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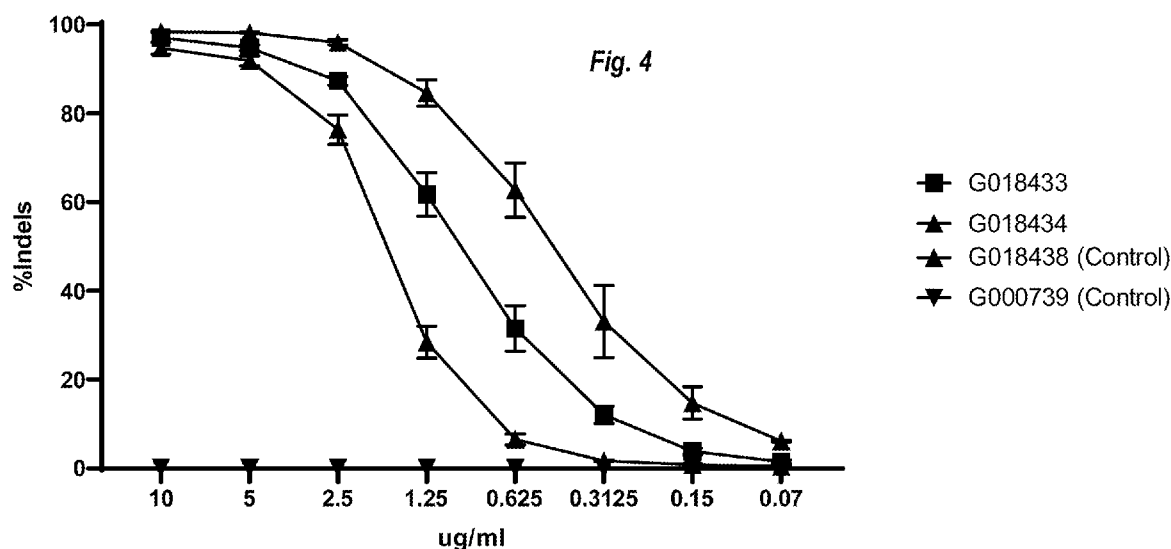
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(54) Title: LYMPHOCYTE ACTIVATION GENE 3 (LAG3) COMPOSITIONS AND METHODS FOR IMMUNOTHERAPY



(57) Abstract: Compositions and methods for editing, e.g., altering a DNA sequence, within a *LAG3* gene are provided. Compositions and methods for immunotherapy are provided.

LYMPHOCYTE ACTIVATION GENE 3 (LAG3) COMPOSITIONS AND METHODS FOR IMMUNOTHERAPY

[001] This application claims the benefit under 35 U.S.C. 119(e) of US Provisional Application No. 63/147,223, filed February 8, 2021, the content of which is incorporated herein by reference in its entirety.

[002] This application is filed with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled "01155-0040-00PCT_Seq_List_ST25.txt" created on January 31, 2022, which is 129,024 bytes in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

INTRODUCTION AND SUMMARY

[003] T cell exhaustion is a broad term that has been used to describe the response of T cells to chronic antigen stimulation. This was first observed in the setting of chronic viral infection but has also been studied in the immune response to tumors. The features and characteristics of the T-cell exhaustion mechanism may have crucial implications for the success of checkpoint blockade and adoptive T cell transfer therapies.

[004] T cell exhaustion is a progressive loss of effector function due to prolonged antigen stimulation, characteristic of chronic infections and cancer. In addition to continuous antigen stimulation, antigen presenting cells and cytokines present in the microenvironment can also contribute to this exhausted phenotype. Thus T cell exhaustion is a state of T cell dysfunction in which T cells present poor effector function and sustained expression of inhibitory receptors. This prevents optimal control of infections or tumours. Additionally, exhausted T cells have a transcriptional state distinct from that of functional effector or memory T cells. Therapeutic treatments have the potential to rescue exhausted T cells (Goldberg, M.V. & Drake, C.G., 2011, Wherry, E.J. & Kurachi M., 2015).

[005] Exhausted T cells typically express co-inhibitory receptors such as programmed cell death 1 (PDCD1 or PD-1). The gene product acts as a component of an immune checkpoint system. T cell exhaustion may be reversed by blocking these receptors.

[006] *LAG3* Lymphocyte Activation Gene-3 (*LAG3*) is part of the Immunoglobulin (Ig) superfamily (Treibel *et al.*, 1990). *LAG3* is a cell surface protein that is expressed on activated CD4⁺ and CD8⁺ T cells, regulatory T cells (Tregs), B cells, natural killer (NK) cells, and dendritic cells (DC). *LAG3* is also expressed on the cell membranes of tumor-infiltrating lymphocytes (TILs). *LAG3* acts as an immune checkpoint in T cells, where it has been shown to have negative regulatory function. During chronic infection, T cells express *LAG3* as well

as other immune checkpoint genes which downregulate the immune response of T cells. The mechanism by which *LAG3* downregulates T cell response is not clear. However, the inhibitory effects due to the binding of MHC II to LAG3 are dependent on the intracellular KIEELE domain of LAG3.

[007] Provided herein are compounds and compositions for use, for example, in methods of preparation of cells with genetic modifications (e.g., insertions, deletions, substitutions) in a LAG3 sequence, e.g., a genomic locus, generated, for example, using the CRISPR/Cas system; and the cells with genetic modifications in the LAG3 sequence and their use in various methods, e.g., to promote an immune response e.g., in immunooncology and infectious disease. The cells with LAG3 genetic modifications that may reduce LAG3 expression, may include genetic modifications in additional genomic sequences including, T-cell receptor (TCR) loci, e.g., TRAC or TRBC loci, to reduce TCR expression; genomic loci that reduce expression of MHC class I molecules, e.g., B2M and HLA-A loci; genomic loci that reduce expression of MHC class II molecules, e.g., CIITA loci; and checkpoint inhibitor loci, e.g., CD244 (2B4) loci, TIM3 loci, and PD-1 loci. The present disclosure relates to populations of cells including cells with genetic modification of the LAG3 sequence, and optionally other genomic loci as provided herein. The cells may be used in adoptive T cell transfer therapies. The present disclosure relates to compositions and uses of the cells with genetic modification of the LAG3 sequence for use in therapy, e.g., cancer therapy and immunotherapy. The present disclosure relates to and provides gRNA molecules, CRISPR systems, cells, and methods useful for genome editing of cells.

[008] Provided herein is an engineered cell comprising a genetic modification in a human LAG3 sequence, within the genomic coordinates of chr12: 6772483-6778455. Further embodiments are provided throughout and described in the claims and Figures.

[009] Also disclosed is the use of a composition or formulation of a cell of any of the foregoing embodiments for the preparation of a medicament for treating a subject. The subject may be human or animal (e.g. human or non-human animal, e.g., cynomolgus monkey). Preferably the subject is human.

[0010] Also disclosed are any of the foregoing compositions or formulations for use in producing a genetic modification (e.g., an insertion, a substitution, or a deletion) a *LAG3* gene sequence. In certain embodiments, the genetic modification within the sequence results in a change in the nucleic acid sequence that prevents translation of a full-length protein prior to genetic modification of the genomic locus, e.g., by forming a frameshift or nonsense mutation, such that translation is terminated prematurely. The genetic modification

can include insertion, substitution, or deletion at a splice site, i.e., a splice acceptor site or a splice donor site, such that the abnormal splicing results in a frameshift mutation, nonsense mutation, or truncated mRNA, such that translation is terminated prematurely. Genetic modifications can also disrupt translation or folding of the encoded protein resulting in premature translation termination.

[0011] Compositions provided herein for use in producing a genetic modification within the sequence preferably results in reduced expression of a protein, e.g., cell surface expression of the protein, from the sequence.

[0012] In another aspect, the invention provides a method of providing an immunotherapy to a subject, the method including administering to the subject an effective amount of a cell as described herein, for example, a cell of any of the aforementioned cell aspects and embodiments.

[0013] In embodiments of the methods, the method includes lymphodepletion prior to administering a cell or population of cells as described herein. In embodiments of the methods, the method includes administering a lymphodepleting agent or immunosuppressant prior to administering to the subject an effective amount of the cell as described herein, for example, a cell of any of the aforementioned cell aspects and embodiments. In another aspect, the invention provides a method of preparing cells (e.g., a population of cells).

[0014] Immunotherapy is the treatment of disease by activating or suppressing the immune system. Immunotherapies designed to elicit or amplify an immune response are classified as activation immunotherapies. Cell-based immunotherapies have been demonstrated to be effective in the treatment of some cancers. Immune effector cells such as lymphocytes, macrophages, dendritic cells, natural killer cells (NK Cell), cytotoxic T lymphocytes (CTL) can be programmed to act in response to abnormal antigens expressed on the surface of tumor cells. Thus, cancer immunotherapy allows components of the immune system to destroy tumors or other cancerous cells.

[0015] Immunotherapy can also be useful for the treatment of chronic infectious disease, e.g., hepatitis B and C virus infection, human immunodeficiency virus (HIV) infection, tuberculosis infection, and malarial infection. Immune effector cells comprising a targeting receptor such as a transgenic TCR or CAR are useful in immunotherapies, such as those described herein.

[0016] In another aspect, the invention provides a method of preparing cells (e.g., a population of cells) for immunotherapy, the method including: (a) modifying cells by reducing or eliminating expression of one or more or all components of a T-cell receptor

(TCR), for example, by introducing into said cells a gRNA molecule (as described herein), or more than one gRNA molecule, as disclosed herein; and (b) expanding said cells. Cells of the invention are suitable for further engineering, e.g. by introduction of a heterologous sequence coding for a targeting receptor, e.g. a polypeptide that mediates TCR/CD3 zeta chain signalling. In some embodiments, the polypeptide is a targeting receptor selected from a non-endogenous TCR or CAR sequence. In some embodiments, the polypeptide is a wild-type or variant TCR. Cells of the invention may also be suitable for further engineering by introduction of a heterologous sequence coding for an alternative antigen binding moiety, e.g. by introduction of a heterologous sequence coding for an alternative (non-endogenous) T cell receptor, e.g. a chimeric antigen receptors (CAR) engineered to target a specific protein. CAR are also known as chimeric immunoreceptors, chimeric T cell receptors or artificial T cell receptors).

[0017] In another aspect, the invention provides a method of treating a subject that includes administering cells (e.g., a population of cells) prepared by a method of preparing cells described herein, for example, a method of any of the aforementioned aspects and embodiments of methods of preparing cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] Fig. 1 shows the extent of editing for samples from each of 4 donors (“018”, “100”, “315” and “797”) as measured by next generation (NGS) sequencing.

[0019] Figs. 2A and 2B show the extent of LAG3 protein expression on restimulated T-cells as measured by flow cytometry. The y-axis shows the percentage of LAG3 positive cells with the error bars showing the standard deviation (SD) of this measurement. FIG 2A shows the results for samples derived from donors “018” and “100”. FIG 2B shows the results for samples derived from donors “315” and “797.”

[0020] Fig. 3A shows the extent of editing in restimulated T-cells as measured by NGS sequencing. Fig. 3B shows the percent of LAG3 + cells as measured by flow cytometry with the error bars showing the SD of this measurement.

[0021] Fig. 4 shows a dose response curve of editing with LAG3 guide RNAs in T cells.

[0022] Fig. 5A shows stem cell memory T cells (Tscm) among CD8+ WT1 TCR expressing engineered cells.

[0023] Fig. 5B shows central memory T cells (Tcm) among CD8+ WT1 TCR expressing engineered cells

[0024] Fig. 5C shows effector memory T cells (Tem) among CD8+ WT1 TCR expressing engineered cells

[0025] Fig. 6A shows indel frequency as determined with a first primer set via NGS for the third sequential edit in engineered T cells.

[0026] Fig. 6B shows indel frequency as determined with a second, distinct primer set via NGS for the third sequential edit in engineered T cells.

[0027] Figs. 7A-7I show the mean image area fluorescing in both red and green after WT1 expressing AML cells are exposed to engineered T cells. Fig. 7A, Fig. 7B, and Fig. 7C show assays using an E:T of 5:1 with AML cell lines pAML1, pAML2 or pAML3, respectively. Fig. 7D, Fig. 7E, and Fig. 7F show assays using an E:T of 1:1 with AML cell lines pAML1, pAML2 or pAML3, respectively. Fig. 7G, Fig. 7F, and Fig. 7I show assays using an E:T of 1:5 with AML cell lines pAML1, pAML2 or pAML3, respectively.

DETAILED DESCRIPTION

[0028] Reference will now be made in detail to certain embodiments of the invention, examples of which are illustrated in the accompanying drawings. While the present teachings are described in conjunction with various embodiments, it is not intended to limit the present teachings to those embodiments. On the contrary, the present teaching encompass various alternatives, modifications, and equivalents, as will be appreciated by those of skill in the art.

[0029] 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 claims, 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 of cells (e.g., a population of cells) and the like.

[0030] 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. In some embodiments a population of cells refers to a population of at least 10^3 , 10^4 , 10^5 or 10^6 cells, preferably 10^7 , 2×10^7 , 5×10^7 , or 10^8 cells.

[0031] 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. 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).

[0032] The term “or” is used in an inclusive sense in the specification, i.e., equivalent to “and/or,” unless the context clearly indicates otherwise.

[0033] The term “about”, when used before a list, modifies each member of the list. The term “about” is understood to encompass tolerated variation or error within the art, e.g., 2 standard deviations from the mean, or the sensitivity of the method used to take a measurement. When “about” is present before the first value of a series, it can be understood to modify each value in the series.

[0034] Ranges are understood to include the numbers at the end of the range and all logical values therebetween. For example, 5-10 nucleotides is understood as 5, 6, 7, 8, 9, or 10 nucleotides, whereas 5-10% is understood to contain 5% and all possible values through 10%.

[0035] At least 17 nucleotides of a 20 nucleotide sequence is understood to include 17, 18, 19, or 20 nucleotides of the sequence provided, thereby providing an upper limit even if one is not specifically provided as it would be clearly understood. Similarly, up to 3 nucleotides would be understood to encompass 0, 1, 2, or 3 nucleotides, providing a lower limit even if one is not specifically provided. When “at least”, “up to”, or other similar language modifies a number, it can be understood to modify each number in the series.

[0036] 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.

[0037] As used herein, ranges include both the upper and lower limit.

[0038] 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.

[0039] In the event of a conflict between a chemical name and a structure, the structure predominates.

[0040] As used herein, “detecting an analyte” and the like is understood as performing an assay in which the analyte can be detected, if present, wherein the analyte is present in an amount above the level of detection of the assay.

[0041] 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.

[0042] 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

[0043] Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings:

[0044] “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 or 2' halide substitutions. An RNA may comprise one or more deoxyribose nucleotides, e.g. as modifications, and similarly a DNA may comprise one or more ribonucleotides. 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 nucleosides with 2' methoxy substituents, or polymers containing both conventional nucleosides and one or more nucleoside 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). RNA and DNA have different sugar moieties and can differ by the presence of uracil or analogs thereof in RNA and thymine or analogs thereof in DNA.

[0045] “Guide RNA”, “gRNA”, and simply “guide” are used herein interchangeably to refer to, for example, either a single guide RNA, or, for example, the combination of a crRNA and a trRNA (also known as tracrRNA). The crRNA and trRNA may be associated as a single RNA molecule (as a single guide RNA, sgRNA) or in two separate RNA strands (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.

[0046] As used herein, a “guide sequence” refers to a sequence within a guide RNA that is complementary to a target sequence and functions to direct a guide RNA to a target sequence for binding or modification (e.g., cleavage) by an RNA-guided DNA binding agent. A “guide sequence” may also be referred to as a “targeting sequence,” or a “spacer sequence.” A guide sequence can be 20 base pairs in length, e.g., in the case of *Streptococcus pyogenes* (i.e., Spy Cas9) 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. For example, in some embodiments, the guide sequence comprises at least 17, 18, 19, or 20 contiguous nucleotides of a sequence selected from SEQ ID NOs: 1–88. In some embodiments, the target sequence is in a gene 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 is at least 75%, 80%, 85%, 90%, or 95%, or is 100%. For example, in some embodiments, the guide sequence comprises a sequence with at least 75%, 80%, 85%, 90%, or 95%, or 100% identity to at least 17, 18, 19, or 20 contiguous nucleotides of a sequence selected from SEQ ID NOs: 1–88. 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, 3, or 4 mismatches, where the total length of the target sequence is 17, 18, 19, 20 nucleotides, or more. 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 nucleotides, or more. 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. That is, the guide sequence and the target region may form a duplex region having 17, 18, 19, 20 base pairs, or more. In certain embodiments, the duplex region may include 1, 2, 3, or 4 mismatches such that guide strand and target sequence are not fully complementary. For example, a guide strand and target sequence may be complementary over a 20 nucleotide region, including 2 mismatches, such that the guide sequence and target sequence are 90% complementary providing a duplex region of 18 base pairs out of 20.

[0047] 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 reverse complement of the sequence), 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 sense or antisense strand (e.g. 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.

[0048] 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. Exemplary RNA-guided DNA binding agents include Cas cleavases/nickases and inactivated forms thereof (“dCas DNA binding agents”). “Cas nuclease”, as used herein, encompasses Cas cleavases, Cas nickases, and dCas DNA binding agents. The dCas DNA binding agent may be a dead nuclease comprising non-functional nuclease domains (RuvC or HNH domain). In some embodiments the Cas cleavase or Cas nickase encompasses a dCas DNA binding agent modified to permit DNA cleavage, e.g. via fusion with a FokI domain. Cas cleavases/nickases and dCas DNA binding agents 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 cleavase/nickase activity is inactivated. 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), is homologous to Cas9, and 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).

[0049] 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, amino acid sequences, or nucleic acid sequences encoding the amino acid sequences provided herein are also contemplated.

