and APC-Fire 750 anti-human CD4 (Biolegend, Cat. 300560). The splenocytes were processed on a Cytoflex flow cytometer (Beckman Coulter) and analyzed using the FlowJo software package. Cells were gated on mouse CD45-, human CD45+. tTregs were gated as GFP+ and engineered T cells as mScarlet+GFP+. To determine the number of cells of individual populations, the percentage of each population was applied to the total number of human splenocytes recovered. Data shown in **Table 9** and **Figure 3**.

Table 9. Quantification of human lymphocytes in spleens of mice

Sample	% of Total Human Lymphocytes (CD45+)		
	Mean %	SEM	n
tTregs	5.21	3.39	8
DKI DKO	2.90	2.23	6
TKI DKO	0.54	0.28	3
TKI TKO	40.86	28.07	8

Example 3.3. In vivo cytokine profile analysis of engineered T cells

[000422] Plasma for cytokine analysis was isolated by extraction of blood via cardiac puncture, preserved via addition of EDTA. Collected blood is centrifuged for 10min at 1000xg and plasma stored at -80C until further analysis.

[000423] Plasma samples were assessed for levels of cytokine release by MSD. Plasma samples were thawed for subsequent cytokine quantification using a custom U-PLEX Biomarker kit (Meso Scale Diagnostics, Cat. K15067L-2), according to manufacturer's instructions. The U-PLEX Biomarker plates were read using the Meso Quickplex SQ120 instrument (Meso Scale Discovery)

and the data were analyzed with the Discovery Workbench 4.0 software package (Meso Scale Discovery). Results are shown in **Table 10** and **Figure 4**.

Table 10. IL-10 levels (pg/mL) in plasma

Sample	IL10 (pg/mL)		n
	Mean	SEM	
Vehicle	0	0	1
PBMC	7	7	5
tTregs	11	10	8
DKI DKO	107	80	5
TKI DKO	31	24	6
TKI TKO	924	617	8

Example 4. In vivo study for CD70-CAR targeting of synTregs using a xenoGvHD mouse model

[000424] The *in vivo* suppressive function of the engineered T cells was assessed using a graft versus host disease (GvHD) mouse model. In particular, CD70-CAR targeted and untargeted triple-KI (dmTGFB1/IL10DE/CTLA4)/triple-KO (TGFB2/IFNG/TNFA) synTregs were compared to unedited tTregs and controls. CD70 is upregulated by activated lymphocytes, including autoreactive T cells and B cells.

Example 4.1 T cell engineering

[000425] Generally, T cells were prepared as described in Example 2. CD4⁺ and CD4⁺ CD25⁺ T cells were isolated from human PBMCs using commercially available kits (Miltenyi) following the manufacturer's protocols. Cells were activated with Immunocult and edited according to **Table 11**.

Table 11. T cell editing treatment groups. "LV" is for lentivirus. "AAV" is for adeno-associated virus.

Group	Starting Cell	LNP IFNg, & LNP TNFa	LV CTLA4 & IL-10DE	LV dmTGFB1	AAV CD70-CAR; LNP TRAC, LNP CD70, LNP UGI	LNP TGFB2
tTreg	CD4 ⁺ CD25 ⁺	-	-	-	-	-
synTreg	CD4 ⁺	+	+	+	-	+
CD70 CAR-synTreg	CD4 ⁺	+	+	+	+	+

[000426] On Day 0, CD4⁺ and CD4⁺CD25⁺ cells were activated with Immunocult. CD4⁺ cells were treated with LNPs carrying SpCas9 mRNA and gRNA G019753 targeting IFN γ and gRNA G019757 targeting TNF α to disrupt these genes. Day 1, double knockout cells were harvested, resuspended and treated with lentivirus encoding CTLA4 and IL10DE. Day 2, the resulting double knockout, double knockin cells were harvested, resuspended and treated with lentivirus encoding dmTGFB1. Day 3, a portion of the resulting double knockout, triple knockin cells were harvested, resuspended and treated with AAV encoding CD70 CAR with approximately 500 nucleotide homology arms with sequences flanking the TRAC guide cut site, LNP carrying mRNA encoding SpCas9 and gRNA G013006 targeting TRAC for insertion of the CAR into the TRAC locus. The same cells were also treated with LNP carrying mRNA encoding an NmeCas9 base editor (SEQ ID No.365) and gRNA G034199 targeting CD70, and LNP carrying mRNA encoding UGI for disruption of the CD70 gene. On Day 4, CD70 edited cells were harvested, resuspended and cultured overnight. On Day 5, double knockout, triple knockin cells and CD70 CAR-targeted, double knockout, triple knockin cells were harvested, resuspended, and treated with LNPs carrying SpCas9 mRNA and gRNA G029528 targeting TGFBR2 for the disruption of TGFBR2. On Day 6 Thymic Treg, synTreg and CD70 CAR synTreg cells were washed and culture with the regular addition of cytokines.

Example 4.2 In vivo functional characterization

[000427] The in vivo suppressive function of the engineered T cells was assessed using a graft versus host disease (GvHD) mouse model. Sorted T cells and PBMCs for in vivo injections were harvested and resuspended in media. Female NOG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug}/JicTac; Taconic, Cat No. NOG-F) were conditioned for cellular transplant by sublethal irradiation (150 rads) using X-rays (RS-2000 irradiator; Rad Source Technologies) one day before injection. Cohorts of irradiated NOG mice were injected intravenously with four million PBMC cells and 0.4 million T cells as described in **Table 11**. Eight irradiated mice injected with injection media were used as the irradiation only control (Vehicle). Body weight was monitored daily. Upon 20% weight loss, mice were sacrificed, and the cellular composition of their spleens was assessed. Survival data is plotted in **Table 12** and **Figure 5**.

Table 12. Percent survival of mice in each cohort surviving at each timepoint after injection

Days	Vehicle	PBMC only	tTreg	synTreg	CAR-synTreg
0	100%	100%	100%	100%	100%
14	100%	88%	100%	100%	100%
23	100%	75%	100%	88%	100%
26	100%	75%	100%	88%	100%
34	100%	75%	88%	88%	100%
36	100%	63%	88%	88%	100%

39	100%	63%	75%	88%	100%
48	100%	50%	63%	88%	100%
50	100%	13%	63%	88%	100%
54	100%	0%	63%	75%	100%
56	100%	0%	50%	63%	100%
60	100%	0%	50%	50%	100%
63	100%	0%	38%	50%	100%
67	100%	0%	25%	38%	100%

[000428] The persistence of Treg cells was assessed generally as described in Example 3.

Table 13 and **Figure 6** show the mean percentage of synTregs among all human CD45+ cells recovered from mouse spleen at time of sacrifice. The percentage of tTreg in the spleen were not measured in this experiment, but typically have ranged from 1-5% in similar experiments.

Table 13. Mean percentage of synTregs among all human CD45+ cells recovered from mouse spleen at time of sacrifice.

Treatment	Mean	SD	n
PBMC only	0.0	0.0	8
synTreg	10.0	6.7	7
CAR synTreg	37.2	16.6	8

[000429] Mice were assessed for phenotypic signs of GvHD at the time of sacrifice based on the method described in Lai, et al. Cell Transplantation. 2012;21(9):2033-2045. For each mouse, each phenotypic criterion was graded 0-2 as described in **Table 14** and grades for a single mouse were summed to get a GvHD score. The mean GvHD score for each experimental condition is shown in **Table 15** and **Figure 7**. Mice treated with untargeted tTregs or synTregs were statistically significantly improved in GvHD score over the PBMC-only control. CD70-CAR synTreg treated mice were asymptomatic throughout the experiment -indistinguishable from mice in the vehicle control sample. GvHD scores for CD70-CAR synTreg treated mice were significantly distinguished from the PBMC only control and untargeted synTregs.

Table 14. GvHD scoring rubric

Criteria	Grade 0	Grade 1	Grade 2
Weight Loss	<10%	10-20%	>20%
Activity	Normal	Mild to moderately decreased	Stationary until stimulated
Posture	Normal	Hunching only at rest	Severe gait, impaired movement
Fur Texture	Normal	Mild to moderate ruffling	Severe ruffling/poor grooming
Skin Integrity	Normal	Scaling of paws and/or tail	Obvious areas of denuded skin

Table 15. Mean GvHD score at time of sacrifice.

Treatment	Mean	SD	n
PBMC only	4.6	0.7	8
tTreg	3.4	2.2	8
synTreg	3.0	2.2	8
CAR synTreg	0.0	0.0	8

[000430] Cytokine profile was assessed generally as described in Example 3. **Table 16** and **Figure 8** show the mean IL-10 serum levels for mice in each treatment group.

Table 16. Mean IL-10 levels in pg/ml of serum at time of sacrifice.