[0050] Exemplary open reading frame for Cas9 (SEQ ID NO: 93)

[0051] AUGGACAAGAAGUACUCCAUCGGCCUGGACAUCGGCACCAACUCCGUGG
GCUGGGCCGUGAUCACCGACGAGUACAAGGUGCCCUCCAAGAAGUUAAGGUG
CUGGGCAACACCGACCGGCACUCCAUCAAGAAGAACCUGAUCGGCGCCCUGCU
GUUCGACUCCGGCGAGACCGCCGAGGCCACCCGGCUGAAGCGGACCGCCCGGC
GGCGGUACACCCGGCGGAAGAACCGGAUCUGCUACCUGCAGGAGAUCUUCUCC
AACGAGAUGGCCAAGGUGGACGACUCCUUCUUCACCGGCUGGAGGAGUCCU
CCUGGUGGAGGAGGACAAGAAGCAGAGCGGCACCCCAUCUUCGGCAACAUCG
UGGACGAGGUGGCCUACCACGAGAAGUACCCACCAUCUACCACCUGCGGAAG
AAGCUGGUGGACUCCACCGACAAGGCCGACCUGCGGCUGAUCUACCUGGCCCU
GGCCCACAUGAUCUAGUUCGGGGCCACUUCUGAUCGAGGGCGACCUGAACC
CCGACAACUCCGACGUGGACAAGCUGUUAUCCAGCUGGUGCAGACCUACAAC
CAGCUGUUCGAGGAGAACCCCAUCAACGCCUCCGGCGUGGACGCCAAGGCCAU

CCUGUCCGCCCCGGCUGUCCAAGUCCCCGGCGGCUGGAGAACCUGAUCGCCCAGC
UGCCCCGGCGAGAAGAAGAACGGCCUGUUCGGCAACCUGAUCGCCCUGUCCCUG
GGCCUGACCCCCAACUUAAGUCCAACUUCGACCUGGCCGAGGACGCCAAGCU
GCAGCUGUCCAAGGACACCUACGACGACGACCUGGACAACCUGCUGGCCCCAGA
UCGGCGACCAGUACGCCGACCUGUUCUGGCCGCCAAGAACCUGUCCGACGCC
AUCCUGCUGUCCGACAUCUCCUGCGGGUGAACACCGAGAUACCAAGGCCCCCU
GUCCGCCUCCAUGAUCAAGCGGUACGACGAGCACCACCAGGACCUGACCCUGC
UGAAGGCCCUGGUGCGGCAGCAGCUGCCCCGAGAAGUACAAGGAGAUCUUCUUC
GACCAGUCCAAGAACGGCUACGCCGGCUACAUCGACGGCGGGCGCCUCCAGGA
GGAGUUCUACAAGUUAUCAAGCCCAUCCUGGAGAAGAUGGACGGCACCGAGG
AGCUGCUGGUGAAGCUGAACCGGGAGGACCUGCUGCGGAAGCAGCGGACCUUC
GACAACGGCUCCAUCCCCCACCAGAUCCACCUGGGCGAGCUGCACGCCAUCCU
GCGGCGGCAGGAGGACUUCUACCCCUUCCUGAAGGACAACCGGGAGAAGAUCG
AGAAGAUCUCCUGACCUUCCGGAUCCCCUACUACGUGGGCCCCCUGGCCCGGGGC
AACUCCCGGUUCGCCUGGAUGACCCGGAAGUCCGAGGAGACCAUCACCCCCUG
GAACUUCGAGGAGGUGGUGGACAAGGGCGCCUCCGCCAGUCCUUAUCGAGC
GGAUGACCAACUUCGACAAGAACCUGCCCAACGAGAAGGUGCUGCCCAAGCAC
UCCUGCUGUACGAGUACUUCACCGUGUACAACGAGCUGACCAAGGUGAAGUA
CGUGACCGAGGGCAUGCGGAAGCCCGCCUUCUGUCCGGCGAGCAGAAGAAGG
CCAUCGUGGACCUGCUGUUAAGACCAACCGGAAGGUGACCGUGAAGCAGCUG
AAGGAGGACUACUUAAGAAGAUCGAGUGCUUCGACUCCGUGGAGAUCUCCG
GCGUGGAGGACCGGUUCAACGCCUCCCUGGGCACCUACCACGACCUGCUGAAG
AUCAUCAAGGACAAGGACUUCUGGACAACGAGGAGAACGAGGACAUCCUGG
AGGACAUCGUGCUGACCCUGACCCUGUUCGAGGACCGGGAGAUGAUCGAGGAG
CGGCUGAAGACCUACGCCACCUGUUCGACGACAAGGUGAUGAAGCAGCUGAA
GCGGCGGCGGUACACCGGCUGGGGCGGCUGUCCCGGAAGCUGAUCAACGGCA
UCCGGGACAAGCAGUCCGGCAAGACCAUCCUGGACUUCUGAAGUCCGACGGC
UUCGCCAACCGGAACUUAUGCAGCUGAUCCACGACGACUCCUGACCUUCAA
GGAGGACAUCAGAAAGGCCAGGUGUCCGGCCAGGGCGACUCCUGCAGCAGC
ACAUCGCCAACCGGGCCGCUCCCCGCCAUCAAGAAGGGCAUCCUGCAGACC
GUGAAGGUGGUGGACGAGCUGGUGAAGGUGAUGGGCCGGCACAAGCCCGAGA
ACAUCGUGAUCGAGAUGGCCCCGGGAGAACCAGACCACCAGAAGGGCCAGAAG
AACUCCCGGGAGCGGAUGAAGCGGAUCGAGGAGGGCAUCAAGGAGCUGGGCU
CCCAGAUCCUGAAGGAGCACCCCGUGGAGAACACCAGCUGCAGAACGAGAAG

CUGUACCUGUACUACCUGCAGAACGGCCGGGACAUGUACGUGGACCAGGAGCU
GGACAUCAACCGGCUGUCCGACUACGACGUGGACCACAUCGUGCCCCAGUCCU
UCCUGAAGGACGACUCCAUCGACAACAAGGUGCUGACCCGGUCCGACAAGAAC
CGGGGCAAGUCCGACAACGUGCCCUCGAGGAGGUGGUGAAGAAGAUGAAGA
ACUACUGGCGGCAGCUGCUGAACGCCAAGCUGAUCACCCAGCGGAAGUUCGAC
AACCUGACCAAGGCCGAGCGGGGCGGCCUGUCCGAGCUGGACAAGGCCGGCUU
CAUCAAGCGGCAGCUGGUGGAGACCCGGCAGAUACCAAGCACGUGGCCCAGA
UCCUGGACUCCCGGAUGAACACCAAGUACGACGAGAACGACAAGCUGAUCCGG
GAGGUGAAGGUGAUCACCCUGAAGUCCAAGCUGGUGUCCGACUUCCGGAAGG
ACUUCCAGUUCUACAAGGUGCGGGAGAUAACAACUACCACCACGCCCACGAC
GCCUACCUGAACGCCGUGGUGGGCACCGCCUGAUAAGAAGUACCCCAAGCU
GGAGUCCGAGUUCGUGUACGGCGACUACAAGGUGUACGACGUGCGGAAGAUG
AUCGCCAAGUCCGAGCAGGAGAUCCGGCAAGGCCACCGCCAAGUACUUCUUCUA
CUCCAACAUAUGAACUUCUUAAGACCGAGAUAACCCUGGCCAACGGCGAGA
UCCGGAAGCGGCCCCUGAUCGAGACCAACGGCGAGACCGGCGAGAUUGUGUGG
GACAAGGGCCGGGACUUCGCCACCGUGCGGAAGGUGCUGUCCAUGCCCCAGGU
GAACAUCGUGAAGAAGACCGAGGUGCAGACCGGCGGCUUCUCCAAGGAGUCCA
UCCUGCCCAAGCGGAACUCCGACAAGCUGAUCGCCCCGGAAGAAGGACUGGGAC
CCCAAGAAGUACGGCGGCUUCGACUCCCCACCGUGGCCUACUCCGUGCUGGU
GGUGGCCAAGGUGGAGAAGGGCAAGUCCAAGAAGCUGAAGUCCGUGAAGGAG
CUGCUGGGCAUCACCAUCAUGGAGCGGUCCUCCUUCGAGAAGAACCCCAUCGA
CUUCCUGGAGGCCAAGGGCUACAAGGAGGUGAAGAAGGACCUGAUAUCAAG
CUGCCCAAGUACUCCUGUUCGAGCUGGAGAACGGCCGGAAGCGGAUGCUGGC
CUCCGCCGGCGAGCUGCAGAAGGGCAACGAGCUGGCCCUGCCUCCAAGUACG
UGAACUUCCUGUACCUGGCCUCCCACUACGAGAAGCUGAAGGGCUCCCCGAG
GACAACGAGCAGAAGCAGCUGUUCGUGGAGCAGCACAAGCACUACCUGGACGA
GAUCAUCGAGCAGAUCUCCGAGUUCUCCAAGCGGGUGAUCUCCUGGCCGACGCCA
ACCUGGACAAGGUGCUGUCCGCCUACAACAAGCACCGGGACAAGCCCAUCCGG
GAGCAGGCCGAGAACAUAUCCACCUGUUCACCCUGACCAACCUGGGCGCCCC
CGCCGCCUUAAGUACUUCGACACCACCAUCGACCGGAAGCGGUACACCUCCA
CCAAGGAGGUGCUGGACGCCACCCUGAUCCACCAGUCCAUCACCGGCCUGUAC
GAGACCCGGAUCGACCUGUCCAGCUGGGCGGCGACGGCGGCGGCUCCCCCAA
GAAGAAGCGGAAGGUGUGA

[0052] Exemplary amino acid sequence for Cas9 (SEQ ID NO: 94)

[0053] MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLF
 DSGETAETRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHRLEESFLVEED
 KKHHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRILIYALAHMIKFRGH
 FLIEGDLNPDNSDVKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLI
 AQLPGEKKNGLFGNLIASLGLTPNFKSNFDLAEDAKLQLSKDQYDDDLDNLLAQIG
 DQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQ
 QLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLL
 RKQRTFDNGSIPHQIHLGELHAILRRQEDFYFPLKDNREKIEKILTRIPYYVGPLARG
 NSRFAWMTRKSEETITPWNFEVVVDKGASAQSFIERMTNFDKNLPNEKVLPHKSHLLY
 EYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKK
 IECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREM
 IEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGF
 ANRNFQMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVD
 ELVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVE
 NTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVHDHVPQSFLKDDSIDNKVLT
 RSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDK
 AGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSCLVSDFRKDFQ
 FYKVVREINNYHHAHDAYLNAVVGTAIIKKYPKLESEFVYGDYKVYDVRKMIKSE
 QEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVR
 KVLSPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPPKKYGGFDSPTVAY
 SVLVVAKVEKGKSKKLKSVKELLGITIMERSSEFKNPIDFLEAKGYKEVKKDLIIKLP
 KYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDEQK
 QLFVEQHKHYLDEIIEQISEFSKRVLADANLDKVLSAYNKHARDKPIREQAENIIHLFT
 LTNLGAPAAFKYFDTTIDRKRYTSTKEVLDTLHQISITGLYETRIDLSQLGGDGGGS
 PKKKRKV

[0054] Exemplary open reading frame for Cas9 (SEQ ID NO: 95)

[0055] AUGGACAAGAAGUACAGCAUCGGACUGGACAUCGGAACAAACAGCGUC
 GGAUGGGCAGUCAUCACAGACGAAUACAAGGUCCCGAGCAAGAAGUUCAAGG
 UCCUGGGAAACACAGACAGACACAGCAUCAAGAAGAACCUGAUCGGAGCACUG
 CUGUUCGACAGCGGAGAAACAGCAGAAGCAACAAGACUGAAGAGAACAGCAA
 GAAGAAGAUACACAAGAAGAAAGAACAGAAUCUGCUACCUGCAGGAAAUCUU
 CAGCAACGAAAUGGCAAAGGUCGACGACAGCUUCUCCACAGACUGGAAGAAA
 GCUUCCUGGUCGAAGAAGACAAGAAGCACGAAAGACACCCGAUCUUCGGAAAC
 AUCGUCGACGAAGUCGAUACCACGAAAAGUACCCGACAAUCUACCACCUGAG

AAAGAAGCUGGUCGACAGCACAGACAAGGCAGACCUGAGACUGAUCUACCUGG
CACUGGCACACAUGAUCAAGUUCAGAGGACACUUCCUGAUCGAAGGAGACCUG
AACCCGGACAACAGCGACGUCGACAAGCUGUUCAUCCAGCUGGUCCAGACAU
CAACCAGCUGUUCGAAGAAAACCCGAUCAACGCAAGCGGAGUCGACGCAAAGG
CAAUCCUGAGCGCAAGACUGAGCAAGAGCAGAAGACUGGAAAACCUGAUCGCA
CAGCUGCCGGGAGAAAAGAAGAACGGACUGUUCGGAAACCUGAUCGCACUGA
GCCUGGGACUGACACCGAACUUCAAGAGCAACUUCGACCUGGCAGAAGACGCA
AAGCUGCAGCUGAGCAAGGACACAUAACGACGACGACCUGGACAACCUGCUGGC
ACAGAUCGGAGACCAGUACGCAGACCUGUUCCUGGCAGCAAAGAACCUGAGCG
ACGCAAUCCUGCUGAGCGACAUCCUGAGAGUCAACACAGAAAUCACAAAGGCA
CCGCUGAGCGCAAGCAUGAUCAAGAGAUACGACGAACACCACCAGGACCUGAC
ACUGCUGAAGGCACUGGUCAGACAGCAGCUGCCGGAAAAGUACAAGGAAAUC
UUCUUCGACCAGAGCAAGAACGGAUACGCAGGAUACAUCGACGGAGGAGCAA
GCCAGGAAGAAUUCUACAAGUUCAUCAAGCCGAUCCUGGAAAAGAUGGACGG
AACAGAAGAACUGCUGGUCAAGCUGAACAGAGAAGACCUGCUGAGAAAGCAG
AGAACAUUCGACAACGGGAAGCAUCCCGCACCAGAUCCACCUGGGAGAACUGCA
CGCAAUCCUGAGAAGACAGGAAGACUUCUACCCGUUCCUGAAGGACAACAGAG
AAAAGAUCGAAAAGAUAUCCUGACAUAUCAGAAUCCCGUACUACGUCGGACCGCUG
GCAAGAGGAAACAGCAGAUUCGCAUGGAUGACAAGAAAGAGCGAAGAAACAA
UCACACCGUGGAACUUCGAAGAAGUCGUCGACAAGGGAGCAAGCGCACAGAGC
UUCAUCGAAAGAAUGACAAACUUCGACAAGAACCUGCCGAACGAAAAGGUCCU
GCCGAAGCACAGCCUGCUGUACGAAUACUUCACAGUCUACAACGAACUGACAA
AGGUCAAGUACGUCACAGAAGGAAUGAGAAAGCCGGCAUUCCUGAGCGGAGA
ACAGAAGAAGGCAAUCGUCGACCUGCUGUUCAAGACAAACAGAAAGGUCACA
GUCAAGCAGCUGAAGGAAGACUACUUCAAGAAGAUCGAAUGCUUCGACAGCG
UCGAAAUCAGCGGAGUCGAAGACAGAUUCAACGCAAGCCUGGGAACAUACCAC
GACCUGCUGAAGAUCAUCAAGGACAAGGACUUCCUGGACAACGAAGAAAACG
AAGACAUCCUGGAAGACAUCGUCCUGACACUGACACUGUUCGAAGACAGAGAA
AUGAUCGAAGAAAGACUGAAGACAUAACGCACACCUGUUCGACGACAAGGUCA
UGAAGCAGCUGAAGAGAAGAAGAUACACAGGAUGGGGAAGACUGAGCAGAAA
GCUGAUAACGGAAUCAGAGACAAGCAGAGCGGAAAGACAAUCCUGGACUUCC
UGAAGAGCGACGGAUUCGCAAACAGAAACUUCAUGCAGCUGAUCCACGACGAC
AGCCUGACAUAUCAAGGAAGACAUCCAGAAGGCACAGGUCAGCGGACAGGGAG
ACAGCCUGCACGAACACAUCGCAAACCUGGCAGGAAGCCCGGCAAUCAAGAAG

GGAAUCCUGCAGACAGUCAAGGUCGUCGACGAACUGGUCAAGGUCAUGGGAA
GACACAAGCCGGAAAACAUCGUCAUCGAAAUGGCAAGAGAAAACCAGACAACA
CAGAAGGGACAGAAGAACAGCAGAGAAAGAAUGAAGAGAAUCGAAGAAGGAA
UCAAGGAACUGGGAAGCCAGA UCCUGAAGGAACACCCGGUCGAAAACACACAG
CUGCAGAACGAAAAGCUGUACCUGUACUACCUGCAGAACGGAAGAGACAUGU
ACGUCGACCAGGAACUGGACAUCAACAGACUGAGCGACUACGACGUCGACCAC
AUCGUCCCGCAGAGCUUCCUGAAGGACGACAGCAUCGACAACAAGGUCCUGAC
AAGAAGCGACAAGAACAGAGGAAAGAGCGACAACGUCCCGAGCGAAGAAGUC
GUCAAGAAGAUGAAGAACUACUGGAGACAGCUGCUGAACGCAAAGCUGAUCA
CACAGAGAAAGUUCGACAACCUGACAAAGGCAGAGAGAGGAGGACUGAGCGA
ACUGGACAAGGCAGGAUUCAUCAAGAGACAGCUGGUCGAAACAAGACAGAU
ACAAAGCACGUCGCACAGAUCCUGGACAGCAGAAUGAACACAAAGUACGACGA
AAACGACAAGCUGAUCAGAGAAGUCAAGGUCAUCACACUGAAGAGCAAGCUG
GUCAGCGACUUCAGAAAGGACUCCAGUUCUACAAGGUCAGAGAAAUCAACA
ACUACCACCACGCACACGACGCAUACCUGAACGCAGUCGUCGGAACAGCACUG
AUCAAGAAGUACCCGAAGCUGGAAAGCGAAUUCGUCUACGGAGACUACAAGG
UCUACGACGUCAGAAAGAUGAUCGCAAAGAGCGAACAGGAAAUCGGAAAGGC
AACAGCAAAGUACUUCUUCUACAGCAACAUCAUGAACUUCUUAAGACAGAAA
UCACACUGGCAAACGGAGAAAUCAGAAAGAGACCGCUGAUCGAAACAAACGG
AGAAACAGGAGAAAUCGUCUGGGACAAGGGAAGAGACUUCGCAACAGUCAGA
AAGGUCCUGAGCAUGCCGCAGGUCAACAUCGUCAAGAAGACAGAAGUCCAGAC
AGGAGGAUUCAGCAAGGAAAGCAUCCUGCCGAAGAGAAACAGCGACAAGCUG
AUCGCAAGAAAGAAGGACUGGGACCCGAAGAAGUACGGAGGAUUCGACAGCC
CGACAGUCGCAUACAGCGUCCUGGUCGUCGCAAAGGUCGAAAAGGGAAAGAGC
AAGAAGCUGAAGAGCGUCAAGGAACUGCUGGGAAUCACAAUCAUGGAAAGAA
GCAGCUUCGAAAAGAACCCGAUCGACUUCUGGAAGCAAAGGGGAUACAAGGA
AGUCAAGAAGGACCUGAUCAUCAAGCUGCCGAAGUACAGCCUGUUCGAACUGG
AAAACGGAAGAAAGAGAAUGCUGGCAAGCGCAGGAGAACUGCAGAAGGGAAA
CGAACUGGCACUGCCGAGCAAGUACGUCAACUUCUGUACCUGGCAAGCCACU
ACGAAAAGCUGAAGGGAAGCCCGGAAGACAACGAACAGAAGCAGCUGUUCGU
CGAACAGCACAAAGCACUACCUGGACGAAAUCAUCGAACAGAUCAGCGAAUUCA
GCAAGAGAGUCAUCCUGGCAGACGCAAACCUGGACAAGGUCCUGAGCGCAUAC
AACAAAGCACAGAGACAAGCCGAUCAGAGAACAGGCAGAAAACAUCAUCCACCU
GUUCACACUGACAAACCUGGGAGCACCGGCAGCAUUCAAGUACUUCGACACAA

CAAUCGACAGAAAGAGAUACACAAGCACAAAGGAAGUCCUGGACGCAACACUG
 AUCCACCAGAGCAUCACAGGACUGUACGAAACAAGAAUCGACCUGAGCCAGCU
 GGGAGGAGACGGAGGAGGAAGCCCGAAGAAGAAGAGAAAGGUCUAG

[0056] 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 cleaving or nicking.