Treatment	Mean	SD	n
Vehicle	0.3	0.6	7
PBMC only	9.1	5.7	8
tTreg	7.0	3.7	7
synTreg	98.4	172.7	8
CAR synTreg	5.9	7.4	6

Example 4.3. Suppressive activity of synTregs isolated from spleen

[000431] A bead-based suppression assay was used to assay the suppressive function of Tregs isolated from frozen spleen samples of the mice in this Example. The suppression assay was performed generally as described in Example 2 and by methods known in the art using allogeneic APC, human anti-CD3/CD28 beads for T cell stimulation. Unengineered CD4⁺ and CD8⁺ T cells (Tconv) were isolated for use as a negative control from frozen PBMCs of the same donor used to engineer the synTregs. Tregs were isolated from splenic samples by flow cytometry based on their fluorescent labeling: GFP for tTregs and GFP/mScarlet for synTregs. CD3⁺ T cells from frozen PBMCs were isolated using a Miltenyi Biotec kit for use as responder (Tresp) cells.

[000432] Tconv control cells were labelled with CellTrace Yellow and Tresp cells were labelled with CellTrace Violet. Anti-human CD3/CD28 activator beads (Miltenyi Biotec, Cat# 130-091-441) were used to stimulate the coculture of Tresp and Treg cells for five days. Cells were harvested and assayed by flow cytometry. Percent suppression of expansion was calculated using the following formula:

$$100 - \frac{\text{Tresp count (sample)} - \text{unstim. Tresp count}}{\text{stim. Tresp count (without Tregs)} - \text{unstim. Tresp count}} * 100$$

[000433] The calculated suppression of CTV-labelled cell expansion is shown in **Table 17** and **Figure 9**. Spleen-isolated synTregs remained highly suppressive, exceeding spleen-isolated tTreg activity especially at low Treg:Tresp ratios. In line with in vivo persistence data, synTreg survival ex vivo was superior to tTregs.

Table 17. Mean percent suppression of Tresp cell expansion

Treg:Tresp	Tconv ctrl			tTregs			synTregs		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
1:1	66.7	11.5	3	88.4	3.5	3	99.4	1.0	3
1:3	41.2	9.9	2	82.3	4.0	3	94.8	3.5	2
1:9	29.0	8.8	3	48.2	0.6	2	87.6	4.8	2
1:27	0.0	0.0	3	6.3	10.9	3	56.5	12.9	3

[000434] Spleen-isolated synTreg and the control of Tconv cells cultured without Tresp cellswere restimulated via α CD3/CD28 beads. The supernatant was assayed using Meso Scale Discovery MSD -U-PLEX Proinflammatory Combo 1 Human Kit according to the manufacturer's protocol. SynTregs did not produce proinflammatory cytokines but still secreted IL-10 upon bead-mediated stimulation, as shown in **Table 18** and **Figs. 10A-D**.

Table 18. Cytokine levels in cell culture supernatant in pg/ml.

Cytokine	Condition	Tconv		synTreg	
		Mean	SD	Mean	SD
IFN γ	unstim.	0	0	0	0
	Bead-stim	2829	1322	14	0
TFN α	unstim.	0	0	0	0
	Bead-stim	3	1	0	0
IL-2	unstim.	0	0	0	0
	Bead-stim	11	2	0	0
IL-10	unstim.	0	0	4	0
	Bead-stim	1	0	26	3

Example 5. Characterization of mouse MAdCAM-1-CAR synTregs

[000435] Mouse MAdCAM-CAR targeted and untargeted triple-KI (dmTGFB1/IL10DE/CTLA4)/triple-KO (TGFB2/IFNG/TNFA) synTregs were compared to conventional T cell controls when assayed in vitro for cell activation and cytokine secretion in response to activation.

Example 5.1 T cell engineering

[000436] Generally, T cells were prepared as described in Example 2. CD4⁺ T cells were isolated from human PBMCs using a commercially available kit (Miltenyi) following the manufacturer's protocols. Cells were activated with Immunocult and edited according to **Table 19**.

Table 19. T cell editing treatment groups. "LV" is for lentivirus.

Group		Day 2	Day 3	Day 4	Day 6
-------	--	-------	-------	-------	-------

	Starting Cell	LV CTLA4, IL-10DE, dmTGFB1	LV MECA CAR	LNP IFN γ , & LNP TNF α	LNP TGFBR2 & LNP TRAC
Tconv	CD4+	-	-	-	-
synTreg	CD4+	+	-	+	+
CAR synTreg	CD4+	+	+	+	+

[000437] On Day 1, CD4⁺ cells were activated with Immunocult. Day 2, a portion of CD4⁺ were treated with a lentivirus encoding a cassette including CTLA4, IL10DE and dmTGFB1. Day 3, treated cells were harvested and resuspended in media. A portion of triple knockin cells were treated with lentivirus encoding a chimeric antigen receptor based on the monoclonal anti-mouse MAdCAM-1, MECA-367 antibody. Day 4, cells were harvested, resuspended and treated LNPs carrying SpCas9 mRNA and gRNAs targeting IFN γ and TNF to disrupt these genes. Cells were washed and resuspended in media for continued culture the following day. Day 6 cells were sorted by flow cytometry to isolate populations of 1) Tconv, 2) triple knockin/double knockout and 3) CAR + triple knockin/double knockout cells. Following sorting, edited cells were further treated with LNP carrying mRNA encoding SpCas9 and gRNA targeting TRAC and LNP carrying SpCas9 mRNA and gRNA targeting TGFBR2 for the disruption of these genes. Cells were washed and resuspended the following day.

Example 5.2 In vitro functional characterization

[000438] Cells were assessed for activation via co-culture with murine SVEC4-10 endothelial target cells expressing transgenic murine MAdCAM-1, or in wells coated with murine MAdCAM-1. Cells cultured alone served as negative control. After 24 hours, cells were harvested and assessed by flow cytometry for expression of CD69, a marker of T cell activation. CAR-synTregs, but not Tconv or untargeted synTregs were activated by exposure to murine MAdCAM-1 as shown in Table 20 and Figs. 11A-C.

Table 20. Mean percentage of CD69⁺ T cells. “n.a” is “not assayed.”

Sample	culture alone			mMAdCAM-1 coated			SVEC mMAdCAM-1 expressing cells		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
Tconv	3.9	0.3	3	4.2	0.4	3	n.a.		
synTreg	2.1	0.2	3	2.1	0.1	3	n.a.		
CAR-synTreg	2.4	0.4	3	47.2	4.4	3	67.5	3.0	3

[000439] Cytokine secretion upon restimulation with either mouse MAdCAM-1 or Immunocult was assessed. Cells were incubated in wells coated with murine MAdCAM-1 or containing Immunocult for 48 hours. Cells were harvested by centrifugation and supernatant was reserved.

The supernatant was assayed using Meso Scale Discovery MSD -U-PLEX Proinflammatory Combo 1 Human Kit according to the manufacturer's protocol. Only Tconv produced proinflammatory cytokines IFN γ and TNF α upon stimulation, as shown in **Table 21** and **Fig. 12**. Similarly, targeted and untargeted synTregs produce IL-10 and activeTGFB upon appropriate stimulation.

Table 21. Cytokine secretion upon restimulation in pg/ml. Cells were stimulated with murine MAdCAM-1 (MC-1) or Immunocult (IC). "n.a" is "not assayed."

Cytokine	Condition	Tconv ctrl			synTreg			CAR-synTreg		
		Mean	SD	n	Mean	SD	n	Mean	SD	n
IL-10	unstim	1	1	3	113	11	2	4	0	2
	MC-1 stim	n.a.			n.a.			2,336	488	3
	IC stim	510	68	3	642	57	3	868	137	3
TGF- β	unstim	0	0	2	98	4	2	0	0	2
	MC-1 stim	n.d.			n.d.			1,033	96	3
	IC stim	23	34	3	566	29	3	341	57	3
IFN γ	unstim	580	94	2	11	2	2	13	6	2
	MC-1 stim	n.a.			n.a.			2,363	190	3
	IC stim	188,353	8,367	3	1,581	91	3	970	165	3
TNF α	unstim	2	0	2	0	0	2	0	0	2
	MC-1 stim	n.a.			n.a.			3	1	3
	IC stim	321	98	3	2	1	3	1	1	3

Example 6. SynTregs Display Potent *In Vitro* Suppression of Ulcerative colitis patient-derived T cells

[000440] The suppressive capacity of synTregs was assessed using T cells derived from ulcerative colitis patients as responder cells in a mixed lymphocyte reaction. Suppression of responder cell growth and of inflammatory cytokine secretion was measured.

[000441] A mixed lymphocyte reaction (MLR) assay was used to measure the suppressive function of Tregs. The MLR assay was performed generally as described in Example 2 and by methods known in the art. CD4⁺ T cells (Tresp) were isolated from PBMCs from a healthy donor (HD) and 3 donors with ulcerative colitis (UC) using a commercially available kit, (e.g. Miltenyi). SynTregs were engineered generally as the synTreg samples in Example 4, with slight variation in timing of edits. SynTreg cells were engineered from CD4⁺ cells and edited to insert IL-10DE, CTLA4, and dmTGFB1 and to disrupt IFN γ , TNF α , and TGFB2. To assess secretion of inflammatory cytokines into the media, supernatant was collected at 72 hour timepoint in the MLR assay. The supernatant was assayed using Meso Scale Discovery MSD -U-PLEX Proinflammatory Combo 1 Human Kit according to the manufacturer's protocol. SynTregs effectively suppressed

proliferation (Table 22 and Fig. 13) and inflammatory cytokine release of ulcerative colitis patient-derived T cells (Table 22 and Fig. 14A-B).

Table 22. Mean cytokine secretion and mean percent suppression of cell proliferation in a mixed lymphocyte reaction using synTregs and T cells derived from ulcerative colitis patients. HD, healthy donor. UC, ulcerative colitis.

Assay	Treg: Tresp	HD			UC#1			UC#2			UC#3		
		Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
TNFa (pg/ml)	1:1	29	7	3	51	8	3	30	6	3	31	2	3
	1:3	41	2	3	74	10	3	64	13	3	62	16	3
	1:9	60	12	3	84	3	3	54	7	3	65	12	3
IFNg (pg/ml)	1:1	23	6	3	62	21	3	24	10	3	9	2	3
	1:3	31	9	3	53	16	3	23	0	3	14	2	3
	1:9	22	2	3	66	16	3	20	4	3	13	1	3
% Suppression	1:1	74	2	3	59	2	3	65	2	3	82	3	3
	1:3	40	2	3	44	5	3	49	9	3	61	5	3
	1:9	51	7	3	7	7	3	46	9	3	50	5	3

Example 7. SynTregs outperformed tTregs in suppression of mouse colitis symptoms

[000442] SynTregs and tTregs were tested for suppression of gross and histological signs of colitis in a humanized mouse model of colitis generally by the methods described in Cell Mol Gastroenterol Hepatol. 2019 May 9;8(2):193–19. Thymic Tregs and synTregs were prepared generally as described in Example 4, although the timing of edits may vary. SynTregs were engineered using CD4⁺ starting material, lentivirus encoding the same sequences, and the same LNP reagents as used in Example 4. SynTreg cells were engineered from CD4⁺ cells and edited to insert IL-10DE, CTLA4, and dmTGFB1 and to disrupt IFNg, TNFa, and TGFB2.

[000443] T cells for in vivo injections were harvested and resuspended in media. CD34⁺ humanized NSG mice (Jackson Laboratory. Stock #705557) were injected intravenously with 2 million cells from each test cell population described in Table 23. Seven mice were treated in each test group. Two hours after cell injection, mice were anesthetized and injected subcutaneously with 1% v/v 2,4,6-trinitrobenzenesulfonic acid, (TNBS; Millipore Sigma, 92822) in PBS to induce an immune response against this agent. Seven days after initial treatment, mice were rechallenged with 250 ug TNBS via a rectal catheter to induce colitis. For the next three days, body weight was monitored daily. Mice were sacrificed 10 days after cell injection and 3 days after colitis induction. Colon tissue was collected for analysis. Colon length was recorded, stool removed, tissue weight was recorded. Paraffin-embedded colonic sections were stained and analyzed blinded for

inflammation, crypt hyperplasia, ulcerations, bowel wall thickening, and edema. Each histologic parameter was scored in increments of 0.25 from 0 to 1 resulting in a possible maximum score of 5. Results are shown in **Table 23** and **Figs. 15-16**. Polyclonal synTregs outperformed tTregs in their ability to suppress gross and histological signs of colitis as demonstrated by the lower colon weight:length ratio and histology score for synTregs.

Table 23. Tissue analysis in mouse colitis model.

Sample	Colon weight:length (mg/mm)			Histology score		
	Mean	SD	n	Mean	SD	n
Uninduced control	2.7	0.3	5	0.4	0.2	5
colitis induced control	4.7	1.1	5	2.8	1.1	6
tTreg	4.5	0.7	6	2.4	1.0	6
synTreg	3.3	0.7	6	1.1	1.1	6

Example 8. Allo-editing Increased Persistence of synTregs in a Humanized Colitis Model

[000444] CAR-targeted synTregs with and without additional gene disruptions to prevent allogenicity (made “off the shelf” as described herein) were tested in a humanized mouse model of colitis generally by the methods described in Cell Mol Gastroenterol Hepatol. 2019 May 9;8(2):193–19.

[000445] Generally, T cells were prepared as described in Example 4. CD4 T cells were isolated from human PBMCs using a commercially available kit (e.g. Miltenyi) following the manufacturer’s protocols. Cells were activated with Immunocult and edited according to **Table 24**. All synTreg cells were edited to insert IL-10DE, CTLA4, dmTGFB1, and anti-mouse MAdCAM-1 CAR (“MECA-CAR”) and to disrupt IFNg, TNFa, TGFB2, and TRAC. Cells edited to avoid rejection by the immune system of humanized mice were also edited to disrupt HLA-A, HLA-B, and CIITA. IFNg, TNFa and TRAC were disrupted using mRNA encoding SpCas9 and guides G019753, G019757, and G013006, respectively. The MECA-CAR AAV construct included homology arms flanking the TRAC guide cut site. TGFB2, HLA-A, HLA-B and CIITA were disrupted using mRNA encoding NmeCas9 base editor and guides G034983, G034202, G034208, and G034619, respectively, along with a separate mRNA encoding UGI.

Table 24. T cell editing treatment groups. “LV” is for lentivirus. “AAV” is for adeno-associated virus.

Group	CAR-synTreg	allo CAR-synTreg
Starting Cell	CD4+	CD4+
LV CTLA4 & IL-10DE	+	+
LV dmTGFB 1	+	+
LNP IFNg LNP TNFa	+	+
LNP TGFB2	+	+
LNP-HLA-B LNP UGI	-	+
AAV MECA-CAR LNP TRAC	+	+
LNP HLA-A LNP CIITA LNP UGI	-	+

[000446] T cells for in vivo injections were harvested and resuspended in media. CD34+ humanized NSG mice (Jackson Laboratory, Stock #705557) were injected intravenously with 1 million cells from each test cell population described in Table 25. Seven mice were treated in each test group. Body weight was monitored daily. Two hours after cell injection, mice were anesthetized and injected subcutaneously with 100 uL 2% v/v 2,4-dinitrobenzenesulfonic acid dihydrate (DNBS; Sigma Aldrich, R750735) in PBS to induce an immune response against this agent. Six days after initial treatment, mice were rechallenged with 2.5 mg DNBS via a rectal catheter to induce colitis. Mice were sacrificed 9 days after cell injection and 3 days after colitis induction. Spleens, mesenteric lymph nodes (MLNs), and colon tissue were collected for analysis. Colon length was recorded, stool removed, colon weight was recorded. Spleen and MLN tissues were dissociated and residual cells were stained and analyzed by flow cytometry. Results are shown in **Table 25** and **Figs. 17-19**. Both CAR synTregs and allo-edited CAR synTregs protected mice against colitis as demonstrated by the decrease in colon weight:length compared to the colitis-induced control (**Table 25, Fig 17**). Allo-editing increased engineered T cell persistence in spleen and MLN samples (**Table 25, Fig. 18 and Fig 19, respectively**).

Table 25. Tissue analysis in mouse colitis model

Sample	Colon			Spleen			MLN		
	weight:length (mg/mm)			% synTregs of CD4+			% synTregs of CD4+		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
uninduced control	4.1	0.4	7	0.01	0.01	7	0.00	0.00	7
colitis induced control	4.9	0.5	7	0.00	0.00	7	0.00	0.00	7
CAR synTreg	4.2	0.3	6	0.07	0.06	7	0.04	0.04	7

Sample	Colon			Spleen			MLN		
	weight:length (mg/mm)			% synTregs of CD4+			% synTregs of CD4+		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
allo CAR synTreg	4.1	0.4	7	0.22	0.10	7	0.14	0.09	7

Example 9. SynTregs suppress symptoms in chronic colitis model

[000447] The ability of polyclonal synTregs to suppress colitis induction was tested in a chronic, syngeneic mouse colitis model. In this model, adoptive cell transfer of murine naïve CD4⁺ T cells was used to induce colitis. Polyclonal murine synTregs were compared to bulk murine T cells including natural Tregs for their ability to suppress colitis symptoms. SynTregs were prepared generally as described in Example 4, although the timing of edits may vary. Mouse CD4⁺ T cells were isolated from C57BL/6 mouse spleens using a commercially available kit (Miltenyi) following the manufacturer's protocols. SynTregs were engineered using CD4⁺ as starting material, a retrovirus encoding CTLA4, IL-10DE, and dmTGFB1 for gene insertion, and LNP reagents to disrupt IFN γ , TNFa, and TGFBR2.