[0057] 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, i.e., that is sufficiently complementary to the guide sequence to permit specific binding of the guide 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.

[0058] As used herein, a first sequence is considered to be “identical” or have “100% identity” with a second sequence if an alignment of the first sequence to the second sequence shows that all of the positions of the second sequence in its entirety are matched by the first sequence. For example, the sequence AAG has 100% identity to the sequence AAGA because an alignment would give 100% identity in that there are matches, without gaps, to all three positions of the first sequence. Less than 100% identity can be calculated using routine methods. For example ACG would have 67% identity with AAGA as two of the three positions of the first sequence are matches to the second sequence ($2/3 = 67\%$). 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.

[0059] Similarly, as used herein, a first sequence is considered to be “fully complementary” or 100% complementary” to a second sequence when all of the nucleotides of a first sequence are complementary to a second sequence, without gaps. For example, the sequence UCU would be considered to be fully complementary to the sequence AAGA as each of the nucleobases from the first sequence basepair with the nucleotides of the second sequence, without gaps. The sequence UGU would be considered to be 67% complementary to the sequence AAGA as two of the three nucleobases of the first sequence basepair with nucleobases of the second sequence. One skilled in the art will understand that algorithms are available with various parameter settings to determine percent complementarity for any pair of sequences using, e.g., the NCBI BLAST interface (blast.ncbi.nlm.nih.gov/Blast.cgi) or the Needleman-Wunsch algorithm.

[0060] “mRNA” is used herein to refer to a polynucleotide that 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.

[0061] Exemplary guide sequences useful in the guide RNA compositions and methods described herein are shown in Table 1 and throughout the application. For example, where Table 1 shows a guide sequence, this guide sequence may be used in a guide RNA to direct a RNA-guided DNA binding agent, e.g., a nuclease, such as a Cas nuclease, such as Cas9, to a target sequence. Target sequences are provided in Table 1 as genomic coordinates, and include both the positive and negative strands of genomic DNA (i.e., the sequence given and the sequence's reverse complement). 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.

[0062] As used herein, “indels” refer to insertion/deletion mutations consisting of a number of nucleotides that are either inserted or deleted at the site of double-stranded breaks (DSBs) in a target nucleic acid.

[0063] As used herein, “inhibit expression” and the like refer to a decrease in expression of a particular gene product (e.g., protein, mRNA, or both). Expression of a protein (i.e., gene product) can be measured by detecting total cellular amount of the protein from a tissue or cell population of interest by detecting expression of a protein as individual members of a population of cells, e.g., by cell sorting to define percent of cells expressing a protein, or expression of a protein in cells in aggregate, e.g., by ELISA or western blot. Inhibition of expression can result from genetic modification of a gene sequence, e.g., a genomic sequence, such that the full-length gene product, or any gene product, is no longer expressed, e.g. knockdown of the gene. Certain genetic modifications can result in the introduction of frameshift or nonsense mutations that prevent translation of the full-length gene product. Genetic modifications at a splice site, e.g., at a position sufficiently close to a splice acceptor site or a splice donor site to disrupt splicing, can prevent translation of the full-length protein. Inhibition of expression can result from a genetic modification in a regulatory sequence within the genomic sequence required for the expression of the gene product, e.g., a promoter sequence, a 3' UTR sequence, e.g., a capping sequence, a 5' UTR sequence, e.g., a poly A sequence. Inhibition of expression may also result from disrupting expression or activity of regulatory factors required for translation of the gene product, e.g., production of no gene product. For example, a genetic modification in a transcription factor sequence, inhibiting expression of the full-length transcription factor, can have downstream effects and inhibit expression of the expression of one or more gene products controlled by the transcription factor. Therefore, inhibition of expression can be predicted by changes in genomic or mRNA sequences. Therefore, mutations expected to result in inhibition of expression can be detected by known methods including sequencing of mRNA isolated from a tissue or cell population of interest. Inhibition of expression can be determined as the percent of cells in a population having a predetermined level of expression of a protein, i.e., a reduction of the percent or number of cells in a population expressing a protein of interest at at least a certain level. Inhibition of expression can also be assessed by determining a decrease in overall protein level, e.g., in a cell or tissue sample, e.g., a biopsy sample. In certain embodiments, inhibition of expression of a secreted protein can be assessed in a fluid sample, e.g., cell culture media or a body fluid. Proteins may be present in a body fluid, e.g., blood or urine, to permit analysis of protein level. In certain embodiments, protein level may be determined by

protein activity or the level of a metabolic product, e.g., in urine or blood. In some embodiments, “inhibition of expression” may refer to some loss of expression of a particular gene product, for example a decrease in the amount of mRNA transcribed or a decrease in the amount of protein expressed by a population of cells. In some embodiments, “inhibition” may refer to some loss of expression of a particular gene product, for example a *LAG3* gene product at the cell surface. It is understood that the level of knockdown is relative to a starting level in the same type of subject sample. For example, routine monitoring of a protein level is more easily performed in a fluid sample from a subject, e.g., blood or urine, than in a tissue sample, e.g., a biopsy sample. It is understood that the level of knockdown is for the sample being assayed. Similarly, in animal studies where serial tissue samples may be obtained, e.g., liver tissue, the knockdown target may be expressed in other tissues. Therefore, the level of knockdown is not necessarily the level of knockdown systemically, but within the tissue, cell type, or fluid being sampled.

[0064] As used herein, a “genetic modification” is a change at the DNA level, e.g. induced by a CRISPR/Cas9 gRNA and Cas9 system. A genetic modification may comprise an insertion, deletion, or substitution (i.e., base sequence substitution, i.e., mutation), typically within a defined sequence or genomic locus. A genetic modification changes the nucleic acid sequence of the DNA. A genetic modification may be at a single nucleotide position. A genetic modification may be at multiple nucleotides, e.g., 2, 3, 4, 5 or more nucleotides, typically in close proximity to each other, e.g, contiguous nucleotides. A genetic modification can be in a coding sequence, e.g., an exon sequence. A genetic modification can be at a splice site, i.e., sufficiently close to a splice acceptor site or a splice donor site to disrupt splicing. A genetic modification can include insertion of a nucleotide sequence not endogenous to the genomic locus, e.g., insertion of a coding sequence of a heterologous open reading frame or gene. As used herein, preferably a genetic modification prevents translation of a full-length protein having an amino acid sequence of the full-length protein prior to genetic modification of the genomic locus. Prevention of translation of a full-length protein or gene product includes prevention of translation of a protein or gene product of any length. Translation of a full-length protein can be prevented, for example, by a frameshift mutation that results in the generation of a premature stop codon or by generation of a nonsense mutation. Translation of a full-length protein can be prevented by disruption of splicing.

[0065] As used herein, a “heterologous coding sequence” refers to a coding sequence 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 coding

sequence is heterologous with respect to at least its insertion site. A polypeptide expressed from such heterologous coding sequence gene is referred to as a “heterologous polypeptide.” The heterologous coding sequence can be naturally-occurring or engineered, and can be wild-type or a variant. The heterologous coding sequence may include nucleotide sequences other than the sequence that encodes the heterologous polypeptide (*e.g.*, an internal ribosomal entry site). The heterologous coding sequence can be a coding sequence that occurs naturally in the genome, as a wild-type or a variant (*e.g.*, mutant). For example, although the cell contains the coding sequence of interest (as a wild-type or as a variant), the same coding sequence or variant thereof can be introduced as an exogenous source for, *e.g.*, expression at a locus that is highly expressed. The heterologous coding sequence can also be a coding sequence that is not naturally occurring in the genome, or that expresses a heterologous polypeptide that does not naturally occur in the genome. “Heterologous coding sequence”, “exogenous coding sequence”, and “transgene” are used interchangeably. In some embodiments, the heterologous coding sequence 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 coding sequence 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 coding sequence may be heterologous with respect to its insertion site and with respect to its recipient cell.

[0066] 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. In some embodiments, insertions at a locus or loci targeted for knockdown such as a TRC gene, *e.g.*, TRAC gene, is advantageous for cells. Other suitable safe harbor loci are known in the art.

[0067] 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.

[0068] 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.

[0069] 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., the standard of care for the indication to be treated.

[0070] The human wild-type *LAG3* sequence is available at NCBI Gene ID: 3902 (www.ncbi.nlm.nih.gov/gene/3902, in the version available on the date of filing the instant application); Ensembl:ENSG00000089692, chr12:6772483-6778455. Lymphocyte-activation protein 3 belongs to Ig superfamily and contains 4 extracellular Ig-like domains. The *LAG3* gene contains 8 exons. The sequence data, exon/intron organization, and chromosomal localization all indicate a close relationship of *LAG3* to CD4. Lymphocyte activating 3, CD223, and FDC4 are gene synonyms for *LAG3*.

[0071] 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.

[0072] “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.

[0073] “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.

[0074] 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.

[0075] 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.

[0076] 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⁺.

[0077] As used herein, “MHC” or “MHC protein” refers to a major histocompatibility complex molecule (or plural), and includes *e.g.*, MHC class I molecules (*e.g.*, HLA-A, HLA-B, and HLA-C in humans) and MHC class II molecules (*e.g.*, HLA-DP, HLA-DQ, and HLA-DR in humans).

[0078] “*CIITA*” or “CIITA” or “C2TA,” as used herein, refers to the nucleic acid sequence or protein sequence of “class II major histocompatibility complex transactivator;” the human gene has accession number NC_000016.10 (range 10866208..10941562), reference GRCh38.p13. The CIITA protein in the nucleus acts as a positive regulator of MHC class II gene transcription and is required for MHC class II protein expression.

[0079] “ β 2M” or “B2M,” as used herein, refers to nucleic acid sequence or protein sequence of “ β -2 microglobulin;” the human gene has accession number NC_000015 (range 44711492..44718877), reference GRCh38.p13. The B2M protein is associated with MHC

class I molecules as a heterodimer on the surface of nucleated cells and is required for MHC class I protein expression.

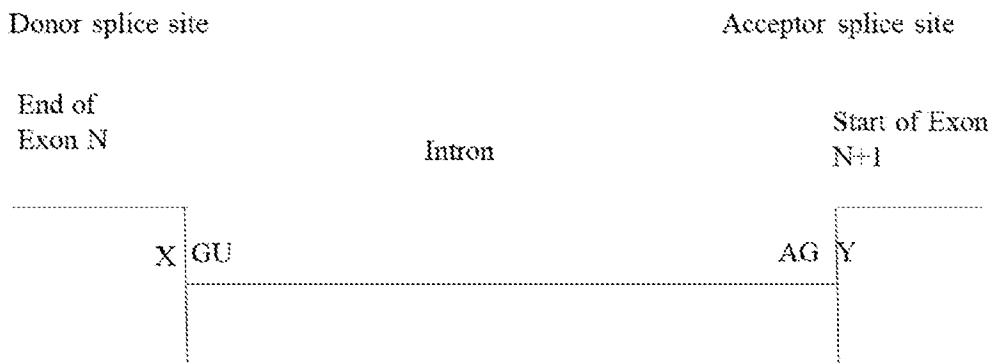
[0080] The term “HLA-A,” as used herein in the context of HLA-A protein, refers to the MHC class I protein molecule, which is a heterodimer consisting of a heavy chain (encoded by the HLA-A gene) and a light chain (*i.e.*, beta-2 microglobulin). The term “HLA-A” or “HLA-A gene,” as used herein in the context of nucleic acids refers to the gene encoding the heavy chain of the HLA-A protein molecule. The HLA-A gene is also referred to as “HLA class I histocompatibility, A alpha chain;” the human gene has accession number NC_000006.12 (29942532..29945870). The HLA-A gene is known to have thousands of different versions (also referred to as “alleles”) across the population (and an individual may receive two different alleles of the HLA-A gene). A public database for HLA-A alleles, including sequence information, may be accessed at IPD-IMGT/HLA: www.ebi.ac.uk/ipd/imgt/hla/. All alleles of HLA-A are encompassed by the terms “HLA-A” and “HLA-A gene.”

[0081] As used herein, the term “within the genomic coordinates” includes the boundaries of the genomic coordinate range given. For example, if chr6:29942854-chr6:29942913 is given, the coordinates chr6:29942854- chr6:29942913 are encompassed. Throughout this application, the referenced genomic coordinates are based on genomic annotations in the GRCh38 (also referred to as hg38) assembly of the human genome from the Genome Reference Consortium, available at the National Center for Biotechnology Information website. Tools and methods for converting genomic coordinates between one assembly and another are known in the art and can be used to convert the genomic coordinates provided herein to the corresponding coordinates in another assembly of the human genome, including conversion to an earlier assembly generated by the same institution or using the same algorithm (e.g., from GRCh38 to GRCh37), and conversion of an assembly generated by a different institution or algorithm (e.g., from GRCh38 to NCBI33, generated by the International Human Genome Sequencing Consortium). Available methods and tools known in the art include, but are not limited to, NCBI Genome Remapping Service, available at the National Center for Biotechnology Information website, UCSC LiftOver, available at the UCSC Genome Browser website, and Assembly Converter, available at the Ensembl.org website.

[0082] A “splice site,” as used herein, refers to the three nucleotides that make up an acceptor splice site or a donor splice site (defined below), or any other nucleotides known in the art that are part of a splice site. *See e.g.*, Burset et al., Nucleic Acids Research

28(21):4364-4375 (2000) (describing canonical and non-canonical splice sites in mammalian genomes). The three nucleotides that make up an “acceptor splice site” are two conserved residues (*e.g.*, AG in humans) at the 3’ of an intron and a boundary nucleotide (*i.e.*, the first nucleotide of the exon 3’ of the AG). The “splice site boundary nucleotide” of an acceptor splice site is designated as “Y” in the diagram below and may also be referred to herein as the “acceptor splice site boundary nucleotide,” or “splice acceptor site boundary nucleotide.” The terms “acceptor splice site,” “splice acceptor site,” “acceptor splice sequence,” or “splice acceptor sequence” may be used interchangeably herein.

[0083] The three nucleotides that make up a “donor splice site” are two conserved residues (*e.g.*, GT (gene) or GU (in RNA such as pre-mRNA) in human) at the 5’ end of an intron and a boundary nucleotide (*i.e.*, the first nucleotide of the exon 5’ of the GT). The “splice site boundary nucleotide” of a donor splice site is designated as “X” in the diagram below and may also be referred to herein as the “donor splice site boundary nucleotide,” or “splice donor site boundary nucleotide.” The terms “donor splice site,” “splice donor site,” “donor splice sequence,” or “splice donor sequence” may be used interchangeably herein.



II. Compositions

A. Compositions Comprising Guide RNA (gRNAs)

[0084] Provided herein are compositions useful for altering a DNA sequence, e.g., inducing a single-stranded (SSB) or double-stranded break (DSB), within a *LAG3* gene, e.g., using a guide RNA with an RNA-guided DNA binding agent (e.g., a CRISPR/Cas system). Guide sequences targeting a *LAG3* gene are shown in Table 1 at SEQ ID NOs: 1–88, as are the genomic coordinates that such guide RNA targets.

[0085] Each of the guide sequences shown in Table 1 at SEQ ID NOs: 1–88 may further comprise additional nucleotides to form a crRNA, e.g., with the following exemplary

nucleotide sequence following the guide sequence at its 3' end:

GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU

[0086] 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:

GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU
GAAAAAGUGGCACCGAGUCGGUGC (SEQ ID NO: 201) in 5' to 3' orientation.

[0087] 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:

GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU
GAAAAAGUGGCACCGAGUCGGUGCUUUU (SEQ ID NO: 202) in 5' to 3' orientation.

[0088] In the case of a sgRNA, the guide sequences may be integrated into the following modified motif.

mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUAGAmGmCmUmAmGmAmAmAmU
mAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAm
AmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
(SEQ ID NO: 300), where "N" may be any natural or non-natural nucleotide, preferably an RNA nucleotide; sugar moieties of the nucleotide can be ribose, deoxyribose, or similar compounds with substitutions; m is a 2'-O-methyl modified nucleotide, and * is a phosphorothioate linkage between nucleotide residues; and wherein the N's are collectively the nucleotide sequence of a guide sequence.

[0089] In the case of a sgRNA, the guide sequences may further comprise a SpyCas9 sgRNA sequence. An example of a SpyCas9 sgRNA sequence is shown in the table below (SEQ ID NO: 201)

GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU
GAAAAAGUGGCACCGAGUCGGUGC "Exemplary SpyCas9 sgRNA-1"), included at the 3' end of the guide sequence, and provided with the domains as shown in the table below. LS is lower stem. B is bulge. US is upper stem. H1 and H2 are hairpin 1 and hairpin 2, respectively. Collectively H1 and H2 are referred to as the hairpin region. A model of the structure is provided in Figure 10A of WO2019237069 which is incorporated herein by reference.

[0090] The nucleotide sequence of Exemplary SpyCas9 sgRNA-1 may serve as a template sequence for specific chemical modifications, sequence substitutions and truncations.

[0091] In certain embodiments, the gRNA is an sgRNA or a dgRNA, for example, and it optionally comprises a chemical modification. In some embodiments, the modified sgRNA comprises a guide sequence and a SpyCas9 sgRNA sequence, e.g., Exemplary SpyCas9 sgRNA-1. A gRNA, such as an sgRNA, may include modifications on the 5' end of the guide sequence or on the 3' end of the guide sequence, such as e.g., Exemplary SpyCas9 sgRNA-1 at one or more of the terminal nucleotides, e.g., at 1, 2, 3, or 4 of the nucleotides at the 3' end or at the 5' end. In certain embodiments, the modified nucleotide is selected from a 2'-O-methyl (2'-OMe) modified nucleotide, a 2'-O-(2-methoxyethyl) (2'-O-moe) modified nucleotide, a 2'-fluoro (2'-F) modified nucleotide, a phosphorothioate (PS) linkage between nucleotides, an inverted abasic modified nucleotide, or a combination thereof. In certain embodiments, the modified nucleotide includes a 2'-OMe modified nucleotide. In certain embodiments, the modified nucleotide includes a PS linkage. In certain embodiments, the modified nucleotide includes a 2'-OMe modified nucleotide and a PS linkage.