On the day of mouse injection, naïve CD4⁺ and total CD4⁺ mouse T cells were isolated from C57BL/6 mouse spleens using a commercially available kit (Miltenyi) following the manufacturer's protocol. T cells for in vivo injections were harvested and resuspended in media. Immunocompromised RAGN12 mice (Taconic Biosciences) were injected peritoneally as described in **Table 26**. Ten mice were treated in each test group. Mice were sacrificed ~48 days after cell injection. Mesenteric lymph nodes (MLNs), and colon tissue were collected for analysis. Colon length was recorded, stool removed, and tissue weight recorded. Paraffin-embedded colonic sections were stained and scored by a histopathologist in a blinded fashion. The morphological criteria used for grading were those recommended by Fabrega et al. (Front Microbiol. 2017 Jul 11;8:1274) and Camuesco et al. (Br J Pharmacol. 2012 Feb;165(3):729-40.). Necrosis and ulceration were graded on a 0-4 scale. The broad areas of scoring were (1) mucosal epithelium and lamina propria, (2) crypts, (3) submucosa, and (4) muscular layer. Results are shown in **Table 26** and **Figs. 20-21**. SynTregs suppressed gross and histological signs of colitis as demonstrated by the lower colon weight:length ratio and histology score for mice treated with synTregs compared to the colitis induction control.

Table 26. Tissue analysis in chronic mouse colitis model.

Sample	Injected	Colon weight:length (mg/mm)			Histology Score		
		Mean	SD	n	Mean	SD	n
Uninduced ctrl	Vehicle (injection media alone)	3.4	0.3	10	3.2	0.4	9
Colitis induction ctrl	1.5 M naïve CD4 ⁺	5.7	2.3	10	29.6	6.7	10

Sample	Injected	Colon weight:length (mg/mm)			Histology Score		
		Mean	SD	n	Mean	SD	n
Colitis suppression ctrl	1M total CD4+	3.3	0.2	10	16.1	1.2	10
synTreg	1.5 M naïve CD4+ 0.5 M synTreg	4.1	0.8	10	22.7	2.5	10

[000448] Other markers of inflammation and colitis were also assessed. Mesenchymal lymph nodes (MLN) were dissociated and cells were stained and analyzed by flow cytometry. Mice injected with synTregs had a significantly decreased percentage of CD4+ T cells producing inflammatory cytokines in the colon-draining lymph nodes as shown in **Table 27** and **Figs. 22A-D**.

Table 27. Mean percentage of CD4+ cells from mesenchymal lymph nodes expressing each inflammatory cytokine.

Sample	TNF			IFN γ			IL-22			IL-17		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Colitis induction ctrl	57.8	8.4	8	68.9	16.1	8	19.9	21.1	8	11.5	4.7	8
Colitis suppression ctrl	80.8	9.9	7	36.2	8.1	7	10.9	6.5	7	19.1	2.6	7
synTreg	39.7	16.5	9	48.6	13.2	10	8.5	7.1	10	4.8	4.2	10

[000449] The colon explant assay assessed cytokine production by immune cells present in colon tissue. One cm of freshly harvested colon tissue was placed in culture medium for 5 hours at 37C followed by quantitation of cytokine levels in the harvested media. Cytokine levels were measured using the MSD -U-PLEX Proinflammatory Combo 1 Murine Kit according to manufacturer's instructions. SynTreg treatment decreased the amount of proinflammatory cytokines IFN γ and TNF produced by colon-resident immune cells, as shown in **Table 28** and **Figs. 23A-B**. Lipocalin-2 is a biomarker that is produced by neutrophils and colon epithelial cells in response to inflammation. Human Lipocalin-2 (NGAL) is reportedly increased in patients with active colitis. Lipocalin levels were assessed in stool samples using a Lipocalin-2 ELISA (Biolegend). Murine synTregs significantly reduced the amount of Lipocalin-2 in the stool of treated mice as shown in **Table 28** and **Fig. 24**.

Table 28. Mean cytokine levels (pg/ml) in media after colon explant culture and mean lipocalin levels in stool samples (ng/ml).

Sample	IFN γ (pg/ml)			TNFa (pg/ml)			Lipocalin (ng/ml)		
	Mean	SD	n	Mean	SD	n	Mean	SD	n

Uninduced ctrl	2.3	3.5	8	10.9	6.2	9	2	1	9
Colitis induction ctrl	141.3	156.0	10	178.3	144.0	10	1589	2337	10
Colitis suppression ctrl	1.9	2.0	8	16.5	7.4	10	2	1	10
synTreg	17.9	9.2	10	59.5	40.7	10	18	23	9

Table 29. Sequences

Description	Sequence	SEQ ID NO
G034199	mC*mU*mC*mAmGCUmAmCGmUCmCCACUmGGAAmGAAmGUUGmUmAmGmCUCCUmGmAmAmAmCmCGUUmGmCUAmCAAUAAAGmGmCCmGmUmCmGmAmAmAmGmAmUGUGCmCGmCAAmCGCUCUmGmCCmUmUmCmUGGCAUCG*mU*mU	353
G013006	mC*mU*mC*UCAGCUGGUACACGGCAGUUUUAGAmGmCmUmAmGmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU	354
G025420	mC*mU*mC*UCAGCUGGUACACGGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCACGAAAGGGCACCGAGUCGmU*mG*mC*mU	355
G034983	mC*mG*mA*mGmAUGmUmCmUUmUCCCAmGAGCmACcmGUUGmUmAmGmCUCCUmGmAmAmAmCmCGUUmGmCUAmCAAUAAAGmGmCCmGmUmCmGmAmAmAmGmAmUGUGCmCGmCAAmCGCUCUmGmCCmUmUmCmUGGCAUCG*mU*mU	356
G034208	mU*mC*mU*mGmGGAmAmAGmGAmGGGAmAGAmGAGmGUUGmUmAmGmCUCCUmGmAmAmAmCmCGUUmGmCUAmCAAUAAAGmGmCCmGmUmCmGmAmAmAmGmAmUGUGCmCGmCAAmCGCUCUmGmCCmUmUmCmUGGCAUCG*mU*mU	357
G034202	mG*mC*mU*mCmUAmCmCmAmCGmGCGCCmCGCmGCUmGUUGmUmAmGmCUCCUmGmAmAmAmCmCGUUmGmCUAmCAAUAAAGmGmCCmGmUmCmGmAmAmAmGmAmUGUGCmCGmCAAmCGCUCUmGmCCmUmUmCmUGGCAUCG*mU*mU	358
G034619	mA*mG*mC*mUmGCCmGmUUmCmUUmGCCCmGUCCmGGGmGUUGmUmAmGmCUCCUmGmAmAmAmCmCGUUmGmCUAmCAAUAAAGmGmCCmGmUmCmGmAmAmAmGmAmUGUGCmCGmCAAmCGCUCUmGmCCmUmUmCmUGGCAUCG*mU*mU	359
MECA-CAR ORF	ATGGACTTCCAGGTGCAGATCTTCAGCTTCCTGCTGATCAGCGCCAGCGTGATCATGAGCCGGATGGCCGATATCCAGATGACCCAGTCCCCTAGCGTGCTGAGCGCCAGCGTGGGCGACAGAGTGACACTGTCTGCAAGACAAGCCAGAACATCAATAAGAATCTGGACTGGTACCAGCAGAAGCTGGGCGAGGCCCCAAAGCTGCTGATCTACTTTACCAATAATCTGCAGACCGGCAATCCCCAGCAGATTTCCGGCTCCGGCTCCGGAACCGATTACACCCTGACCATCTCCAGCCTGCAGCCCGAGGACGTGGCCAATCTACTGCTACCAGTACAATAGCGGCCCCACATTTGG	360

Description	Sequence	SEQ ID NO
	CGCCGGCACCAAGCTGGAGCTGAGAGGAGGCGGCGGCTC CGGAGGAGGAGGATCTGGAGGCGGCGGAAGCCAGGTGCA GCTGAAGGAGTCCGGCCCCGGCTGGTGCAGCCATCTCAG ACACTGAGCCTGACCTGTACCGTGTCCGGCTTCAGCATCA CAAGCAACGGCGTGAGCTGGGTGAGACAGCCCCCGGAA AGGGCCTGGAGTGGATGGGCGCCATCTGGAGCGGCGGCA GCAGAGACTACAATTCCGCCCTGAAGAGCAGGCTGTCCAT CAGCAGGGACACAAGCAAGTCCCAGGTGTTCTGAACCTG AATAGCCTGCAGACAGAGGACACCGCCATCTACTTTTGCA CCAGATCCGATTACCACGATGGCACAAGCCTGTACTACTA CGTGATGGATGCCTGGGGCCAGGGCGCCAGCGTGACAGTT AGCAGCACCCTACCCAGCACCGAGGCCACCCACCCCGG CTCCTACCATCGCCTCCAGCCTCTGTCCCTGCGTCCGGAG GCATGTAGACCCGAGCTGGTGGGGCCCGTGCATACCCGGG GTCTTGACTTCGCCTGCGATATCTATATCTGGGCTCCTTTG GCCGGCACCTGCGGGGTGCTGCTGCTTTCTTGGTGATCAC TCTTTACTGTAAACGGGGCAGAAAGAACTCCTGTATATA TTCAAACAACCATTTATGAGACCAGTACAACTACTCAAG AGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAG AAGGAGGATGTGAACTGCGCGTGAAATTCAGCCGCAGCGC AGATGCTCCAGCCTACAAGCAGGGGCAGAACCCAGCTCTAC AACGAACTCAATCTTGGTCGGAGAGAGGAGTACGACGTGC TGGACAAGCGGAGAGGACGGGACCCAGAAATGGGCGGGA AGCCGCGCAGAAAGAATCCCCAAGAGGGCCTGTACAACG AGCTCCAAAAGGATAAGATGGCAGAAGCCTATAGCGAGA TTGGTATGAAAGGGGAACGCAGAAGAGGCAAAGGCCACG ACGGACTGTACCAGGGACTCAGCACCGCCACCAAGGACAC CTATGACGCTCTTCACATGCAGGCCCTGCCGCTCGG	
MECA-CAR amino acid sequence	MDFQVQIFSLLISASVIMSRMADIQMTQSPSVLSASVGDRV LCKTSQNINKNLDWYQQKLGEAPKLLIYFTNNLQTGIPSRFS GSGGTDYTLTISSLQPEDVATYYCYQYNSGPTFGAGTKLEL RGGGSGGGSGGGGSQVQLKESGPGLVQPSQTLSTCTVS GFSITNGVSWVRQPPGKGLEWMGAIWSSGSRDYNLSALKSR LSISRDTSKSQVFLNLSLQTEDTAIFYFCTRSYHDGTSLYYY VMDAWGQGASVTVSSTTTPAPRPPTPAPTIASQPLSLRPEACR PAAGGAVHTRGLDFACDIYWAPLAGTCGVLLLSLVITLYCK RGRKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELR VKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDRRGR DPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERR RGKGHDGLYQGLSTATKDTYDALHMQALPPR	361
MECA-CAR amino acid sequence	MALPVTALLPLALLLHAARPDIQMTQSPSVLSASVGDRVTL SCKTSQNINKNLDWYQQKLGEAPKLLIYFTNNLQTGIPSRFSG SGSGTDYTLTISSLQPEDVATYYCYQYNSGPTFGAGTKLELR GGGSGGGSGGGGSQVQLKESGPGLVQPSQTLSTCTVSGF SITNGVSWVRQPPGKGLEWMGAIWSSGSRDYNLSALKSRSLI SRDTSKSQVFLNLSLQTEDTAIFYFCTRSYHDGTSLYYYVM DAWGQGASVTVSSTTTPAPRPPTPAPTIASQPLSLRPEACRPA AGGAVHTRGLDPFGFWVLVVVGVLACYLLVTVAFIIFWV RSKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRS LERVRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDR RRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMK GERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	362

Description	Sequence	SEQ ID NO
CD70-CAR ORF	ATGGCCCTGCCTGTGACAGCTCTGCTCCTCCCTCTGGCCCT GCTGCTCCATGCCGCCAGACCCGAGGTGCAGCTCCTGGAG AGTGGTGGAGGCCTGGTCCAGCCAGGGGGCTCCTTGCGTC TGTCTTGCGCCGCTAGCGGCTTCACTTTTTCTATCTACGCC ATGAGTTGGGTGCGCCAGGCTCCTGGTAAAGGCCCTCGAGT GGGTCTCTGCCATCAGCGATTCTGGTGGACGCACCTACTTC GCGGACAGCGTACGTGGCCGCTTCACCATCAGCCGGGACA ACTCCAAGAACACGCTTAGCCTGCAGATGAATTCCCTCCG CGCGGAGGACACCGCAGTCTATTACTGTGCCAAGGTGGAT TACAGCAACTACCTGTTCTTCGACTATTGGGGGCCAGGGAA CATTGGTGACCGTTTCTCTGGTGGCGGGGGTTCCGGCGG CGGCGGCTCGGGGGGTGGGGGGTCCGAGATCGTGCTGACC CAGAGTCCTGGCACTCTTTCGCTGTCGCCGGGGGAGCGCG CCACTCTGTCTATGCCGAGCTTCTCAGTCGATCTCGTCCTCT TACCTGGCCTGGTACCAGCAGAAGCCCGGCCAGGCGCCGA GGCTGTTGATTACGGCGCATCCTCCCGCGCTACCGGCATC CCCACAGATTTTCAGGCAGCGGCTCCGGAACCGACTTCA CACTAACCATTCTCGCCTGGAACCCGAGGACTTTGCCGTG TACTACTGTCAACAGTACGGTTCCAGCCCCTATACCTTCGG CCAGGGCACCAAGCTGGAGATCAAGGGCTCCACCACCACC CCTGCCCCCTAGACCTCCCACCCCAGCCCCAACAAATCGCCA GCCAGCCTCTGTCTCTGCGGCCCGAAGCCTGTAGACCTGCT GCCGGCGGAGCCGTGCACACCAGAGGCCTGGACTTCGCCT GCGACATCTACATCTGGGCCCCCTCTGGCCGGCACCTGTGG CGTGCTGCTGCTGAGCCTGGTGATCACCTGTACTGCAAA CGGGGCAGAAAGAACTCCTGTATATATTCAAACAACCAT TTATGAGACCAGTACAACTACTCAAGAGGAAGATGGCTG TAGTGCCGATTTCCAGAAGAAGAAGAAGGAGGATGTGA ACTGAGAGTGAAGTTCAGCAGATCCGCCGACGCCCCCTGCC TACCAGCAGGGACAGAACCAGCTGTACAACGAGCTGAAC CTGGGCAGACGGGAAGAGTACGACGTGCTGGACAAGCGG AGAGGCCGGGACCCCGAGATGGGCGGAAAGCCCAGACGG AAGAACCCCCAGGAAGGCCTGTATAACGAACTGCAGAAA GACAAGATGGCCGAGGCCTACAGCGAGATCGGCATGAAG GGCGAGCGGAGGCGCGGCAAGGGCCACGATGGCCTGTAC CAGGGCCTGAGCACCGCCACCAAGGACACCTACGACGCCC TGCACATGCAGGCCCTGCCCCCAGA	363
CD70-CAR amino acid sequence	MALPVTALLPLALLHAAARPEVQLLESGGGLVQPGGSLRLS CAASGFTFSIYAMSWVRQAPGKGLEWVSAISDSGGRTYFADS VRGRFTISRDNKNTLSLQMNSLRAEDTAVYYCAKVDYSNY LFFDYWGQGTLVTVSSGGGSGGGGSGGGGSEIVLTQSPGTL SLSPGERATLSCRASQSISSSYLAWYQQKPGQAPRLLIYGASS RATGIPDRFSGSGSDFTLTISRLEPEDFAVYYCQQYGSSPY TFGQGTKLEIKGSTTTPAPRPPTPAPTIASQPLSLRPEACRPAA GGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGR KKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCELRVKFS RSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEM GGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKG HDGLYQGLSTATKDTYDALHMQALPPR	364
NmeCas9 base editor	AGGAAGCUCAGAAUAAACGCUCAACUUUGGCCGGAUCUC CCACCAUGGACGGCUCCGGCGGCGGCUCACCCCAAGAAGA AGCGGAAGGUGGAGGACAAGCGGCCCGCCGCCACCAAGA AGGCCGGCCAGGCCAAGAAGAAGAAGGGCGGCUCGCGCG	365