[0092] In certain embodiments, using (SEQ ID NO: 201, "Exemplary SpyCas9 sgRNA-1") as an example, the Exemplary SpyCas9 sgRNA-1 further includes one or more of:

[0093] A. a shortened hairpin 1 region, or a substituted and optionally shortened hairpin 1 region, wherein

1. at least one of the following pairs of nucleotides are substituted in hairpin 1 with Watson-Crick pairing nucleotides: H1-1 and H1-12, H1-2 and H1-11, H1-3 and H1-10, or H1-4 and H1-9, and the hairpin 1 region optionally lacks
 - a. any one or two of H1-5 through H1-8,
 - b. one, two, or three of the following pairs of nucleotides: H1-1 and H1-12, H1-2 and H1-11, H1-3 and H1-10, and H1-4 and H1-9, or
 - c. 1-8 nucleotides of hairpin 1 region; or
2. the shortened hairpin 1 region lacks 4-8 nucleotides, preferably 4-6 nucleotides; and
 - a. one or more of positions H1-1, H1-2, or H1-3 is deleted or substituted relative to Exemplary SpyCas9 sgRNA-1 or
 - b. one or more of positions H1-6 through H1-10 is substituted relative to Exemplary SpyCas9 sgRNA-1; or

3. the shortened hairpin 1 region lacks 5-10 nucleotides, preferably 5-6 nucleotides, and one or more of positions N18, H1-12, or n is substituted relative to Exemplary SpyCas9 sgRNA-1; or

[0094] B. a shortened upper stem region, wherein the shortened upper stem region lacks 1-6 nucleotides and wherein the 6, 7, 8, 9, 10, or 11 nucleotides of the shortened upper stem region include less than or equal to 4 substitutions relative to Exemplary SpyCas9 sgRNA-1; or

[0095] C. a substitution relative to Exemplary SpyCas9 sgRNA-1 at any one or more of LS6, LS7, US3, US10, B3, N7, N15, N17, H2-2 and H2-14, wherein the substituent nucleotide is neither a pyrimidine that is followed by an adenine, nor an adenine that is preceded by a pyrimidine; or

[0096] D. an Exemplary SpyCas9 sgRNA-1 with an upper stem region, wherein the upper stem modification comprises a modification to any one or more of US1-US12 in the upper stem region, wherein

1. the modified nucleotide is optionally selected from a 2'-O-methyl (2'-OMe) modified nucleotide, a 2'-O-(2-methoxyethyl) (2'-O-moe) modified nucleotide, a 2'-fluoro (2'-F) modified nucleotide, a phosphorothioate (PS) linkage between nucleotides, an inverted abasic modified nucleotide, or a combination thereof; or
2. the modified nucleotide optionally includes a 2'-OMe modified nucleotide.

[0097] In certain embodiments, Exemplary SpyCas9 sgRNA-1 (SEQ ID NO: 201), or an sgRNA, such as an sgRNA comprising an Exemplary SpyCas9 sgRNA-1, further includes a 3' tail, e.g., a 3' tail of 1, 2, 3, 4, or more nucleotides. In certain embodiments, the tail includes one or more modified nucleotides. In certain embodiments, the modified nucleotide is selected from a 2'-O-methyl (2'-OMe) modified nucleotide, a 2'-O-(2-methoxyethyl) (2'-O-moe) modified nucleotide, a 2'-fluoro (2'-F) modified nucleotide, a phosphorothioate (PS) linkage between nucleotides, an inverted abasic modified nucleotide; or a combination thereof. In certain embodiments, the modified nucleotide includes a 2'-OMe modified nucleotide. In certain embodiments, the modified nucleotide includes a PS linkage between nucleotides. In certain embodiments, the modified nucleotide includes a 2'-OMe modified nucleotide and a PS linkage between nucleotides.

[0098] In certain embodiments, the hairpin region includes one or more modified nucleotides. In certain embodiments, the modified nucleotide is selected from a 2'-O-methyl

(2'-OMe) modified nucleotide, a 2'-O-(2-methoxyethyl) (2'-O-moe) modified nucleotide, a 2'-fluoro (2'-F) modified nucleotide, a phosphorothioate (PS) linkage between nucleotides, an inverted abasic modified nucleotide; or a combination thereof. In certain embodiments, the modified nucleotide includes a 2'-OMe modified nucleotide.

[0099] In certain embodiments, the upper stem region includes one or more modified nucleotides. In certain embodiments, the modified nucleotide selected from a 2'-O-methyl (2'-OMe) modified nucleotide, a 2'-O-(2-methoxyethyl) (2'-O-moe) modified nucleotide, a 2'-fluoro (2'-F) modified nucleotide, a phosphorothioate (PS) linkage between nucleotides, an inverted abasic modified nucleotide; or a combination thereof. In certain embodiments, the modified nucleotide includes a 2'-OMe modified nucleotide.

[00100] In certain embodiments, the Exemplary SpyCas9 sgRNA-1 comprises one or more YA dinucleotides, wherein Y is a pyrimidine, wherein the YA dinucleotide includes a modified nucleotide. In certain embodiments, the modified nucleotide selected from a 2'-O-methyl (2'-OMe) modified nucleotide, a 2'-O-(2-methoxyethyl) (2'-O-moe) modified nucleotide, a 2'-fluoro (2'-F) modified nucleotide, a phosphorothioate (PS) linkage between nucleotides, an inverted abasic modified nucleotide, or a combination thereof. In certain embodiments, the modified nucleotide includes a 2'-OMe modified nucleotide.

[00101] In certain embodiments, the Exemplary SpyCas9 sgRNA-1 comprises one or more YA dinucleotides, wherein Y is a pyrimidine, wherein the YA dinucleotide includes a substituted nucleotide, i.e., sequence substituted nucleotide, wherein the pyrimidine is substituted for a purine. In certain embodiments, when the pyrimidine forms a Watson-Crick base pair in the single guide, the Watson-Crick based nucleotide of the substituted pyrimidine nucleotide is substituted to maintain Watson-Crick base pairing.

Attorney Docket No. 01155-0040-00PCT

Exemplary spyCas9 sgRNA-1 (SEQ ID NO: 201)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
G	U	U	U	U	A	G	A	G	C	U	A	G	A	A	A	U	A	G	C	A	A	G	U	U	A	A	A	A	U
LS1-LS6						B1-B2			US1-US12												B2-B6			LS7-LS12					
31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
A	A	G	G	C	U	A	G	U	C	C	G	U	U	A	U	C	A	A	C	U	U	G	A	A	A	A	A	G	U
Nexus																		H1-1 through H1-12											
61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76														
G	G	C	A	C	C	G	A	G	U	C	G	G	U	G	C	H2-1 through H2-15													
N																													

Table 1: LAG3 guide sequences and chromosomal coordinates

SEQ ID NO:	LAG3 ID	Guide Sequence	Genomic Coordinates (hg38)
1	LAG3-1	GCGGUCCCUGAGGUGCACCG	chr12:6773938-6773958
2	LAG3-2	GUUCCGGAACCAAUGCACAG	chr12:6774678-6774698
3	LAG3-3	UUACCUGGAGCCACCCAAAG	chr12:6772894-6772914
4	LAG3-4	GACGUUGAAGCCAUCUCUGU	chr12:6774816-6774836
5	LAG3-5	AGAGGAAGCUUCCGCUAAG	chr12:6774742-6774762
6	LAG3-6	UCCCCCAGGAGGAGUCCACU	chr12:6775380-6775400
7	LAG3-7	GUCCCCCAUCACCACUUAG	chr12:6774727-6774747
8	LAG3-8	UCCGCUAAGUGGUGAUGGG	chr12:6774732-6774752
9	LAG3-9	GCAAUGCCAGCUGUACCAGG	chr12:6777435-6777455
10	LAG3-10	AGAGUCCAUGGGGCUGACUU	chr12:6774771-6774791
11	LAG3-11	CCCGCCAUCCCCGUUUUACC	chr12:6772909-6772929
12	LAG3-12	GCUUCCGCUAAGUGGUGAU	chr12:6774735-6774755
13	LAG3-13	CCACGCUCAGCACCGUGUAG	chr12:6773783-6773803
14	LAG3-14	GCUCCAGCGUACACUGUCAA	chr12:6775292-6775312
15	LAG3-15	UGGCAAUGCCAGCUGUACCA	chr12:6777433-6777453
16	LAG3-16	AGGGUGAAUCCCUUGCUCUA	chr12:6778268-6778288
17	LAG3-17	GGCUCACAUCCUCUAGUCGA	chr12:6775444-6775464
18	LAG3-18	GUGCAUUGGUUCCGGAACCG	chr12:6774680-6774700
19	LAG3-19	UCGACUAGAGGAUGUGAGCC	chr12:6775444-6775464
20	LAG3-20	AGCGCGGGGACUUCUCGCUA	chr12:6773872-6773892
21	LAG3-21	CGCCCCACAUAUCGAGGCC	chr12:6773993-6774013
22	LAG3-22	GCGUACACUGUCAAGGGAGU	chr12:6775286-6775306
23	LAG3-23	CGACUUUACCCUUCGACUAG	chr12:6775432-6775452
24	LAG3-24	UCCAUAGGUGCCCAACGCUC	chr12:6777783-6777803
25	LAG3-25	CACCGCGGCGCGGUACUCGC	chr12:6773923-6773943
26	LAG3-26	CCAUAAGGUGCCCAACGCUCU	chr12:6777784-6777804
27	LAG3-27	UUGGUUCCGGAACCGGGGCC	chr12:6774685-6774705
28	LAG3-28	ACCGUGUAGCGGCGGGGCCU	chr12:6773772-6773792
29	LAG3-29	CGCUACACGGUGCUGAGCGU	chr12:6773781-6773801
30	LAG3-30	CGCGUCCAGCUGGAUGAGCG	chr12:6773844-6773864
31	LAG3-31	UGACCCUGCUCUUCGCAGA	chr12:6773297-6773317
32	LAG3-32	UCCAGCUGGAUGAGCGCGGC	chr12:6773848-6773868
33	LAG3-33	GAUGGGGGGACUCCCGGACA	chr12:6774718-6774738
34	LAG3-34	GCCGGCCGCGCUAUCCAGC	chr12:6773852-6773872
35	LAG3-35	GCAAGGGAUUCACCCUCCGC	chr12:6778272-6778292
36	LAG3-36	GUACGCUGGAGCAGGUUCCA	chr12:6775300-6775320
37	LAG3-37	ACUCGCCGGCGUCCGCGCGC	chr12:6773909-6773929
38	LAG3-38	CGCUCAGCACCGUGUAGCGG	chr12:6773780-6773800
39	LAG3-39	AUGAGCGCGGCCGCGCAGCGC	chr12:6773857-6773877
40	LAG3-40	GCUCACAUCCUCUAGUCGAA	chr12:6775443-6775463

SEQ ID NO:	LAG3 ID	Guide Sequence	Genomic Coordinates (hg38)
41	LAG3-41	UCUAAGGCAGAAAUCGUCU	chr12:6778252-6778272
42	LAG3-42	UGCUCACAGCGUACACUGUCA	chr12:6775293-6775313
43	LAG3-43	CACCGUGUAGCGGCGGGGCC	chr12:6773773-6773793
44	LAG3-44	ACCGCGCCGCGGUGCACCUC	chr12:6773929-6773949
45	LAG3-45	AGCUUCCGCUAAGUGGUGA	chr12:6774736-6774756
46	LAG3-46	CUGUGCAUUGGUUCCGGAAC	chr12:6774678-6774698
47	LAG3-47	UGGGGCUCUCCCAGAGCGUU	chr12:6777796-6777816
48	LAG3-48	CUCAGCACCGUGUAGCGGCG	chr12:6773778-6773798
49	LAG3-49	GCUCAGCACCGUGUAGCGGC	chr12:6773779-6773799
50	LAG3-50	CGCCGGCGAGUACCGCGCCG	chr12:6773918-6773938
51	LAG3-51	UGUACGCUGGAGCAGGUUCC	chr12:6775299-6775319
52	LAG3-52	UUGGCAAUGCCAGCUGUACC	chr12:6777432-6777452
53	LAG3-53	UGAUGGGGGGACUCCCGGAC	chr12:6774719-6774739
54	LAG3-54	UUGACAGUGUACGCUGGAGC	chr12:6775292-6775312
55	LAG3-55	CGCGCUCAUCCAGCUGGACG	chr12:6773846-6773866
56	LAG3-56	CGUCCCGCCCCACAUCUCG	chr12:6773998-6774018
57	LAG3-57	CGUACACUGUCAAGGGAGUU	chr12:6775285-6775305
58	LAG3-58	GCGAGAAGUCCCCGCGCUGC	chr12:6773870-6773890
59	LAG3-59	AAGCGUUCUUGUCCAGAUAC	chr12:6777325-6777345
60	LAG3-60	CUCGCCGGCGUCCGCGCGCC	chr12:6773908-6773928
61	LAG3-61	UGGGCGGUCAGGGCGGCUGA	chr12:6774654-6774674
62	LAG3-62	UGUGCAUUGGUUCCGGAACC	chr12:6774679-6774699
63	LAG3-63	CCGCUACACGGUGCUGAGCG	chr12:6773780-6773800
64	LAG3-64	CCGCGCCGCGGUGCACCUC	chr12:6773930-6773950
65	LAG3-65	GCGCUCAUCCAGCUGGACGC	chr12:6773845-6773865
66	LAG3-66	GAUGAGCGCGGCCGCGCAGCG	chr12:6773856-6773876
67	LAG3-67	GUUCAAAUUGACCCAGUCGG	chr12:6774627-6774647
68	LAG3-68	CCCUGAGGUGCACCGCGGCG	chr12:6773933-6773953
69	LAG3-69	ACUCCCUUGACAGUGUACGC	chr12:6775286-6775306
70	LAG3-70	CCAGCCCGGCGCGCGGACGC	chr12:6773901-6773921
71	LAG3-71	ACGCUUUGUGUGGAGCUCUC	chr12:6777339-6777359
72	LAG3-72	UGUGAGGUGACUCCAGUAUC	chr12:6777310-6777330
73	LAG3-73	ACGGUGCUGAGCGUGGGUCC	chr12:6773787-6773807
74	LAG3-74	GUCCCGGAGGCCUGCGCAGC	chr12:6773803-6773823
75	LAG3-75	CAGCCUCUGUGCAUUGGUUC	chr12:6774672-6774692
76	LAG3-76	AACAGUGAGGUUAUACAUGA	chr12:6774840-6774860
77	LAG3-77	CAAGUCAGCCCCAUGGACUC	chr12:6774770-6774790
78	LAG3-78	AUCUCUCAGAGCCUCCGACU	chr12:6774613-6774633
79	LAG3-79	CUCCCGCUGCGCAGGCCUCC	chr12:6773808-6773828
80	LAG3-80	GGGGCAGCCUCCGCGUGCGC	chr12:6773816-6773836
81	LAG3-81	GCUCAAUGCCACUGUCACAU	chr12:6775513-6775533
82	LAG3-82	CUUCCGCUAAGUGGUGAUG	chr12:6774734-6774754

SEQ ID NO:	LAG3 ID	Guide Sequence	Genomic Coordinates (hg38)
83	LAG3-83	CUGGACAAGAACGCUUUGUG	chr12:6777329-6777349
84	LAG3-84	CUUUGGGUCACCUGGAUCCC	chr12:6777276-6777296
85	LAG3-85	ACAUACUCGAGGCCUGGCCC	chr12:6773987-6774007
86	LAG3-86	CCCGGACAGGGACUCGGCCC	chr12:6774706-6774726
87	LAG3-87	GGGACUCCCGGACAGGGACU	chr12:6774712-6774732
88	LAG3-88	CGCCAUUGUCUCCAGUCACC	chr12:6775414-6775434

Table 2: sgRNA sequences targeting LAG3

Guide ID	SEQ ID NO:	sgRNA Sequence	Genomic Coordinates (hg38)
	89	GUUCCGGAACCAAUGCACAGGU UUUAGAGCUAGAAAUAGCAAG UUAAAAUAAGGCUAGUCCGUU AUCAACUUGAAAAAGUGGCAC CGAGUCGGUGCUUUU	chr12:6774678-6774698
G015068	90	GACGUUGAAGCCAUCUCUGUG UUUUAGAGCUAGAAAUAGCAA GUUAAAAUAAGGCUAGUCCGU UAUCAACUUGAAAAAGUGGCA CCGAGUCGGUGCUUUU	chr12:6774816-6774836
G015069	91	GCGGUCCCUGAGGUGCACCGGU UUUAGAGCUAGAAAUAGCAAG UUAAAAUAAGGCUAGUCCGUU AUCAACUUGAAAAAGUGGCAC CGAGUCGGUGCUUUU	chr12:6773938-6773958
G015070	92	GCAAUGCCAGCUGUACCAGGGU UUUAGAGCUAGAAAUAGCAAG UUAAAAUAAGGCUAGUCCGUU AUCAACUUGAAAAAGUGGCAC CGAGUCGGUGCUUUU	chr12:6777435-6777455
G015071	105	AGAGGAAGCUUUCGCUAAGG UUUUAGAGCUAGAAAUAGCAA GUUAAAAUAAGGCUAGUCCGU UAUCAACUUGAAAAAGUGGCA CCGAGUCGGUGCUUUU	chr12:6774742-6774762
G018433	106	mG*mU*mU*CCGGAACCAAUGC ACAGGUUUUAGAmGmCmUmAm GmAmAmAmUmAmGmCAAGUUA AAAUAAAGGCUAGUCCGUUAUC AmAmCmUmUmGmAmAmAmAm AmGmUmGmGmCmAmCmCmGmA mGmUmCmGmGmUmGmCmU*mU *mU*mU	chr12:6774678-6774698

Guide ID	SEQ ID NO:	sgRNA Sequence	Genomic Coordinates (hg38)
G018434	107	mG*mC*mG*GUCCCUGAGGUGC ACCGGUUUUAGAmGmCmUmAm GmAmAmUmAmGmCAAGUUA AAAUAAAGGCUAGUCCGUUAUC AmAmCmUmUmGmAmAmAmAm AmGmUmGmGmCmAmCmCmGmA mGmUmCmGmGmUmGmCmU*mU *mU*mU	chr12:6773938-6773958

* = PS linkage; m = 2'-O-Me nucleotide; N = any natural or non-natural nucleotide

[00102] In some embodiments, the invention provides a composition comprising one or more guide RNA (gRNA) comprising guide sequences that direct an RNA-guided DNA binding agent, which can be a nuclease (e.g., a Cas nuclease such as Cas9), to a target DNA sequence in *LAG3*. In some embodiments comprising a gRNA, the gRNA comprises a guide sequence shown in Table 1, e.g., an sgRNA. In some embodiments, the gRNA may comprise a guide sequence selected from SEQ ID NOs: 1-27, SEQ ID NOs: 1-15, SEQ ID NOs: 1-11, SEQ ID NOs: 1-4, or SEQ ID NOs: 1, 4, 5, and 9. The gRNA may comprise a crRNA comprising 17, 18, 19, or 20 contiguous nucleotides of a guide sequence shown in Table 1. In some embodiments, the gRNA comprises a guide sequence comprising a sequence with at least 75%, 80%, 85%, 90%, or 95%, or 100% identity to at least 17, 18, 19, or 20 contiguous nucleotides of a guide sequence shown in Table 1, optionally SEQ ID NOs: 1-27, SEQ ID NOs: 1-15, SEQ ID NOs: 1-11, SEQ ID NOs: 1-4, or SEQ ID NOs: 1, 4, 5, and 9. In some embodiments, the gRNA comprises a guide sequence comprising a sequence with at least 75%, 80%, 85%, 90%, or 95%, or 100% identity to a guide sequence shown in Table 1, optionally SEQ ID NOs: 1-27, SEQ ID NOs: 1-15, SEQ ID NOs: 1-11, SEQ ID NOs: 1-4, or SEQ ID NOs: 1, 4, 5, or 9. The gRNA may further comprise a trRNA. In each embodiment described herein, the gRNA may comprise a crRNA and trRNA associated as a single RNA (sgRNA) or 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.