Description	Sequence	SEQ ID NO
	GGGGCGAGGCCUCCCCCGCCUCCGGCCCCCGGCACCUGAU GGACCCCCACAUCUUCACCUCCAACUUAACAACGGCAU CGGCCGGCACAAGACCUACCUUGUCUACGAGGUGGAGCG GCUGGACAACGGCACCUCGUGAAGAUGGACCAGCACCG GGGCUUCCUGCACAACCAGGCCAAGAACCUGCUGUGCGG CUUCUACGGCCGGCACGCCGAGCUGCGGUUCCUGGACCU GGUGCCCUCCUGCAGCUGGACCCCGCCAGAUCUACCG GGUGACCUGGUUCAUCUCCUGGUCCCCUGCUUCUCCUG GGGCUGCGCCGGCGAGGUGCGGGCCUCCUGCAGGAGAA CACCCACGUGCGGCUGCGGAUCUUCGCCGCCCGGAUCUA CGACUACGACCCCUUGUACAAGGAGGCCUGCAGAUGCU GCGGGACGCCGGCGCCAGGUGUCCAUAUGACCUACGA CGAGUUAAGCACUGCUGGGACACCUUCGUGGACCACCA GGGCUGCCCUUCCAGCCUGGGACGGCCUGGACGAGCA CUCCAGGCCCUGUCCGGCCGGCUGCGGGCCAUCCUGCA GAACCAGGGCAACGGCACCAAGGACUCCACCAAGGACAU CCCCGAGACCCCUCCAAGGACGCAGCGUUAACCAAA UCCCAUCAACUACAUCUGGGCCUGGCCAUCGGCAUCGC CUCCGUGGGCUGGGCCAUGGUGGAGAUCCGACGAGGAGGA GAACCCCAUCCGGCUGAUCCGACCGGGCGUGCGGGUGUU CGAGCGGGCCGAGGUGCCCAAGACCGGCGACUCCUGGC CAUGGCCCGGGCGGCUGGCCCGGUCCGUGCGGCGGCUGAC CCGGCGGGCGGGCCACCGGCUGCUGCGGGCCCGGCGGCU GCUGAAGCGGGAGGGCGUGCUGCAGGCCGCCGACUUCGA CGAGAACGGCCUGAUCAAGUCCUGCCCAACACCCCUUG GCAGCUGCGGGCCGCCGCCUGGACCGGAAGCUGACCCC CCUGGAGUGUCCGCCGUGCUGCUGCACCUGAUCAAGCA CCGGGGCUACCUGUCCAGCGGAAGAACGAGGGCGAGAC CGCCGACAAGGAGCUGGGCGCCUGCUGAAGGGCGUGGC CAACAACGCCCACGCCUGCAGACCGGCGACUUCGGAC CCCCGCCGAGCUGGCCCUGAACAAGUUCGAGAAGGAGUC CGGCCACAUCGGAACCAGCGGGGCGACUACUCCACAC CUUCUCCCGGAAGGACCUGCAGGCCGAGCUGAUCCUGCU GUUCGAGAAGCAGAAGGAGUUCGGCAACCCACGUGUC CGGCGGCCUGAAGGAGGGCAUCGAGACCCUGCUGAUGAC CCAGCGGCCCGCCUGUCCGGCGACGCCGUGCAGAAGAU GCUGGGCCACUGCACCUUCGAGCCCGCCGAGCCCAAGGC CGCCAAGAACACCUACACCGCCGAGCGGUUCAUCUGGCU GACCAAGCUGAACAACCUGCGGAUCCUGGAGCAGGGCUC CGAGCGGCCCCUGACCGACACCGAGCGGGCCACCCUGAU GGACGAGCCCUACCGGAAGUCCAAGCUGACCUACGCCCCA GGCCCGGAAGCUGCUGGGCCUGGAGGACACCGCCUUCUU CAAGGGCCUGCGGUACGGCAAGGACAACGCCGAGGCCUC CACCCUGAUGGAGAUGAAGGCCUACCACGCCAUCUCCCG GGCCUGGAGAAGGAGGGCCUGAAGGACAAGAAGUCCCC CCUGAACCUGUCCUCCGAGCUGCAGGACGAGAUCGGCAC CGCCUUCUCCUGUUAAGACCGACGAGGACAUCACCGG CCGGCUGAAGGACCGGGUGCAGCCGAGAUCCUGGAGGC CCUGCUGAAGCACAUCUCCUUCGACAAGUUCGUGCAGAU CUCCUGAAGGCCUGCGGGCGGAUCGUGCCCCUGAUGGA GCAGGGCAAGCGGUACGACGAGGCCUGCGCCGAGAUCUA CGGCGACCACUACGGCAAGAAGAACACCGAGGAGAAGAU CUACCUGCCCCCAUCCCGCCGACGAGAUCCGGAACCC	

Description	Sequence	SEQ ID NO
	GUGGUGCUGCGGGCCCCUGUCCCAGGCCCCGGAAGGUGAUC AACGGCGUGGUGCGGGCGGUACGGCUCCCCCGCCGGAUC CACAU CGAGACCGCCCGGGAGGUGGGCAAGUCCUUCAAG GACCGGAAGGAGAU CGAGAAGCGGCAGGAGGAGAACCG GAAGGACCGGGAGAAGGCCCGCCCAAGUCCGGGAGUA CUUCCCCAACUUCGUGGGCGAGCCCAAGUCCAAGGACAU CCUGAAGCUGCGGCUGUACGAGCAGCAGCACGGCAAGUG CCUGUACUCCGGCAAGGAGAUCAACCUGGUGCGGCUGAA CGAGAAGGGCUACGUGGAGAU CGACCACGCCUGCCCUU CUCCCCGACCUGGGACGACUCCUUCAACAACAAGGUGCU GGUGCUGGGCUCCGAGAACCAGAACAAGGGCAACCAGAC CCCCUACGAGUACUUCAACGGCAAGGACAACUCCCCGGA GUGGCAGGAGUUCAAGGCCCGGGUGGAGACCUCCCCGUU CCCCCGGUCCAAGAAGCAGCGGAUCCUGCUGCAGAAGUU CGACGAGGACGGCUUCAAGGAGUGCAACCUGAACGACAC CCGGUACGUGAACCUCUUCUGUGCCAGUUCGUGGCCGA CCACAUCCUGCUGACCGGCAAGGGCAAGCGGCGGGUGUU CGCCUCCAACGGCCAGAU CACCAACCUGCUGCGGGGCUU CUGGGGCCUGCGGAAGGUGCGGGCCGAGAACGACCGGCA CCACGCCUGGACGCCGUGGUGGUGGCCUGCUCCACCGU GGCCAUGCAGCAGAAGAUCACCCGGUUCGUGCGGUACAA GGAGAUGAACGCCUUCGACGGCAAGACCAUCGACAAGGA GACCGGCAAGGUGCUGCACCAGAAGACCCACUUCCCCCA GCCUGGGAGUUCUUCGCCCAGGAGGUGAUGAUCCGGGU GUUCGGCAAGCCCGACGGCAAGCCCGAGUUCGAGGAGGC CGACACCCCGAGAAGCUGCGGACCCUGCUGGCCGAGAA GCUGUCCUCCCGGCCGAGGCCGUGCAGGAGUACGUGAC CCCCUGUUCGUGUCCCGGGCCCCCAACCGGAAGAUGUC CGGCGCCACAAAGGACACCCUGCGGUCCGCCAAGCGGUU CGUGAAGCACAACGAGAAGAUCUCCGUGAAGCGGGUGUG GCUGACCGAGAUCAAGCUGGCCGACCUGGAGAACAUGGU GAACUACAAGAACGGCCGGGAGAU CGAGCUGUACGAGGC CCUGAAGGCCCGGCUGGAGGCCUACGGCGGCAACGCCAA GCAGGCCUUCGACCCCAAGGACAACCCCUUCUACAAGAA GGGCGGCCAGCUGGUGAAGGCCGUGCGGGUGGAGAAGAC CCAGGAGUCCGGCGUGCUGCUGAACAAGAAGAACGCCUA CACCAUCGCCGACAACGGCGACAUGGUGCGGGUGGACGU GUUCUGCAAGGUGGACAAGAAGGGCAAGAACCAGUACU UCAUCGUGCCCAUCUACGCCUGGACGGUGGCCGAGAAC UCCUGCCCGACAUCGACUGCAAGGGCUACCGGAUCGACG ACUCCUACACCUUCUGCUUCUCCUGCACAAGUACGACC UGAUCGCCUUC CAGAAGGACGAGAAGUCCAAGGUGGAGU UCGCCUACUACAUAACUGCGACUCCUCCAACGGCCGGU UCUACCUGGCCUGGCACGACAAGGGCUCCAAGGAGCAGC AGUUCCGGAUCUCCACCCAGAACCUGGUGCUGAUCCAGA AGUACCAGGUGAACGAGCUGGGCAAGGAGAUCCGGCCCU GCCGGCUGAAGAAGCGGCCCCCCGUGCGGUAGCUAGCAC CAGCCUCAAGAACACCCGAAUGGAGUCUCUAAGCUACAU AAUACCAACUUAACUUUACAAAAUGUUGUCCCCCAAAA UGUAGCCAUUCGUUUCUGCUCCUAAUAAAAAGAAAGUU UCUUCACAUUCUCUCGAGAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	

Description	Sequence	SEQ ID NO
	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAUCUAG	