[00103] In each embodiment 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 in Table 1, 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.

[00104] In each embodiment 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 in Table 1, or a guide sequence selected from SEQ ID NOs: 1-27, SEQ ID NOs: 1-15, SEQ ID NOs: 1-11, SEQ ID NOs: 1-4, or SEQ ID NOs: 1, 4, 5, and 9, covalently linked to a trRNA. The sgRNA may comprise 17, 18, 19, or 20 contiguous nucleotides of a guide sequence shown in Table 1, or a guide sequence selected from SEQ ID NOs: 1-27, SEQ ID NOs: 1-15, SEQ ID NOs: 1-11, SEQ ID NOs: 1-4, or SEQ ID NOs: 1, 4, 5, and 9. 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.

[00105] 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 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 70, 80, 90, 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.

[00106] In some embodiments, the invention provides a composition comprising one or more guide RNAs comprising a guide sequence of any one of SEQ ID NOs: 1–88, preferably SEQ ID NOs: 1-27, SEQ ID NOs: 1-15, SEQ ID NOs: 1-11, SEQ ID NOs: 1-4, or SEQ ID NOs: 1, 4, 5, and 9.

[00107] In some embodiments, the invention provides a composition comprising one or more sgRNAs comprising any one of SEQ ID NOs: 89-92, 105, 106, and 107.

[00108] In one aspect, the invention provides a composition comprising a gRNA that comprises a guide sequence that is 100% or at least 95% or 90% identical to any of the nucleic acids of SEQ ID NOs: 1–88, preferably SEQ ID NOs: 1-27, SEQ ID NOs: 1-15, SEQ ID NOs: 1-11, SEQ ID NOs: 1-4, or SEQ ID NOs: 1, 4, 5, and 9.

[00109] In other embodiments, the composition comprises at least one, e.g., at least two gRNA's comprising guide sequences selected from any two or more of the guide sequences

of SEQ ID NOs: 1–88 , preferably SEQ ID NOs: 1-27, SEQ ID NOs: 1-15, SEQ ID NOs: 1-11, SEQ ID NOs: 1-4, or SEQ ID NOs: 1, 4, 5, and 9. In some embodiments, the composition comprises at least two gRNA's that each comprise a guide sequence 100%, or at least 95% or 90% identical to any of the nucleic acids of SEQ ID NOs: 1–88 , preferably SEQ ID NOs: 1-27, SEQ ID NOs: 1-15, SEQ ID NOs: 1-11, SEQ ID NOs: 1-4, or SEQ ID NOs: 1, 4, 5, and 9.

[00110] The guide RNA compositions of the present invention are designed to recognize (e.g., hybridize to) a target sequence in a *LAG3* gene. For example, the *LAG3* target sequence may be recognized and cleaved by a provided Cas cleavase comprising a guide RNA. In some embodiments, an RNA-guided DNA binding agent, such as a Cas cleavase, may be directed by a guide RNA to a target sequence of a *LAG3* gene, where the guide sequence of the guide RNA hybridizes with the target sequence and the RNA-guided DNA binding agent, such as a Cas cleavase, cleaves the target sequence.

[00111] In some embodiments, the selection of the one or more guide RNAs is determined based on target sequences within a *LAG3* gene.

[00112] Without being bound by any particular theory, mutations (e.g., frameshift mutations resulting from indels, i.e., insertions or deletions, occurring as a result of a nuclease-mediated DSB) in certain regions of the gene may be less tolerable than mutations in other regions of the gene, thus the location of a DSB is an important factor in the amount or type of protein knockdown that may result. In some embodiments, a gRNA complementary or having complementarity to a target sequence within *LAG3* is used to direct the RNA-guided DNA binding agent to a particular location in the appropriate *LAG3* gene. In some embodiments, gRNAs are designed to have guide sequences that are complementary or have complementarity to target sequences in exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, exon 7, or exon 8 of *LAG3*.

[00113] In some embodiments, the guide sequence is 100% or at least 95% or 90% identical to a target sequence or to the reverse complement of a target sequence present in a human *LAG3* gene. In some embodiments, the target sequence 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 at least 80%, 85%, 90%, or 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 at least one mismatch. For example, the target sequence and the guide sequence of the gRNA

may contain 1, 2, 3, or 4 mismatches, where the total length of the guide sequence is 20. In some embodiments, the target sequence and the guide sequence of the gRNA may contain 1-4 mismatches where the guide sequence is 20 nucleotides.

[00114] In some embodiments, a composition or formulation disclosed herein comprises an mRNA comprising an open reading frame (ORF) encoding an RNA-guided DNA binding agent, such as a Cas nuclease as described herein. In some embodiments, an mRNA comprising an ORF encoding an RNA-guided DNA binding agent, such as a Cas nuclease, is provided, used, or administered.

B. Modified gRNAs and mRNAs

[00115] 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) wholesale replacement of the phosphate moiety with “dephospho” linkers (an exemplary backbone modification); (iv) modification or replacement of a naturally occurring nucleobase, including with a non-canonical nucleobase (an exemplary base modification); (v) replacement or modification of the ribose-phosphate backbone (an exemplary backbone modification); (vi) modification of the 3' end or 5' end of the oligonucleotide, *e.g.*, removal, modification or replacement of a terminal phosphate group or conjugation of a moiety, cap or linker (such 3' or 5' cap modifications may comprise a sugar or backbone modification); and (vii) modification or replacement of the sugar (an exemplary sugar modification).

[00116] Chemical modifications such as those listed above can be combined to provide modified gRNAs or mRNAs 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 a 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.

[00117] In some embodiments, the gRNA comprises one, two, three or more modified residues. In some embodiments, at least 5% (*e.g.*, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100%) of the positions in a modified gRNA are modified nucleosides or nucleotides.

[00118] Unmodified nucleic acids can be prone to degradation by, *e.g.*, intracellular nucleases or those found in serum. For example, nucleases can hydrolyze nucleic acid phosphodiester bonds. Accordingly, in one aspect the gRNAs described herein can contain one or more modified nucleosides or nucleotides, *e.g.*, to introduce stability toward intracellular or serum-based nucleases. In some embodiments, the modified gRNA molecules described herein can exhibit a reduced innate immune response when introduced into a population of cells, both *in vivo* and *ex vivo*. The term “innate immune response” includes a cellular response to exogenous nucleic acids, including single stranded nucleic acids, which involves the induction of cytokine expression and release, particularly the interferons, and cell death.

[00119] In some embodiments of a backbone modification, the phosphate group of a modified residue can be modified by replacing one or more of the oxygens with a different substituent. Further, the modified residue, *e.g.*, modified residue present in a modified nucleic acid, can include the wholesale replacement of an unmodified phosphate moiety with a modified phosphate group as described herein. In some embodiments, the backbone modification of the phosphate backbone can include alterations that result in either an uncharged linker or a charged linker with unsymmetrical charge distribution.

[00120] Examples of modified phosphate groups include, phosphorothioate, phosphoroselenates, borano phosphates, borano phosphate esters, hydrogen phosphonates, phosphoroamidates, alkyl or aryl phosphonates and phosphotriesters. The phosphorous atom in an unmodified phosphate group is achiral. However, replacement of one of the non-

bridging oxygens with one of the above atoms or groups of atoms can render the phosphorous atom chiral. The stereogenic phosphorous atom can possess either the “R” configuration (herein Rp) or the “S” configuration (herein Sp). The backbone can also be modified by replacement of a bridging oxygen, (*i.e.*, the oxygen that links the phosphate to the nucleoside), with nitrogen (bridged phosphoroamidates), sulfur (bridged phosphorothioates) and carbon (bridged methylenephosphonates). The replacement can occur at either linking oxygen or at both of the linking oxygens.

[00121] The phosphate group can be replaced by non-phosphorus containing connectors in certain backbone modifications. In some embodiments, the charged phosphate group can be replaced by a neutral moiety. Examples of moieties which can replace the phosphate group can include, without limitation, *e.g.*, methyl phosphonate, hydroxylamino, siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino.

[00122] Scaffolds that can mimic nucleic acids can also be constructed wherein the phosphate linker and ribose sugar are replaced by nuclease resistant nucleoside or nucleotide surrogates. Such modifications may comprise backbone and sugar modifications. In some embodiments, the nucleobases can be tethered by a surrogate backbone. Examples can include, without limitation, the morpholino, cyclobutyl, pyrrolidine and peptide nucleic acid (PNA) nucleoside surrogates.

[00123] The modified nucleosides and modified nucleotides can include one or more modifications to the sugar group, *i.e.* at sugar modification. For example, the 2' hydroxyl group (OH) can be modified, *e.g.* replaced with a number of different “oxy” or “deoxy” substituents. In some embodiments, modifications to the 2' hydroxyl group can enhance the stability of the nucleic acid since the hydroxyl can no longer be deprotonated to form a 2'-alkoxide ion.

[00124] Examples of 2' hydroxyl group modifications can include alkoxy or aryloxy (OR, wherein “R” can be, *e.g.*, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or a sugar); polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$ wherein R can be, *e.g.*, H or optionally substituted alkyl, and n can be an integer from 0 to 20 (*e.g.*, from 0 to 4, from 0 to 8, from 0 to 10, from 0 to 16, from 1 to 4, from 1 to 8, from 1 to 10, from 1 to 16, from 1 to 20, from 2 to 4, from 2 to 8, from 2 to 10, from 2 to 16, from 2 to 20, from 4 to 8, from 4 to 10, from 4 to 16, and from 4 to 20). In some embodiments, the 2' hydroxyl group

modification can be 2'-O-Me. In some embodiments, the 2' hydroxyl group modification can be a 2'-fluoro modification, which replaces the 2' hydroxyl group with a fluoride. In some embodiments, the 2' hydroxyl group modification can include "locked" nucleic acids (LNA) in which the 2' hydroxyl can be connected, *e.g.*, by a C₁₋₆ alkylene or C₁₋₆ heteroalkylene bridge, to the 4' carbon of the same ribose sugar, where exemplary bridges can include methylene, propylene, ether, or amino bridges; O-amino (wherein amino can be, *e.g.*, NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diarylamino, heteroarylamino, or diheteroarylamino, ethylenediamine, or polyamino) and aminoalkoxy, O(CH₂)_n-amino, (wherein amino can be, *e.g.*, NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diarylamino, heteroarylamino, or diheteroarylamino, ethylenediamine, or polyamino). In some embodiments, the 2' hydroxyl group modification can include "unlocked" nucleic acids (UNA) in which the ribose ring lacks the C2'-C3' bond. In some embodiments, the 2' hydroxyl group modification can include the methoxyethyl group (MOE), (OCH₂CH₂OCH₃, *e.g.*, a PEG derivative).

[00125] "Deoxy" 2' modifications can include hydrogen (*i.e.* deoxyribose sugars, *e.g.*, at the overhang portions of partially dsRNA); halo (*e.g.*, bromo, chloro, fluoro, or iodo); amino (wherein amino can be, *e.g.*, NH₂; alkylamino, dialkylamino, heterocyclyl, arylamino, diarylamino, heteroarylamino, diheteroarylamino, or amino acid); NH(CH₂CH₂NH)_nCH₂CH₂- amino (wherein amino can be, *e.g.*, as described herein), -NHC(O)R (wherein R can be, *e.g.*, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino as described herein.

[00126] The sugar modification can comprise a sugar group which may also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a modified nucleic acid can include nucleotides containing *e.g.*, arabinose, as the sugar. The modified nucleic acids can also include abasic sugars. These abasic sugars can also be further modified at one or more of the constituent sugar atoms. The modified nucleic acids can also include one or more sugars that are in the L form, *e.g.* L- nucleosides.

[00127] The modified nucleosides and modified nucleotides described herein, which can be incorporated into a modified nucleic acid, can include a modified base, also called a nucleobase. Examples of nucleobases include, but are not limited to, adenine (A), guanine (G), cytosine (C), and uracil (U). These nucleobases can be modified or wholly replaced to

provide modified residues that can be incorporated into modified nucleic acids. The nucleobase of the nucleotide can be independently selected from a purine, a pyrimidine, a purine analog, or pyrimidine analog. In some embodiments, the nucleobase can include, for example, naturally-occurring and synthetic derivatives of a base.

[00128] In embodiments employing a dual guide RNA, each of the crRNA and the tracrRNA can contain modifications. Such modifications may be at one or both ends of the crRNA or tracrRNA. In embodiments comprising an sgRNA, one or more residues at one or both ends of the sgRNA may be chemically modified, or internal nucleosides may be modified, or the entire sgRNA may be chemically modified. Certain embodiments comprise a 5' end modification. Certain embodiments comprise a 3' end modification. Certain embodiments comprise a 5' end modification and a 3' end modification.

[00129] In some embodiments, the guide RNAs disclosed herein comprise one of the modification patterns disclosed in WO2018/107028 A1, filed December 8, 2017, titled "Chemically Modified Guide RNAs," the contents of which are hereby incorporated by reference in their entirety. In some embodiments, the guide RNAs disclosed herein comprise one of the structures/modification patterns disclosed in US20170114334, the contents of which are hereby incorporated by reference in their entirety. In some embodiments, the guide RNAs disclosed herein comprise one of the structures/modification patterns disclosed in WO2017/136794, the contents of which are hereby incorporated by reference in their entirety.

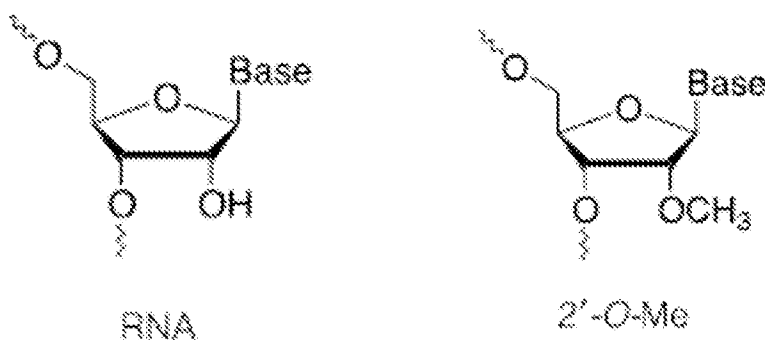
[00130] In some embodiments, the sgRNA comprises any of the modification patterns shown herein, where N is any natural or non-natural nucleotide, and wherein the totality of the N's comprise a *LAG3* guide sequence as described herein in Table 1. In some embodiments, the modified sgRNA comprises the following sequence:

mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU (SEQ ID NO: 300), where "N" may be any natural or non-natural nucleotide, and wherein the totality of N's comprise an *LAG3* guide sequence as described in Table 1, for example. For example, where the N's are replaced with any of the guide sequences disclosed herein in Table 1 optionally wherein the N's are replaced with SEQ ID NOs: 1-88; or SEQ ID NOs: 1-27, SEQ ID NOs: 1-15, SEQ ID NOs: 1-11, SEQ ID NOs: 1-4, or SEQ ID NOs: 1, 4, 5, and 9.

[00131] Any of the modifications described below may be present in the gRNAs and mRNAs described herein.

[00132] The terms “mA,” “mC,” “mU,” or “mG” may be used to denote a nucleotide that has been modified with 2'-O-Me.

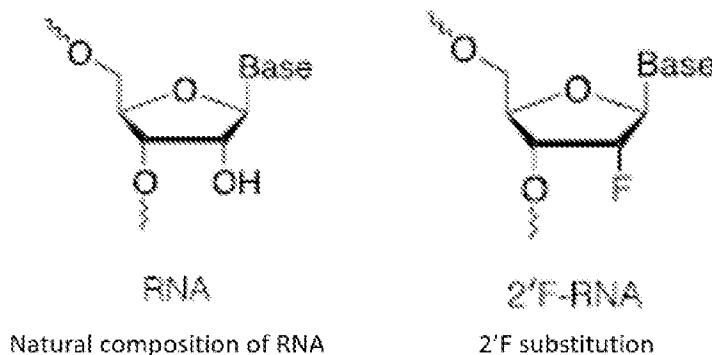
[00133] Modification of 2'-O-methyl can be depicted as follows:



[00134] Another chemical modification that has been shown to influence nucleotide sugar rings is halogen substitution. For example, 2'-fluoro (2'-F) substitution on nucleotide sugar rings can increase oligonucleotide binding affinity and nuclease stability.

[00135] In this application, the terms “fA,” “fC,” “fU,” or “fG” may be used to denote a nucleotide that has been substituted with 2'-F.

[00136] Substitution of 2'-F can be depicted as follows:

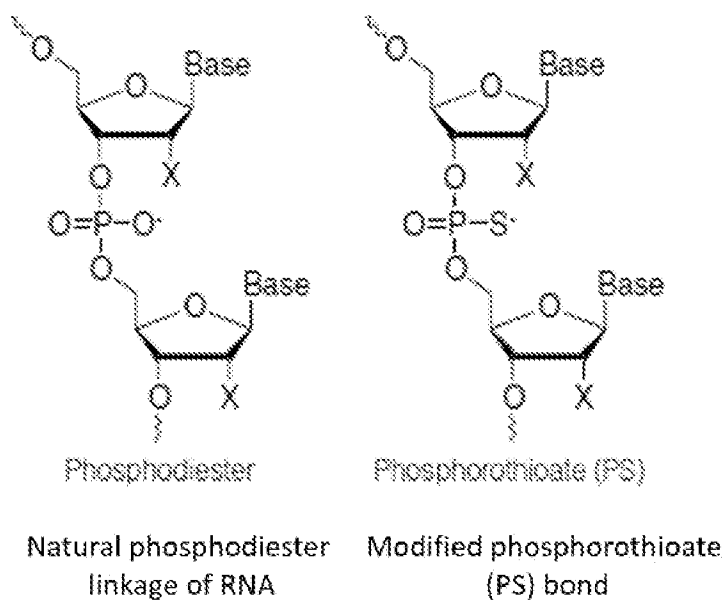


[00137] Phosphorothioate (PS) linkage or bond refers to a bond where a sulfur is substituted for one non-bridging phosphate oxygen in a phosphodiester linkage, for example in the bonds between nucleotides bases. When phosphorothioates are used to generate oligonucleotides, the modified oligonucleotides may also be referred to as S-oligos.