CLAIMS

1. An engineered T cell, comprising:
 - i) a heterologous nucleic acid sequence encoding a dual mutant transforming growth factor beta 1 (dmTGFB1) under control of a promoter sequence; and
 - ii) a modification of an endogenous a nucleic acid sequence encoding a transforming growth factor beta receptor 2 (TGFB2) wherein the modification knocks down expression of TGFB2.
2. The engineered T cell of claim 1, wherein the dmTGFB1 comprises a mutation at two or more amino acids selected from F198, D199, V200, L208, F217, L219, R218, H222, C223, and C225 relative to a wild type human TGFB1.
3. The engineered T cell of claims 1 or 2, wherein the dmTGFB1 comprises a mutation at two or more amino acids selected from R218, H222, C223, and C225 relative to a wild type human TGFB1.
4. The engineered T cell of claims 1, 2, or 3, wherein the dmTGFB1 comprises two or more amino acid mutations selected from R218C/H, H222D, C223S/R/G, and C225R relative to a wild type human TGFB1.
5. The engineered T cell of any one of claims 1-4, wherein the dmTGFB1 comprises amino acid mutations R218C/H and C225R relative to a wild type human TGFB1.
6. The engineered T cell of any one of claims 1-5, wherein the engineered T cell is a CD4+ T cell.
7. The engineered T cell of any one of claims 1-6, further comprising:
 - i) a modification of an endogenous a nucleic acid sequence encoding a tumor necrosis factor alpha (TNFA) wherein the modification knocks down expression of TNFA;
 - ii) a modification of an endogenous nucleic acid sequence encoding an interferon-gamma (IFNG) wherein the modification knocks down expression of the IFNG; or
 - iii) a heterologous nucleic acid sequence encoding a regulatory T cell promoting molecule under control of a promoter sequence.
8. The engineered T cell of any one of claims 1-7, comprising:
 - i) a modification of an endogenous a nucleic acid sequence encoding a tumor necrosis factor alpha (TNFA) wherein the modification knocks down expression of TNFA;
 - ii) a modification of an endogenous nucleic acid sequence encoding an interferon-gamma (IFNG) wherein the modification knocks down expression of the IFNG; and

iii) a heterologous nucleic acid sequence encoding a regulatory T cell promoting molecule under control of a promoter sequence.

9. The engineered T cell of claim 7 or claim 8, wherein the regulatory T cell promoting molecule is a selected from interleukin-10 (IL10), cytotoxic T-lymphocyte associated protein 4 (CTLA4), T Cell immunoreceptor with Ig And ITIM domains (TIGIT), indoleamine 2,3-dioxygenase 1 (IDO1), ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), 5'-nucleotidase ecto (NT5E), interleukin-22 (IL-22), amphiregulin (AREG), interleukin-35 (IL35), GARP, CD274 molecule (CD274), forkhead box P3 (FOXP3), IKAROS family zinc finger 2 (IKZF2), eosinophilia familial (EOS), interferon regulatory factor 4 (IRF4), lymphoid enhancer binding factor 1 (LEF1), BTB domain and CNC homolog 2 (BACH2), and interleukin 2 receptor subunit alpha (IL2RA).

10. The engineered T cell of claim 9, wherein the regulatory T cell promoting molecule is IL10 or CTLA4.

11. The engineered T cell of claims 9 or 10, wherein the regulatory T cell promoting molecule is a first regulatory T cell promoting molecule, and further comprising a heterologous nucleic acid sequence encoding a second regulatory T cell promoting molecule under control of a promoter sequence.

12. The engineered T cell of claim 11, wherein the first and the second regulatory T cell promoting molecules are IL10 and CTLA4.

13. The engineered T cell of any one of claims 9-12, wherein the IL10 comprises a mutation at one or more amino acids selected from D25, E96, N21, and R104 relative to a mature wild type human IL10.

14. The engineered T cell of any one of claims 9-13, wherein the IL10 comprises one or more amino acid mutations selected from D25A/K, E96A, N21A, and R104A relative to a mature wild type human IL10.

15. The engineered T cell of any one of claims 9-14, wherein the IL10 comprises a combination of amino acid mutations selected from D25K, D25A, D25A/E96A, D25K/E96A, N21A/R104A, D25A/N21A/R104A, D25K/N21A/R104A relative to a mature wild type human IL10.

16. The engineered T cell of any one of claims 9-12, wherein the IL10 comprises a mature wild type human IL10.

17. The engineered T cell of any one of claims 1-16, further comprising a modification of an endogenous nucleic acid sequence encoding a protein selected from TRAC, HLA-A, HLA-B, and CIITA, wherein the modification knocks down expression of the protein.

18. The engineered T cell of claim 17, wherein the protein is TRAC, and the modification knocks down expression of TRAC.
19. The engineered T cell of claim 17 of claim 18, wherein the protein is HLA-A, and the modification knocks down expression of HLA-A.
20. The engineered T cell of any one of claims 17-19, wherein the protein is HLA-B, and the modification knocks down expression of HLA-B.
21. The engineered T cell of any one of claims 17-20, wherein the protein is CIITA, and the modification knocks down expression of CIITA.
22. The engineered T cell of any one of claims 1-16, further comprising:
- a) a modification of an endogenous nucleic acid sequence encoding TRAC, wherein the modification knocks down expression of TRAC;
 - b) a modification of an endogenous nucleic acid sequence encoding HLA-A, wherein the modification knocks down expression of HLA-A;
 - c) a modification of an endogenous nucleic acid sequence encoding HLA-B, wherein the modification knocks down expression of HLA-B; and
 - d) a modification of an endogenous nucleic acid sequence encoding CIITA, wherein the modification knocks down expression of CIITA.
23. An engineered T cell comprising:
- i) a modification of a first endogenous nucleic acid sequence encoding a tumor necrosis factor alpha (TNFA), wherein the modification of the first endogenous nucleic acid knocks down expression of TNFA;
 - ii) a modification of a second endogenous nucleic acid sequence encoding an interferon-gamma (IFNG), wherein the modification of the second endogenous nucleic acid knocks down expression of the IFNG;
 - iii) a modification of a third endogenous nucleic acid sequence encoding a protein selected from TRAC, HLA-A, HLA-B, and CIITA, wherein the modification of the third endogenous nucleic acid knocks down expression of the protein; and
 - iv) a heterologous nucleic acid sequence encoding a regulatory T cell promoting molecule under control of a promoter sequence.
24. The engineered T cell of claim 23, wherein the protein is TRAC, and the third modification knocks down expression of TRAC.

25. The engineered T cell of claim 23 or claim 24, wherein the protein is HLA-A, and the third modification knocks down expression of HLA-A.
26. The engineered T cell of any one of claims 23-25, wherein the protein is HLA-B, and the third modification knocks down expression of HLA-B.
27. The engineered T cell of any one of claims 23-26, wherein the protein is CIITA, and the third modification knocks down expression of CIITA.
28. An engineered T cell comprising:
- i) a modification of a first endogenous nucleic acid sequence encoding a tumor necrosis factor alpha (TNFA) wherein the modification knocks down expression of TNFA;
 - ii) a modification of a second endogenous nucleic acid sequence encoding an interferon-gamma (IFNG) wherein the modification knocks down expression of the IFNG;
 - iii) a modification of a third endogenous nucleic acid sequence encoding TRAC, wherein the modification of the third endogenous nucleic acid sequence knocks down expression of TRAC;
 - iv) a modification of a fourth endogenous nucleic acid sequence encoding HLA-A, wherein the modification of the fourth endogenous nucleic acid sequence knocks down expression of HLA-A;
 - v) a modification of a fifth endogenous nucleic acid sequence encoding HLA-B, wherein the modification of the fifth endogenous nucleic acid sequence knocks down expression of HLA-B;
 - vi) a modification of a sixth endogenous nucleic acid sequence encoding CIITA, wherein the modification of the sixth endogenous nucleic acid sequence knocks down expression of CIITA; and
 - vii) a heterologous nucleic acid sequence encoding a regulatory T cell promoting molecule under control of a promoter sequence.
29. The engineered T cell of any one of claims 23-28, wherein the regulatory T cell promoting molecule is a selected from interleukin-10 (IL10), cytotoxic T-lymphocyte associated protein 4 (CTLA4), T Cell immunoreceptor with Ig And ITIM domains (TIGIT), indoleamine 2,3-dioxygenase 1 (IDO1), ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), 5'-nucleotidase ecto (NT5E), interleukin-22 (IL-22), amphiregulin (AREG), interleukin-35 (IL35), GARP, CD274 molecule (CD274), forkhead box P3 (FOXP3), IKAROS family zinc finger 2 (IKZF2), eosinophilia familial (EOS), interferon regulatory factor 4 (IRF4), lymphoid enhancer binding factor 1 (LEF1), BTB domain and CNC homolog 2 (BACH2), and interleukin 2 receptor subunit alpha (IL2RA).