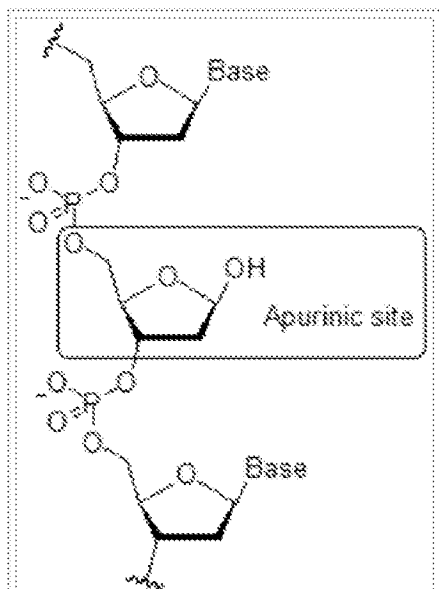
[00138] A “*” may be used to depict a PS modification. In this application, the terms A*, C*, U*, or G* may be used to denote a nucleotide that is linked to the next (e.g., 3') nucleotide with a PS bond.

[00139] In this application, the terms “mA*,” “mC*,” “mU*,” or “mG*” may be used to denote a nucleotide that has been substituted with 2'-O-Me and that is linked to the next (e.g., 3') nucleotide with a PS bond.

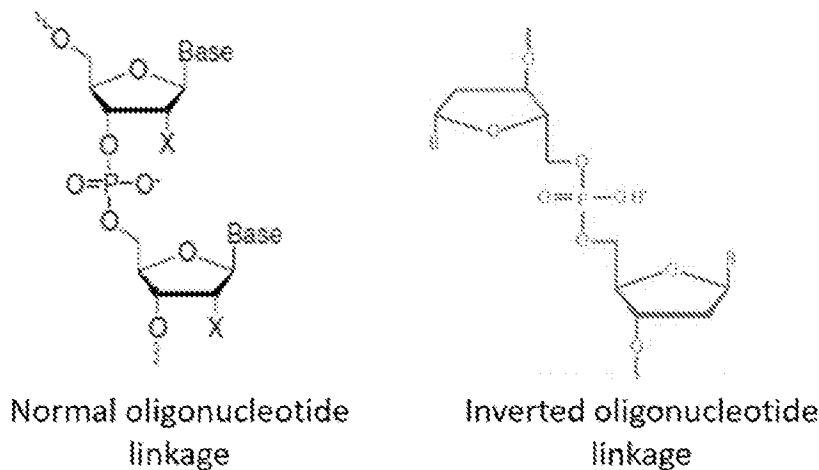
[00140] The diagram below shows the substitution of S- into a non-bridging phosphate oxygen, generating a PS bond in lieu of a phosphodiester bond:



[00141] Abasic nucleotides refer to those which lack nitrogenous bases. The figure below depicts an oligonucleotide with an abasic (also known as apurinic) site that lacks a base:



[00142] Inverted bases refer to those with linkages that are inverted from the normal 5' to 3' linkage (i.e., either a 5' to 5' linkage or a 3' to 3' linkage). For example:



[00143] An abasic nucleotide can be attached with an inverted linkage. For example, an abasic nucleotide may be attached to the terminal 5' nucleotide via a 5' to 5' linkage, or an abasic nucleotide may be attached to the terminal 3' nucleotide via a 3' to 3' linkage. An inverted abasic nucleotide at either the terminal 5' or 3' nucleotide may also be called an inverted abasic end cap.

[00144] In some embodiments, one or more of the first three, four, or five nucleotides at the 5' terminus, and one or more of the last three, four, or five nucleotides at the 3' terminus are modified. In some embodiments, the modification is a 2'-O-Me, 2'-F, inverted abasic nucleotide, PS bond, or other nucleotide modification well known in the art to increase stability or performance.

[00145] In some embodiments, the first four nucleotides at the 5' terminus, and the last four nucleotides at the 3' terminus are linked with phosphorothioate (PS) bonds.

[00146] In some embodiments, the first three nucleotides at the 5' terminus, and the last three nucleotides at the 3' terminus comprise a 2'-O-methyl (2'-O-Me) modified nucleotide. In some embodiments, the first three nucleotides at the 5' terminus, and the last three nucleotides at the 3' terminus comprise a 2'-fluoro (2'-F) modified nucleotide. In some embodiments, the first three nucleotides at the 5' terminus, and the last three nucleotides at the 3' terminus comprise an inverted abasic nucleotide.

[00147] In some embodiments, the guide RNA comprises a modified sgRNA. In some embodiments, the sgRNA comprises the modification pattern shown in

mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUAGAmGmCmUmAmGmAmAmAmU

mAmGmCAAGUUA AAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU (SEQ ID NO: 300), where N is any natural or non-natural nucleotide, and where the totality of the N's comprise a guide sequence that directs a nuclease to a target sequence in *LAG3*, e.g., the genomic coordinates shown in Table 1.

[00148] In some embodiments, the guide RNA comprises a sgRNA comprising any one of the guide sequences of SEQ ID NOs: 1–88 and a conserved portion of an sgRNA shown in for example, the conserved portion of sgRNA shown as Exemplary SpyCas9 sgRNA-1 or the conserved portions of the gRNAs shown in Table 2 and throughout the specification. In some embodiments, the guide RNA comprises a sgRNA comprising any one of the guide sequences of SEQ ID NOs: 1–88 and the nucleotides of

GUUUUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAACUU GAAAAAGUGGCACCGAGUCGGUGCUUUU (SEQ ID NO: 202), wherein the nucleotides are on the 3' end of the guide sequence, and wherein the sgRNA may be modified as shown herein or in the sequence

mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUA AAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU (SEQ ID NO: 300). In some embodiments, the sgRNA comprises Exemplary SpyCas9 sgRNA-1 and the modified versions thereof provided herein, or a version as provided in the table TABLE 2B below, where the totality of the N's comprise a guide sequence that directs a nuclease to a target sequence. Each N is independently modified or unmodified. In certain embodiments, in the absence of an indication of a modification, the nucleotide is an unmodified RNA nucleotide residue, i.e., a ribose sugar and a phosphodiester backbone.

[00149] **TABLE 2B. Exemplary sgRNA sequences (modified and unmodified versions)**

Guide Scaffold (unmodified/modified)	sgRNA unmodified sequence	sgRNA modified sequence
81/181	(N)20GUUUUAGAGCUA GAAAUAGCAAGUUA AAUAAGGCUAGUCCGUU AUCACGAAAGGGCACC GAGUCGGUGC (SEQ ID NO: 401)	mN*mN*mN*(N)17GUUU UAGAmGmCmUmAmGm AmAmAmUmAmGmCAA GUUAAAAUAAGGCUAG UCCGUUAUCACGAAAG GGCACCGAGUCGG*mU *mG*mC (SEQ ID NO: 402)

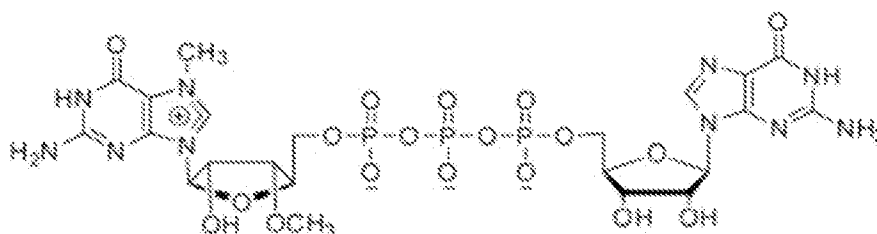
Guide Scaffold (unmodified/modified)	sgRNA unmodified sequence	sgRNA modified sequence
94/194	(N)20GUUUUAGAGCUA GAAAUAGCAAGUAAA AUAAGGCUAGUCCGUU AUCAACUUGGCACCGA GUCGGUGC (SEQ ID NO: 403)	mN*mN*mN*(N)17GUUU UAGAmGmCmUmAmGm AmAmAmUmAmGmCAA GUUAAAAUAAGGCUAG UCCGUUAUCAACUUGG CACCGAGUCGG*mU*m G*mC (SEQ ID NO: 404)
95/195	(N)20GUUUUAGAGCUA GAAAUAGCAAGUAAA AUAAGGCUAGUCCGUU AUCAACUUGGCACCGA GUCGGUGC (SEQ ID NO: 405)	mN*mN*mN*(N)17GUUU UAGAmGmCmUmAmGm AmAmAmUmAmGmCAA GUUAAAAUAAGGCUAG UCCGUUAUCAACUUGG CACCGAGUCGG*mU*m G*mC (SEQ ID NO: 406)
871/971	(N)20GUUUUAGAGCUA GAAAUAGCAAGUAAA AUAAGGCUAGUCCGUU AUCACGAAAGGGCACC GAGUCGGUGC (SEQ ID NO: 407)	mN*mN*mN*(N)17mGUU UfUAGmAmGmCmUmAm GmAmAmAmUmAmGmC mAmAGUfUmAfAmAfAm UAmAmGmGmCmUmAG UmCmCGUfUAmUmCmAm CmGmAmAmAmGmGmG mCmAmCmCmGmAmGm UmCmGmG*mU*mG*mC (SEQ ID NO: 408)
872/972	(N)20GUUUUAGAGCUA GAAAUAGCAAGUAAA AUAAGGCUAGUCCGUU AUCACGAAAGGGCACC GAGUCGGUGC (SEQ ID NO: 409)	mN*mN*mN*(N)17GUUU UAGAmGmCmUmAmGm AmAmAmUmAmGmCAA GUUAAAAUAAGGCUAG UCCGUUAUCACGAAAG GGCACCGAGUCGG*mU *mG*mC (SEQ ID NO: 410)

[00150] As noted above, in some embodiments, a composition or formulation disclosed herein comprises an mRNA comprising an open reading frame (ORF) encoding an RNA-guided DNA binding agent, such as a Cas nuclease, e.g. Cas9 nuclease, as described herein. In some embodiments, an mRNA comprising an ORF encoding an RNA-guided DNA binding agent, such as a Cas nuclease, e.g. Cas9 nuclease, is provided, used, or administered. In some embodiments, the ORF encoding an RNA-guided DNA nuclease is a “modified RNA-guided DNA binding agent ORF” or simply a “modified ORF,” which is used as shorthand to indicate that the ORF is modified.

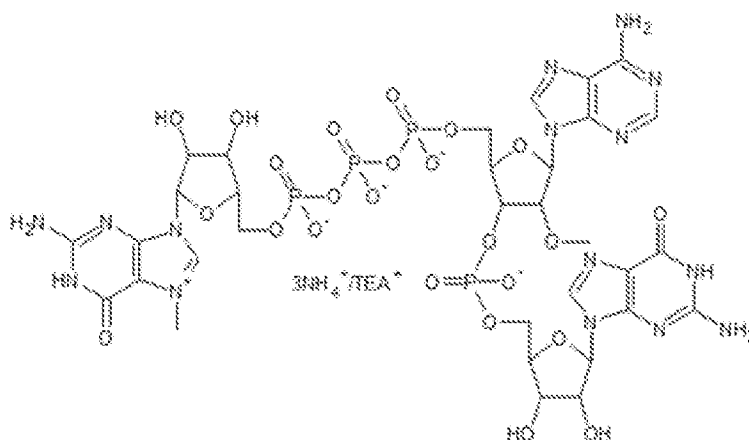
[00151] In some embodiments, the mRNA or modified ORF may comprise a modified uridine at least at one, a plurality of, or all uridine positions. In some embodiments, the modified uridine is a uridine modified at the 5 position, e.g., with a halogen, methyl, or ethyl. In some embodiments, the modified 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.

[00152] 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, as discussed below e.g. with respect to ARCA) 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. 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. Most endogenous higher eukaryotic mRNAs, including mammalian mRNAs such as human mRNAs, comprise Cap1 or Cap2. Cap0 and other cap structures differing from Cap1 and Cap2 may be immunogenic in mammals, such as humans, due to recognition as "non-self" by components of the innate immune system such as IFIT-1 and IFIT-5, which can result in elevated cytokine levels including type I interferon. Components of the innate immune system such as IFIT-1 and IFIT-5 may also compete with eIF4E for binding of an mRNA with a cap other than Cap1 or Cap2, potentially inhibiting translation of the mRNA.

[00153] A cap can be included co-transcriptionally. 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 which can be incorporated in vitro into a transcript at initiation. ARCA results in a Cap0 cap in which the 2' position of the first cap-proximal nucleotide is hydroxyl. See, e.g., Stepinski et al., (2001) "Synthesis and properties of mRNAs containing the novel 'anti-reverse' cap analogs 7-methyl(3'-O-methyl)GpppG and 7-methyl(3'deoxy)GpppG," *RNA* 7: 1486–1495. The ARCA structure is shown below.



[00154] 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) can be used to provide a Cap1 structure co-transcriptionally. 3'-O-methylated versions of CleanCap™ AG and CleanCap™ GG are also available from TriLink Biotechnologies as Cat. Nos. N-7413 and N-7433, respectively. The CleanCap™ AG structure is shown below.



[00155] Alternatively, a cap can be added to an RNA post-transcriptionally. For example, Vaccinia capping enzyme is commercially available (New England Biolabs Cat. No. M2080S) and has RNA triphosphatase and guanylyltransferase activities, provided by its D1 subunit, and guanine methyltransferase, provided by its D12 subunit. As such, it can add a 7-methylguanine to an RNA, so as to give Cap0, in the presence of S-adenosyl methionine and

GTP. See, e.g., Guo, P. and Moss, B. (1990) *Proc. Natl. Acad. Sci. USA* 87, 4023-4027; Mao, X. and Shuman, S. (1994) *J. Biol. Chem.* 269, 24472-24479.

[00156] In some embodiments, the mRNA further comprises a poly-adenylated (poly-A) tail. In some embodiments, the poly-A tail comprises at least 20, 30, 40, 50, 60, 70, 80, 90, or 100 adenines, optionally up to 300 adenines. In some embodiments, the poly-A tail comprises 95, 96, 97, 98, 99, or 100 adenine nucleotides.

C. Ribonucleoprotein complex

[00157] In some embodiments, a composition is encompassed comprising one or more gRNAs comprising one or more guide sequences from Table 1 or one or more sgRNAs from Table 2 and an RNA-guided DNA binding agent, e.g., a nuclease, such as a Cas nuclease, such as Cas9. In some embodiments, the RNA-guided DNA-binding agent has cleavage activity, which can also be referred to as double-strand endonuclease activity. In some embodiments, the RNA-guided DNA-binding agent comprises a Cas nuclease. Examples of Cas9 nucleases include those of the type II CRISPR systems of *S. pyogenes*, *S. aureus*, and other prokaryotes (see, e.g., the list in the next paragraph), and modified (e.g., engineered or mutant) versions thereof. See, e.g., US20160312198; US 20160312199. Other examples of Cas nucleases include a Csm or Cmr complex of a type III CRISPR system or the Cas10, Csm1, or Cmr2 subunit thereof; and a Cascade complex of a type I CRISPR system, or the Cas3 subunit thereof. In some embodiments, the Cas nuclease may be from a Type-IIA, Type-IIB, or Type-IIC system. For discussion of various CRISPR systems and Cas nucleases see, e.g., Makarova et al., NAT. REV. MICROBIOL. 9:467-477 (2011); Makarova et al., NAT. REV. MICROBIOL. 13: 722-36 (2015); Shmakov et al., MOLECULAR CELL, 60:385-397 (2015).

[00158] 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 becsicii*, *Candidatus Desulforudis*, *Clostridium botulinum*, *Clostridium difficile*, *Finegoldia magna*, *Natranaerobius thermophilus*, *Pelotomaculum thermopropionicum*, *Acidithiobacillus caldus*, *Acidithiobacillus ferrooxidans*, *Allochromatium 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*.

[00159] 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*.

[00160] 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.

[00161] 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.

[00162] 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.

[00163] 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.

[00164] 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. 8,889,356 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.

[00165] 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.

[00166] 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))).

[00167] In some embodiments, an mRNA encoding 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, use of double nicking may improve specificity and reduce off-target effects. 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.

[00168] In some embodiments, the RNA-guided DNA-binding agent lacks cleavase and nickase activity. In some embodiments, the RNA-guided DNA-binding agent comprises a dCas DNA-binding polypeptide. A dCas polypeptide has DNA-binding activity while essentially lacking catalytic (cleavase/nickase) activity. In some embodiments, the dCas polypeptide is a dCas9 polypeptide. In some embodiments, the RNA-guided DNA-binding agent lacking cleavase and nickase activity or the dCas DNA-binding polypeptide is a version of a Cas nuclease (e.g., a Cas nuclease discussed above) in which its endonucleolytic active sites are inactivated, e.g., by one or more alterations (e.g., point mutations) in its catalytic domains. *See, e.g.*, US 20140186958; US 20150166980.

[00169] In some embodiments, the RNA-guided DNA-binding agent comprises one or more heterologous functional domains (e.g., is or comprises a fusion polypeptide).

[00170] 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 1-10 NLS(s). 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 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. 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 some embodiments, the RNA-guided DNA-binding agent may be fused with 2, 3, 4, or 5 NLSs. In some embodiments, the RNA-guided DNA-binding agent may be fused with two NLSs. In certain circumstances, the two NLSs may be the same (*e.g.*, two SV40 NLSs) or different. 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: 96) or PKKKRRV (SEQ ID NO: 97). In some embodiments, the NLS may be a bipartite sequence, such as the NLS of nucleoplasmin, KRPAATKKAGQAKKKK (SEQ ID NO: 98). In a specific embodiment, a single PKKKRKV (SEQ ID NO: 96) 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.

[00171] In some embodiments, the heterologous functional domain may be capable of modifying the intracellular half-life of the RNA-guided DNA binding agent. In some embodiments, the half-life of the RNA-guided DNA binding agent may be increased. In some embodiments, the half-life of the RNA-guided DNA-binding agent may be reduced. In some embodiments, the heterologous functional domain may be capable of increasing the stability of the RNA-guided DNA-binding agent. In some embodiments, the heterologous functional domain may be capable of reducing the stability of the RNA-guided DNA-binding agent. In some embodiments, the heterologous functional domain may act as a signal peptide for protein degradation. In some embodiments, the protein degradation may be mediated by proteolytic enzymes, such as, for example, proteasomes, lysosomal proteases, or calpain proteases. In some embodiments, the heterologous functional domain may comprise a PEST sequence. In some embodiments, the RNA-guided DNA-binding agent may be modified by addition of ubiquitin or a polyubiquitin chain. In some embodiments, the ubiquitin may be a ubiquitin-like protein (UBL). Non-limiting examples of ubiquitin-like proteins include small

ubiquitin-like modifier (SUMO), ubiquitin cross-reactive protein (UCRP, also known as interferon-stimulated gene-15 (ISG15)), ubiquitin-related modifier-1 (URM1), neuronal-precursor-cell-expressed developmentally downregulated protein-8 (NEDD8, also called Rub1 in *S. cerevisiae*), human leukocyte antigen F-associated (FAT10), autophagy-8 (ATG8) and -12 (ATG12), Fau ubiquitin-like protein (FUB1), membrane-anchored UBL (MUB), ubiquitin fold-modifier-1 (UFM1), and ubiquitin-like protein-5 (UBL5).