30. The engineered T cell of claim 29, wherein the regulatory T cell promoting molecule is IL10 or CTLA4.
31. The engineered T cell of claims 29 or 30, wherein the regulatory T cell promoting molecule is a first regulatory T cell promoting molecule, and further comprising a heterologous nucleic acid sequence encoding a second regulatory T cell promoting molecule under control of a promoter sequence.
32. The engineered T cell of claim 31, wherein the first and the second regulatory T cell promoting molecules are IL10 and CTLA4.
33. The engineered T cell of any one of claims 29-32, wherein the IL10 comprises a mutation at one or more amino acids selected from D25, E96, N21, and R104 relative to a mature wild type human IL10.
34. The engineered T cell of any one of claims 29-33, wherein the IL10 comprises one or more amino acid mutations selected from D25A/K, E96A, N21A, and R104A relative to a mature wild type human IL10.
35. The engineered T cell of any one of claims 29-34, wherein the IL10 comprises a combination of amino acid mutations selected from D25K, D25A, D25A/E96A, D25K/E96A, N21A/R104A, D25A/N21A/R104A, D25K/N21A/R104A relative to a mature wild type human IL10.
36. The engineered T cell of any one of claims 29-35, wherein the IL10 comprises a mature wild type human IL10.
37. The engineered T cell of any one of claims 1-36, further comprising a modification of an endogenous nucleic acid sequence encoding an interleukin 17A (IL17A), an interleukin-2 (IL2), an interleukin 6 (IL6), a perforin 1 (PRF1), a granzyme A (GZMA), a granzyme B (GZMB), a natural killer cell granule protein 7 (NKG7), a Fas ligand (FASL, NF superfamily, member 6), a ryanodine receptor 2 (RYSR2), and colony stimulating factor 2 (CSF2) wherein the modification knocks down expression of the IL17A, the IL2, the IL6, the PRF1, the GZMA, the GZMB, the FASL, the RYSR2, or the CSF2 respectively.
38. The engineered T cell of any one of claims 1-37, further comprising a modification of an endogenous nucleic acid sequence encoding an endogenous T cell receptor (TCR), wherein the modification knocks down expression of the endogenous TCR.
39. The engineered T cell of any one of claims 1-38, further comprising a heterologous coding sequence for a targeting receptor under control of a promoter sequence.

40. The engineered T cell of claim 39, wherein the targeting receptor is targeted to a ligand selected from mucosal vascular addressin cell adhesion molecule 1 (MADCAM1), tumor necrosis factor alpha (TNFA), CD70 molecule (CD70), CEA cell adhesion molecule 6 (CEACAM6), vascular cell adhesion molecule 1 (VCAM1), citrullinated vimentin, myelin basic protein (MBP), MOG (myelin oligodendrocyte glycoprotein), proteolipid protein 1 (PLP1), CD19 molecule (CD19), CD20 molecule (CD20), TNF (ligand) super family member 7 (TNFSF7), TNF receptor superfamily member 17 (TNFRSF17), dipeptidyl peptidase like 6 (DPP6), solute carrier family 2 member 2 (SCL2A2), glutamate decarboxylase (GAD2), desmoglein 3 (DSG3), and MHC class I HLA-A (HLA-A*02).
41. The engineered T cell of claim 39 or 40, wherein the targeting receptor comprises a chimeric antigen receptor (CAR) or a T cell receptor (TCR).
42. The engineered T cell of any one of claims 39-41, wherein the heterologous nucleic acid sequence encoding the targeting receptor is incorporated into an expression construct.
43. The engineered T cell of any one of claims 39-42, wherein the heterologous nucleic acid sequence encoding a targeting receptor is in an expression construct that does not comprise a nucleic acid sequence encoding a regulatory T cell promoting molecule.
44. The engineered T cell of any one of claims 39-42, wherein the heterologous nucleic acid sequence encoding a targeting receptor is in an expression construct that comprises a nucleic acid sequence encoding a regulatory T cell promoting molecule.
45. The engineered T cell of any one of claims 11-22 or 31-44, wherein the heterologous nucleic acid sequence encoding the first regulatory T cell promoting molecule is incorporated into an expression construct and the heterologous nucleic acid sequence encoding the second regulatory T cell promoting molecule is incorporated in an expression construct.
46. The engineered T cell of any one of claims 11-22 or 31-45, wherein the heterologous nucleic acid sequence encoding the first regulatory T cell promoting molecule and the heterologous nucleic acid sequence encoding the second regulatory T cell promoting molecule are incorporated into separate expression constructs.
47. The engineered T cell of any one of claims 11-22 or 31-46, wherein the heterologous nucleic acid sequence encoding the first regulatory T cell promoting molecule and the heterologous nucleic acid sequence encoding the second regulatory T cell promoting molecule are incorporated into a single expression construct.
48. The engineered T cell of any one of claims 1-47, wherein at least one heterologous coding sequence is in an episomal expression construct.

49. The engineered T cell of any one of claims 1-48, wherein at least one heterologous nucleic acid sequence is inserted into the genome.
50. The engineered T cell of claim 49, wherein the insertion into the genome is an untargeted insertion.
51. The engineered T cell of claim 49, wherein the insertion is a targeted insertion.
52. The engineered T cell of claim 51, wherein the targeted insertion is into a site selected from a TCR gene locus, a TNF gene locus, an IFNG gene locus, IL17A gene locus, IL6 gene locus, IL2 gene locus, an adeno-associated virus integration site 1 (AAVS1) locus.
53. The engineered T cell of claim 52, wherein the TCR gene locus is a T cell receptor alpha constant (TRAC) locus.
54. The engineered T cell of any one of claims 7-53, wherein the modification that knocks down expression of a gene comprises one or more of an insertion, a deletion, or a substitution.
55. The engineered T cell of any of claims 1-54, wherein the engineered T cell is a CD4+ T cell.
56. A population of engineered T cells comprising the engineered T cell of any one of claims 1-55.
57. A population of engineered T cells comprising the engineered T cells of any one of claims 1-22 or 37-56, wherein
- at least 30% or at least 40%, of cells of the population comprise a heterologous nucleic acid sequence encoding a dmTGFB1 under control of a promoter sequence; and
 - at least 50% or at least 70%, of cells of the population comprise a modification of an endogenous nucleic acid sequence encoding a TGFBR2.
58. The population of engineered T cells of claim 56 or 57, wherein
- at least 50%, or at least 70%, of cells of the population comprise a modification of an endogenous nucleic acid sequence encoding a TNFA;
 - at least 50% or at least 70%, of cells of the population comprise a modification of an endogenous nucleic acid sequence encoding an IFNG; or
 - at least 30% or at least 40%, of cells of the population comprise a heterologous nucleic acid sequence encoding a regulatory T cell promoting molecule under control of a promoter sequence.

59. The population of engineered T cells of claim 57 or claim 58, wherein the percent of cells comprising an insertion or a modification is determined by the percent of reads by next generation sequencing (NGS).
60. A pharmaceutical composition comprising the engineered T cells of any one of claims 1-55 or population of engineered T cells of claims 56-59.
61. A method or use of administering the engineered T cell of any one of claims 1-55, a population of engineered T cells of any one of claims 56-59, or a pharmaceutical composition of claim 60 to a subject.
62. The method or use of claim 61, wherein the subject is in need of immunosuppression.
63. The method or use of claim 61 or claim 62, for treatment of an immune disorder.
64. The method or use of any one of claims 61-63, for treatment of an autoimmune disease.
65. The method or use of claim 64, wherein the autoimmune disease is selected from ulcerative colitis, Crohn's disease, rheumatoid arthritis, psoriasis, multiple sclerosis, systemic lupus erythematosus, and type 1 diabetes.
66. The method or use of any one of claims 61-65, for treatment of graft versus host disease (GvHD).
67. The method or use of any one of claims 61, 62, or 66, wherein the subject receives a transplant or transfusion.
68. The method or use of claim 67, wherein the transplant or transfusion comprises a solid organ or a population of cells.
69. The method or use of claim 67 or claim 68, wherein the transplant or transfusion comprises cells from a donor that is not the subject.
70. The method or use of any one of claims 67-69, wherein the subject is administered the engineered T cell of any one of claims 1-55, the population of engineered T cells of any one of claims 56-59, or the pharmaceutical composition of claim 60 prior to receiving the transplant or transfusion.
71. The method or use of any one of claims 67-70, wherein the subject is administered the engineered T cell of any one of claims 1-55, the population of engineered T cells of any one of claims 56-59, or the pharmaceutical composition of claim 60 concurrently with receiving the transplant or transfusion.
72. The method or use of any one of claims 67-71, wherein the subject is administered the engineered T cell of any one of claims 1-55, the population of engineered T cells of any one of

claims 56-59, or the pharmaceutical composition of claim 60 after receiving the transplant or transfusion.