[00172] In some embodiments, the heterologous functional domain may be a marker domain. Non-limiting examples of marker domains include fluorescent proteins, purification tags, epitope tags, and reporter gene sequences. In some embodiments, the marker domain may be a fluorescent protein. Non-limiting examples of suitable fluorescent proteins include green fluorescent proteins (*e.g.*, GFP, GFP-2, tagGFP, turboGFP, sfGFP, EGFP, Emerald, Azami Green, Monomeric Azami Green, CopGFP, AceGFP, ZsGreen1), yellow fluorescent proteins (*e.g.*, YFP, EYFP, Citrine, Venus, YPet, PhiYFP, ZsYellow1), blue fluorescent proteins (*e.g.*, EBFP, EBFP2, Azurite, mKalamal, GFPuv, Sapphire, T-sapphire), cyan fluorescent proteins (*e.g.*, ECFP, Cerulean, CyPet, AmCyan1, Midoriishi-Cyan), red fluorescent proteins (*e.g.*, mKate, mKate2, mPlum, DsRed monomer, mCherry, mRFP1, DsRed-Express, DsRed2, DsRed-Monomer, HcRed-Tandem, HcRed1, AsRed2, eqFP611, mRaspberry, mStrawberry, Jred), and orange fluorescent proteins (mOrange, mKO, Kusabira-Orange, Monomeric Kusabira-Orange, mTangerine, tdTomato) or any other suitable fluorescent protein. In other embodiments, the marker domain may be a purification tag or an epitope tag. Non-limiting exemplary tags include glutathione-S-transferase (GST), chitin binding protein (CBP), maltose binding protein (MBP), thioredoxin (TRX), poly(NANP), tandem affinity purification (TAP) tag, myc, AcV5, AU1, AU5, E, ECS, E2, FLAG, HA, nus, Softag 1, Softag 3, Strep, SBP, Glu-Glu, HSV, KT3, S, S1, T7, V5, VSV-G, 6xHis, 8xHis, biotin carboxyl carrier protein (BCCP), poly-His, and calmodulin. Non-limiting exemplary reporter genes include glutathione-S-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT), beta-galactosidase, beta-glucuronidase, luciferase, or fluorescent proteins.

[00173] In additional embodiments, the heterologous functional domain may target the RNA-guided DNA-binding agent to a specific organelle, cell type, tissue, or organ. In some embodiments, the heterologous functional domain may target the RNA-guided DNA-binding agent to mitochondria.

[00174] In further embodiments, the heterologous functional domain may be an effector domain. When the RNA-guided DNA-binding agent is directed to its target sequence, *e.g.*,

when a Cas nuclease is directed to a target sequence by a gRNA, the effector domain may modify or affect the target sequence. In some embodiments, the effector domain may be chosen from a nucleic acid binding domain, a nuclease domain (e.g., a non-Cas nuclease domain), an epigenetic modification domain, a transcriptional activation domain, or a transcriptional repressor domain. In some embodiments, the heterologous functional domain is a nuclease, such as a FokI nuclease. See, e.g., US Pat. No. 9,023,649. In some embodiments, the heterologous functional domain is a transcriptional activator or repressor. See, e.g., Qi et al., “Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression,” *Cell* 152:1173-83 (2013); Perez-Pinera et al., “RNA-guided gene activation by CRISPR-Cas9-based transcription factors,” *Nat. Methods* 10:973-6 (2013); Mali et al., “CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering,” *Nat. Biotechnol.* 31:833-8 (2013); Gilbert et al., “CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes,” *Cell* 154:442-51 (2013). As such, the RNA-guided DNA-binding agent essentially becomes a transcription factor that can be directed to bind a desired target sequence using a guide RNA. In some embodiments, the heterologous functional domain is a deaminase, such as a cytidine deaminase or an adenine deaminase. In certain embodiments, the heterologous functional domain is a C to T base converter (cytidine deaminase), such as an apolipoprotein B mRNA editing enzyme (APOBEC) deaminase.

D. Determination of efficacy of gRNAs

[00175] In some embodiments, the efficacy of a gRNA is determined when delivered or expressed together with other components forming an RNP. In some embodiments, the gRNA is expressed together with an RNA-guided DNA binding agent, such as a Cas protein, e.g. Cas9. In some embodiments, the gRNA is delivered to or expressed in a cell line that already stably expresses an RNA-guided DNA nuclease, such as a Cas nuclease or nickase, e.g. Cas9 nuclease or nickase. In some embodiments the gRNA is delivered to a cell as part of a RNP. In some embodiments, the gRNA is delivered to a cell along with a mRNA encoding an RNA-guided DNA nuclease, such as a Cas nuclease or nickase, e.g. Cas9 nuclease or nickase.

[00176] As described herein, use of an RNA-guided DNA nuclease and a guide RNA disclosed herein can lead to double-stranded breaks in the DNA which can produce errors in the form of insertion/deletion (indel) mutations upon repair by cellular machinery. Many mutations due to indels alter the reading frame or introduce premature stop codons and,

therefore, produce a non-functional protein. In some embodiments, the efficacy of particular gRNAs is determined based on *in vitro* models. In some embodiments, the *in vitro* model is HEK293 cells stably expressing Cas9 (HEK293_Cas9). In some embodiments the *in vitro* model is a peripheral blood mononuclear cell (PBMC). In some embodiments, the *in vitro* model is a T cell, such as primary human T cells. With respect to using primary cells, commercially available primary cells can be used to provide greater consistency between experiments. In some embodiments, the number of off-target sites at which a deletion or insertion occurs in an *in vitro* model (e.g., in T cell) is determined, e.g., by analyzing genomic DNA from transfected cells *in vitro* with Cas9 mRNA and the guide RNA. In some embodiments, such a determination comprises analyzing genomic DNA from the cells transfected *in vitro* with Cas9 mRNA, the guide RNA, and a donor oligonucleotide. Exemplary procedures for such determinations are provided in the working examples in which HEK293 cells, PBMCs, and human CD3⁺ T cells are used.

[00177] In some embodiments, the efficacy of particular gRNAs is determined across multiple *in vitro* cell models for a gRNA selection process. In some embodiments, a cell line comparison of data with selected gRNAs is performed. In some embodiments, cross screening in multiple cell models is performed.

[00178] In some embodiments, the efficacy of a guide RNA is measured by percent indels or percent genetic modifications of *LAG3*. In some embodiments, the efficacy of a guide RNA is measured by percent indels or percent genetic modifications at a *LAG3* locus. In some embodiments, the efficacy of a guide RNA is measured by percent indels or percent genetic modifications of *LAG3* at genomic coordinates of Table 1 or Table 2. In some embodiments, the percent editing of *LAG3* is compared to the percent indels or genetic modifications necessary to achieve knockdown of the *LAG3* protein products. In some embodiments, the efficacy of a guide RNA is measured by reduced or eliminated expression of LAG3 protein. In embodiments, said reduced or eliminated expression of LAG3 protein is as measured by flow cytometry, e.g., as described herein.

[00179] In some embodiments, the LAG3 protein expression is reduced or eliminated in a population of cells using the methods and compositions disclosed herein. In some embodiments, the population of cells is at least 55%, 60%, 65%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% LAG3 negative as measured by flow cytometry relative to a population of unmodified cells.

[00180] An “unmodified cell” (or “unmodified cells”) refers to a control cell (or cells) of the same type of cell in an experiment or test, wherein the “unmodified” control cell has not been

contacted with a LAG3 guide. Therefore, an unmodified cell (or cells) may be a cell that has not been contacted with a guide RNA, or a cell that has been contacted with a guide RNA that does not target LAG3.

[00181] In some embodiments, the efficacy of a guide RNA is measured by the number or frequency of indels or genetic modifications at off-target sequences within the genome of the target cell type, such as a T cell. In some embodiments, efficacious guide RNAs are provided which produce indels at off target sites at very low frequencies (e.g., <5%) in a cell population or relative to the frequency of indel creation at the target site. Thus, the disclosure provides for guide RNAs which do not exhibit off-target indel formation in the target cell type (e.g., a T cell), or which produce a frequency of off-target indel formation of <5% in a cell population or relative to the frequency of indel creation at the target site. In some embodiments, the disclosure provides guide RNAs which do not exhibit any off target indel formation in the target cell type (e.g., T cell). In some embodiments, guide RNAs are provided which produce indels at less than 5 off-target sites, e.g., as evaluated by one or more methods described herein. In some embodiments, guide RNAs are provided which produce indels at less than or equal to 4, 3, 2, or 1 off-target site(s) e.g., as evaluated by one or more methods described herein. In some embodiments, the off-target site(s) does not occur in a protein coding region in the target cell (e.g., hepatocyte) genome.

[00182] In some embodiments, detecting gene editing events, such as the formation of insertion/deletion (“indel”) mutations and insertion or homology directed repair (HDR) events in target DNA utilize linear amplification with a tagged primer and isolating the tagged amplification products (herein after referred to as “LAM-PCR,” or “Linear Amplification (LA)” method). In some embodiments, the efficacy of a guide RNA is measured by the levels of functional protein complexes comprising the expressed protein product of the gene. In some embodiments, the efficacy of a guide RNA is measured by flow cytometric analysis of TCR expression by which the live population of edited cells is analyzed for loss of the TCR.

E. T Cell Receptors (TCR)

[00183] In some embodiments, the engineered cells or population of cells comprising a genetic modification, e.g., of an endogenous nucleic acid sequence encoding LAG3, further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s), e.g., TRAC or TRBC.

[00184] In some embodiments, the engineered cells or population of cells comprising a genetic modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding LAG3 and insertion into the cell of heterologous sequence(s) encoding a targeting receptor, further comprise a modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s), e.g., TRAC or TRBC.

[00185] 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.

[00186] In some embodiments, the engineered cells or population of cells comprise a genetic modification of an endogenous nucleic acid sequence encoding LAG3, a genetic modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s), e.g., TRAC or TRBC; and modification, e.g., knockdown of an MHC class I gene, e.g., B2M or HLA-A. In some embodiments, an MHC class I gene is an HLA-B gene or an HLA-C gene.

[00187] In some embodiments, the engineered cells or population of cells comprise a genetic modification of an endogenous nucleic acid sequence encoding LAG3 and a genetic modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s), e.g., TRAC or TRBC; and a genetic modification, e.g., knockdown of an MHC class II gene, e.g., CIITA.

[00188] In some embodiments, the engineered cells or population of cells comprise a modification of an endogenous nucleic acid sequence encoding LAG3, a genetic modification, e.g., knockdown, of an endogenous nucleic acid sequence encoding TCR gene sequence(s), e.g., TRAC or TRBC; and a genetic modification, e.g. knockdown of a checkpoint inhibitor gene, e.g., TIM3, 2B4, or PD-1.

[00189] In some embodiments, the engineered cells or population of cells comprise a genetic modification of a LAG3 gene as assessed by sequencing, e.g., NGS, wherein at least 50%, 55%, 60%, 65%, preferably at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of cells comprise an insertion, deletion, or substitution in the endogenous LAG3 sequence. In some embodiments, at least 50% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence. In some embodiments, at least 55% of cells in the population comprise a

modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence. In some embodiments, at least 60% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence. In some embodiments, at least 65% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence. In some embodiments, at least 70% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence. In some embodiments, at least 75% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence. In some embodiments, at least 85% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence. In some embodiments, at least 90% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence. In some embodiments, at least 95% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence. In some embodiments, LAG3 is decreased by at least 50%, 55%, 60%, 65%, preferably at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified. In some embodiments, expression of LAG3 is decreased by at least 50%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified. In some embodiments, expression of LAG3 is decreased by at least 55%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified. In some embodiments, expression of LAG3 is decreased by at least 60%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified. In some embodiments, expression of LAG3 is decreased by at least 65%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified. In some embodiments, expression of LAG3 is decreased by at least 70%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified. In some embodiments, expression of LAG3 is decreased by at least 80%, or to below the limit of detection of the

assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified. In some embodiments, expression of LAG3 is decreased by at least 90%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified. In some embodiments, expression of LAG3 is decreased by at least 95%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified. Assays for LAG3 protein and mRNA expression are known in the art.

[00190] In some embodiments, the engineered cells or population of cells comprise a modification, e.g., knockdown, of a TCR gene sequence by gene editing, e.g., as assessed by sequencing, e.g., NGS, wherein at least 50%, 55%, 60%, 65%, preferably at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of cells comprise an insertion, deletion, or substitution in the endogenous TCR gene sequence. In some embodiments, TCR is decreased by at least 50%, 55%, 60%, 65%, preferably at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the TCR gene has not been modified. In certain embodiments, the TCR is TRAC or TRBC. Assays for TCR protein and mRNA expression are known in the art.

[00191] In some embodiments, the engineered cells or population of cells comprise an insertion of sequence(s) encoding a targeting receptor by gene editing, e.g., as assessed by sequencing, e.g., NGS.

[00192] 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.

[00193] 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.

[00194] 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.

III. Methods and Uses Including Therapeutic Methods and Uses and Methods of Preparing Engineered Cells or Immunotherapy Reagents

[00195] The gRNAs and associated methods and compositions disclosed herein are useful for making immunotherapy reagents, such as engineered cells.

[00196] In some embodiments, the gRNAs comprising the guide sequences of Table 1 together with an RNA-guided DNA nuclease such as a Cas nuclease induce DSBs, and non-homologous ending joining (NHEJ) during repair leads to a modification in a *LAG3* gene. In some embodiments, NHEJ leads to a deletion or insertion of a nucleotide(s), which induces a frame shift or nonsense mutation in a *LAG3* gene. In certain embodiments, gRNAs comprising guide sequences targeted to TCR sequences, e.g., TRAC and TRBC, are also delivered to the cell together with RNA-guided DNA nuclease such as a Cas nuclease, either together or separately, to make a genetic modification in a TCR sequence to inhibit the expression of a full-length TCR sequence. In certain embodiments, the gRNAs are sgRNAs.

[00197] In some embodiments, the subject is mammalian. In some embodiments, the subject is human. In some embodiments, the subject is a non-human primate

[00198] In some embodiments, the guide RNAs, compositions, and formulations are used to produce a cell ex vivo, e.g., an immune cell, e.g., a T cell with a genetic modification in a *LAG3* gene. The modified T cell may be a natural killer (NK) T-cell. The modified T cell may express a T-cell receptor, such as a universal TCR or a modified TCR. The T cell may express a CAR or a CAR construct with a zeta chain signaling motif.

[00199] Delivery of gRNA Compositions

[00200] Lipid nanoparticles (LNPs) are a well-known means for delivery of nucleotide and protein cargo, and may be used for delivery of the guide RNAs and compositions disclosed herein ex vivo and in vitro. In some embodiments, the LNPs deliver nucleic acid, protein, or nucleic acid together with protein.

[00201] In some embodiments, the invention comprises a method for delivering any one of the cells or populations of cells disclosed herein to a subject, wherein the gRNA is delivered via an LNP. In some embodiments, the gRNA/LNP is also associated with a Cas9 or an mRNA encoding Cas9.

[00202] In some embodiments, the invention comprises a composition comprising any one of the gRNAs disclosed and an LNP. In some embodiments, the composition further comprises a Cas9 or an mRNA encoding Cas9.

[00203] In some embodiments, LNPs associated with the gRNAs disclosed herein are for use in preparing cells as a medicament for treating a disease or disorder.

[00204] Electroporation is a well-known means for delivery of cargo, and any electroporation methodology may be used for delivery of any one of the gRNAs disclosed herein. In some embodiments, electroporation may be used to deliver any one of the gRNAs disclosed herein and Cas9 or an mRNA encoding Cas9.

[00205] In some embodiments, the invention comprises a method for delivering any one of the gRNAs disclosed herein to an ex vivo cell, wherein the gRNA is associated with an LNP or not associated with an LNP. In some embodiments, the gRNA/LNP or gRNA is also associated with a Cas9 or an mRNA encoding Cas9.

[00206] In some embodiments, the guide RNA compositions described herein, alone or encoded on one or more vectors, are formulated in or administered via a lipid nanoparticle; see e.g., WO2017/173054 and WO2021/222287, the contents of each of which are hereby incorporated by reference in their entirety.

[00207] In certain embodiments, the invention comprises DNA or RNA vectors encoding any of the guide RNAs comprising any one or more of the guide sequences described herein. In some embodiments, in addition to guide RNA sequences, the vectors further comprise nucleic acids that do not encode guide RNAs. Nucleic acids that do not encode guide RNA include, but are not limited to, promoters, enhancers, regulatory sequences, and nucleic acids encoding an RNA-guided DNA nuclease, which can be a nuclease such as Cas9. 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, 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 nuclease, which can be a Cas protein, such as, Cas9. In one embodiment, the Cas9 is from *Streptococcus pyogenes* (i.e., Spy 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.

[00208] In some embodiments, the components can be introduced as naked nucleic acid, as nucleic acid complexed with an agent such as a liposome or poloxamer, or they can be

delivered by viral vectors (e.g., adenovirus, AAV, herpesvirus, retrovirus, lentivirus). 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.

[00209] This description and exemplary embodiments should not be taken as limiting. For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing quantities, percentages, or proportions, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term “about,” to the extent they are not already so modified. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

EXAMPLES

[00210] 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 – Materials and Methods

Next-generation sequencing (“NGS”) and analysis for on-target cleavage efficiency

[00211] Genomic DNA was extracted using QuickExtract™ DNA Extraction Solution (Lucigen, Cat. No. QE09050) according to manufacturer's protocol.

[00212] To quantitatively determine the efficiency of editing at the target location in the genome, deep sequencing was utilized to identify the presence of insertions and deletions introduced by gene editing. PCR primers were designed around the target site within the gene of interest (e.g. *LAG3*), and the genomic area of interest was amplified. Primer sequence design was done as is standard in the field.

[00213] Additional PCR was performed according to the manufacturer's protocols (Illumina) to add chemistry for sequencing. The amplicons were sequenced on an Illumina MiSeq instrument. The reads were aligned to the human reference genome (e.g., hg38) after

eliminating those having low quality scores. The resulting files containing the reads were mapped to the reference genome (BAM files), where reads that overlapped the target region of interest were selected and the number of wild type reads versus the number of reads which contain an insertion or deletion (“indel”) was calculated.

[00214] The editing percentage (e.g., the “editing efficiency” or “indel percent”) as used in the examples is defined as the total number of sequence reads with insertions or deletions (“indels”) over the total number of sequence reads, including wild type.

Preparation of lipid nanoparticles.

[00215] Unless otherwise specified, 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.

[00216] 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 50:38:9:3 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, unless otherwise specified.

[00217] Lipid nanoparticles (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. The lipids in ethanol were mixed through a mixing cross with the two volumes of RNA solution. A fourth stream of water was mixed with the outlet stream of the cross through an inline tee (*See* WO2016010840 Figure 2.). The LNPs were held for 1 hour at room temperature (RT), 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 LNP's 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.

In vitro transcription (“IVT”) of mRNA

[00218] 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. Plasmid DNA containing a T7 promoter, a sequence for transcription, and a polyadenylation sequence was linearized by incubating at 37°C for 2 hours with XbaI with the following conditions: 200 ng/μL plasmid, 2 U/μL XbaI (NEB), and 1x reaction buffer. The XbaI was inactivated by heating the reaction at 65°C for 20 min. The linearized plasmid was purified from enzyme and buffer salts. The IVT reaction to generate modified mRNA was performed by incubating at 37°C for 1.5-4 hours in the following conditions: 50 ng/μL linearized plasmid; 2-5 mM each of GTP, ATP, CTP, and N1-methyl pseudo-UTP (Trilink); 10-25 mM ARCA (Trilink); 5 U/μL T7 RNA polymerase (NEB); 1 U/μL Murine RNase inhibitor (NEB); 0.004 U/μL Inorganic E. coli pyrophosphatase (NEB); and 1x reaction buffer. TURBO DNase (ThermoFisher) was added to a final concentration of 0.01 U/μL, and the reaction was incubated for an additional 30 minutes to remove the DNA template. The mRNA was purified using a MegaClear Transcription Clean-up kit (ThermoFisher) or a RNeasy Maxi kit (Qiagen) per the manufacturers' protocols. Alternatively, the mRNA was purified through a precipitation protocol, which in some cases was followed by HPLC-based purification. Briefly, after the DNase digestion, mRNA is purified using LiCl precipitation, ammonium acetate precipitation and sodium acetate precipitation. For HPLC purified mRNA, after the LiCl precipitation and reconstitution, the mRNA was purified by RP-IP HPLC (see, *e.g.*, Kariko, et al. Nucleic Acids Research, 2011, Vol. 39, No. 21 e142). The fractions chosen for pooling were combined and desalted by sodium acetate/ethanol precipitation as described above. In a further alternative method, mRNA was purified with a LiCl precipitation method followed by further purification by tangential flow filtration. RNA concentrations were determined by measuring the light absorbance at 260 nm (Nanodrop), and transcripts were analyzed by capillary electrophoresis by Bioanalyzer (Agilent).

[00219] *Streptococcus pyogenes* (“Spy”) Cas9 mRNA was generated from plasmid DNA encoding an open reading frame according to SEQ ID NOs: 801-803 (see sequences in Table 11). When SEQ ID NOs: 801-803 are referred to below with respect to RNAs, it is understood that Ts should be replaced with Us (which were N1-methyl pseudouridines as described above). Messenger RNAs used in the Examples include a 5' cap and a 3' poly-A tail, *e.g.*, up to 100 nts, and are identified by the SEQ ID NOs: 801-803 in Table 11.

Example 2 – LAG3 and Control Guide Design and Screening in HEK Cells**Table 3A: Human *LAG3* guide sequences**

Guide ID	SID	Guide Sequence	Genomic Coord (hg38)
CR007086	18	GUGCAUUGGUUCCGGAACCG	chr12:6774680-6774700
CR007087	12	GCUUUCCGCUAAGUGGUGAU	chr12:6774735-6774755
CR007088	19	UCGACUAGAGGAUGUGAGCC	chr12:6775444-6775464
CR007089	17	GGCUCACAUCCUCUAGUCGA	chr12:6775444-6775464
CR007090	20	AGCGCGGGGACUUCUCGCUA	chr12:6773872-6773892
CR007091	21	CGCCCCACAUAUCGAGGCC	chr12:6773993-6774013
CR007092	22	GCGUACACUGUCAAGGGAGU	chr12:6775286-6775306
CR007093	14	GCUCCAGCGUACACUGUCAA	chr12:6775292-6775312
CR007094	23	CGACUUUACCCUUCGACUAG	chr12:6775432-6775452
CR007095	24	UCCAUAGGUGCCCAACGCUC	chr12:6777783-6777803
CR007096	25	CACCGCGGCGCGGUACUCGC	chr12:6773923-6773943
CR007097	26	CCAUAGGUGCCCAACGCUCU	chr12:6777784-6777804
CR007098	27	UUGGUUCCGGAACCGGGGCC	chr12:6774685-6774705
CR007099	28	ACCGUGUAGCGGCGGGGCCU	chr12:6773772-6773792
CR007100	29	CGCUACACGGUGCUGAGCGU	chr12:6773781-6773801
CR007101	30	CGCGUCCAGCUGGAUGAGCG	chr12:6773844-6773864
CR007102	11	CCCGCCAUCCCCGUUUUACC	chr12:6772909-6772929
CR007103	31	UGACCCCUGCUCUUCGCAGA	chr12:6773297-6773317
CR007104	8	UUCCGCUAAGUGGUGAUGGG	chr12:6774732-6774752
CR007105	32	UCCAGCUGGAUGAGCGCGGC	chr12:6773848-6773868
CR007106	1	GCGGUCCCUGAGGUGCACCG	chr12:6773938-6773958
CR007107	33	GAUGGGGGGACUCCCGGACA	chr12:6774718-6774738
CR007108	34	GCCGGCCGCGCUCAUCCAGC	chr12:6773852-6773872
CR007109	35	GCAAGGGAUUCACCCUCCGC	chr12:6778272-6778292
CR007110	36	GUACGCUGGAGCAGGUUCCA	chr12:6775300-6775320
CR007111	37	ACUCGCCGCGGUCCGCGCGC	chr12:6773909-6773929
CR007112	38	CGCUCAGCACCGUGUAGCGG	chr12:6773780-6773800
CR007113	39	AUGAGCGCGGCCGCGCAGCGC	chr12:6773857-6773877
CR007114	40	GCUCACAUCCUCUAGUCGAA	chr12:6775443-6775463
CR007115	41	UCUAAGGCAGAAAAUCGUCU	chr12:6778252-6778272
CR007116	42	UGCUCAGCGUACACUGUCA	chr12:6775293-6775313
CR007117	4	GACGUUGAAGCCAUCUCUGU	chr12:6774816-6774836
CR007118	43	CACCGUGUAGCGGCGGGGCC	chr12:6773773-6773793
CR007119	44	ACCGCGCCGCGGUGCACCUC	chr12:6773929-6773949
CR007120	45	AGCUUCCGCUAAGUGGUGA	chr12:6774736-6774756
CR007121	46	CUGUGCAUUGGUUCCGGAAC	chr12:6774678-6774698
CR007122	47	UGGGGCUCUCCAGAGCGUU	chr12:6777796-6777816
CR007123	5	AGAGGAAGCUUCCGCUAAG	chr12:6774742-6774762
CR007124	15	UGGCAAUGCCAGCUGUACCA	chr12:6777433-6777453

Guide ID	SID	Guide Sequence	Genomic Coord (hg38)
CR007125	48	CUCAGCACCGUGUAGCGGCG	chr12:6773778-6773798
CR007126	49	GCUCAGCACCGUGUAGCGGC	chr12:6773779-6773799
CR007127	50	CGCCGGCGAGUACCGCGCCG	chr12:6773918-6773938
CR007128	51	UGUACGCUGGAGCAGGUUCC	chr12:6775299-6775319
CR007129	52	UUGGCAAUGCCAGCUGUACC	chr12:6777432-6777452
CR007130	53	UGAUGGGGGGACUCCCGGAC	chr12:6774719-6774739
CR007131	2	GUUCCGGAACCAAUGCACAG	chr12:6774678-6774698
CR007132	54	UUGACAGUGUACGCUGGAGC	chr12:6775292-6775312
CR007133	55	CGCGCUCAUCCAGCUGGACG	chr12:6773846-6773866
CR007134	13	CCACGCUCAGCACCGUGUAG	chr12:6773783-6773803
CR007135	56	CGUCCCGCCCCACAUACUCG	chr12:6773998-6774018
CR007136	57	CGUACACUGUCAAGGGAGUU	chr12:6775285-6775305
CR007137	58	GCGAGAAGUCCCCGCGCUGC	chr12:6773870-6773890
CR007138	59	AAGCGUUCUUGUCCAGAUAC	chr12:6777325-6777345
CR007139	60	CUCGCCGGCGUCCGCGCGCC	chr12:6773908-6773928
CR007140	16	AGGGUGAAUCCCUUGCUCUA	chr12:6778268-6778288
CR007141	61	UGGGCGGUCAGGGCGGCUGA	chr12:6774654-6774674
CR007142	62	UGUGCAUUGGUUCCGGAACC	chr12:6774679-6774699
CR007143	63	CCGCUACACGGUGCUGAGCG	chr12:6773780-6773800
CR007144	64	CCGCGCCGCGGUGCACCUCU	chr12:6773930-6773950
CR007145	65	GCGCUCAUCCAGCUGGACGC	chr12:6773845-6773865
CR007146	66	GAUGAGCGCGGCCGGCAGCG	chr12:6773856-6773876
CR007147	67	GUUCAAAAUGACCCAGUCGG	chr12:6774627-6774647
CR007148	7	GUCCCCCAUACCCACUUAG	chr12:6774727-6774747
CR007149	68	CCCUGAGGUGCACC GCGCGC	chr12:6773933-6773953
CR007150	69	ACUCCCUUGACAGUGUACGC	chr12:6775286-6775306
CR007151	70	CCAGCCCGGCGCGCGGACGC	chr12:6773901-6773921
CR007152	71	ACGCUUUGUGUGGAGCUCUC	chr12:6777339-6777359
CR007153	72	UGUGAGGUGACUCCAGUAUC	chr12:6777310-6777330
CR007154	10	AGAGUCCAUGGGGCUGACUU	chr12:6774771-6774791
CR007155	73	ACGGUGCUGAGCGUGGGUCC	chr12:6773787-6773807
CR007156	74	GUCCCGGAGGCCUGCGCAGC	chr12:6773803-6773823
CR007157	75	CAGCCUCUGUGCAUUGGUUC	chr12:6774672-6774692
CR007158	76	AACAGUGAGGUUAUACAUGA	chr12:6774840-6774860
CR007159	77	CAAGUCAGCCCCAUGGACUC	chr12:6774770-6774790
CR007160	3	UUACCUGGAGCCACCCAAAG	chr12:6772894-6772914
CR007161	78	AUCUCUCAGAGCCUCCGACU	chr12:6774613-6774633
CR007162	79	CUCCCGCUGCGCAGGCCUCC	chr12:6773808-6773828
CR007163	80	GGGGCAGCCUCCCGCUGCGC	chr12:6773816-6773836
CR007164	9	GCAAUGCCAGCUGUACCAGG	chr12:6777435-6777455
CR007165	81	GCUCAAUGCCACUGUCACAU	chr12:6775513-6775533
CR007166	82	CUUCCGCUAAGUGGUGAUG	chr12:6774734-6774754

Guide ID	SID	Guide Sequence	Genomic Coord (hg38)
CR007167	83	CUGGACAAGAACGCUUUGUG	chr12:6777329-6777349
CR007168	6	UCCCCAGGAGGAGUCCACU	chr12:6775380-6775400
CR007169	84	CUUUGGGUCACCUGGAUCCC	chr12:6777276-6777296
CR007170	85	ACAUACUCGAGGCCUGGCC	chr12:6773987-6774007
CR007171	86	CCCGGACAGGGACUCGGCCC	chr12:6774706-6774726
CR007172	87	GGGACUCCCGGACAGGGACU	chr12:6774712-6774732
CR007173	88	CGCCAUUGUCUCCAGUCACC	chr12:6775414-6775434

[00220] For each crRNA, the indicated 20 nt guide sequence is included within an N20GUUUUAGAGCUAUGCUGUUUUG (SEQ ID NO: 99) nucleic acid sequence, where “N20” represents the guide sequence.

[00221] Initial guide selection was performed *in silico* using a human reference genome (e.g., hg38) and user defined genomic regions of interest (e.g., *LAG3*), for identifying PAMs in the regions of interest. For each identified PAM, analyses were performed and statistics reported. gRNA molecules were further selected and rank-ordered based on a number of criteria known in the art (e.g., GC content, predicted on-target activity, and potential off-target activity).

[00222] A total of 88 guide RNAs targeting the protein exonic coding regions of *LAG3* (ENSG00000089692) were tested. Guide sequences and corresponding genomic coordinates are provided (Table 1).

[00223] Guides were initially screened for editing efficiency in HEK293_Cas9 cells. A human embryonic kidney adenocarcinoma cell line HEK293 constitutively expressing Spy Cas9 (“HEK293_Cas9”) was cultured in DMEM media supplemented with 10% fetal bovine serum. Cells were plated at a density of 10,000 cells/well in a 96-well plate about 24 hours prior to transfection (~70% confluent at time of transfection). Cells were transfected with Lipofectamine RNAiMAX (ThermoFisher, Cat. 13778150) according to the manufacturer’s protocol. Cells were transfected with a lipoplex containing individual guide (25 nM), trRNA (25 nM), Lipofectamine RNAiMAX (0.3 µL/well) and OptiMem. DNA isolation and NGS analysis were performed as described in Example 1. Table 3B shows indel % at the *LAG3* locus by these guides in HEK293_Cas9 cells using two primer sets. “No data” indicates that a primer set failed to generate a calculated editing percentage.

Table 3B – Mean percent editing at the LAG3 locus in HEK293 cells.

Guide ID	Mean	SD	Guide ID	Mean	SD	Guide ID	Mean	SD
CR007086	31.60	4.46	CR007115	65.10	5.11	CR007145	4.73	0.33
CR007087	68.47	1.72	CR007116	16.37	0.98	CR007146	9.20	1.93
CR007088	2.23	0.26	CR007117	60.97	2.48	CR007147	11.93	1.44
CR007089	40.70	4.65	CR007118	0.17	0.05	CR007148	45.27	5.44
CR007090	17.43	2.81	CR007119	1.20	0.22	CR007149	19.57	3.04
CR007091	0.50	0.08	CR007120	19.03	1.80	CR007150	4.17	0.83
CR007092	24.70	10.53	CR007121	No data		CR007151	5.27	0.42
CR007093	61.47	3.44	CR007122	23.23	4.37	CR007152	45.20	3.86
CR007094	2.63	0.33	CR007123	44.80	3.35	CR007153	8.77	2.15
CR007095	40.30	1.27	CR007124	78.90	2.90	CR007154	61.23	5.60
CR007096	30.63	4.19	CR007125	9.57	2.15	CR007155	17.80	3.12
CR007097	50.17	2.81	CR007126	6.07	1.17	CR007156	3.83	0.39
CR007098	0.17	0.05	CR007127	1.23	0.12	CR007157	21.60	0.73
CR007099	0.23	0.12	CR007128	3.70	1.16	CR007158	33.67	9.77
CR007100	21.93	2.62	CR007129	45.30	1.76	CR007159	5.20	0.43
CR007101	7.87	1.84	CR007130	2.30	0.29	CR007160	78.97	3.24
CR007102	60.87	7.04	CR007131	68.03	3.29	CR007161	25.07	3.41
CR007103	27.60	7.59	CR007132	2.57	1.03	CR007162	48.60	7.08
CR007104	73.63	5.00	CR007133	5.17	2.07	CR007163	0.30	0.08
CR007105	47.33	13.32	CR007134	34.70	6.35	CR007164	75.03	2.08
CR007106	64.97	11.23	CR007135	6.93	2.16	CR007165	49.33	4.42
CR007107	3.57	0.21	CR007136	8.37	0.76	CR007166	23.03	4.57
CR007108	3.67	0.68	CR007137	8.53	1.47	CR007167	75.10	3.74
CR007109	34.23	2.28	CR007138	43.73	13.66	CR007168	73.63	2.67
CR007110	14.40	2.33	CR007139	0.93	0.21	CR007169	10.30	1.08
CR007111	7.57	2.56	CR007140	68.83	4.39	CR007170	3.07	0.58
CR007112	11.43	0.60	CR007141	53.33	4.81	CR007171	2.77	0.58
CR007113	1.50	0.41	CR007142	9.07	0.98	CR007172	15.40	2.47
CR007114	27.13	5.92	CR007143	5.13	0.82	CR007173	23.47	4.01
			CR007144	15.23	2.04			

Example 3 – LAG3 guide screening in human CD3⁺ T cells

[00224] Guides from the editing screen in HEK293_Cas9 cells from Example 2 were screened for editing efficiency in human CD3⁺ T cells. CD3⁺ T cells are comprised of multiple T cell populations including CD4⁺ T helper cells and CD8⁺ cytotoxic T cells. These cells can be isolated from whole blood or from leukaphoresis samples. T cells can be modified to specifically target cancerous cells and to be less immunogenic, by engineering T cells using Cas9-mediated editing.

Example 3.1. Delivery of RNPs to T cells

[00225] T cells were either obtained commercially (e.g. Human Peripheral Blood CD4⁺CD45RA⁺ T Cells, Frozen, Stem Cell Technology, Cat. 70029) or prepared internally from a leukopak. For internal preparation, T cells were first enriched from a leukopak using a commercially available kit (e.g., EasySep™ Human T Cell Isolation Kit, Stem Cell Technology). Enriched T cells were aliquoted and frozen down (at 5x10⁶/vial) for future use. Vials were subsequently thawed as needed, and activated by addition of 3:1 ratio of CD3/CD28 beads (Dynabeads, Life Technologies) in T cell media (RPMI 1640, FBS, L-glutamine, non-essential amino acids, sodium pyruvate, HEPES buffer, 2-mercaptoethanol and optionally IL2). RNP was generated by pre-annealing individual crRNA and trRNA by mixing equivalent amounts of reagent and incubating at 95°C for 2 min and cooling to room temperature. The dual guide (dgRNA) consisting of pre-annealed crRNA and trRNA, was incubated with Spy Cas9 protein to form a ribonucleoprotein (RNP) complex. CD3⁺ T cells were transfected in triplicate with an RNP containing Spy Cas9 (10nM), individual guide (10 nM) and tracer RNA (10 nM) nucleofected using the P3 Primary Cell 96-well Nucleofector™ Kit (Lonza, Cat. V4SP-3960) using the manufacturer's Amaxa™ 96-well Shuttle™ Protocol for Stimulated Human T Cells. T cell media was added to cells immediately post-nucleofection and cultured for 2 days or more.

[00226] Two days post nucleofection, genomic DNA was prepared as described in Example 1 and NGS analysis performed. Table 4A and FIG. 1 show % editing NGS data in CD3⁺ T cells.

Table 4A: Mean percent editing at the LAG3 locus in primary T Cells.

	Donor 018		Donor 100		Donor 315		Donor 797	
Guide ID	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CR007106	47.23	1.21	52.63	4.74	68.47	2.12	62.63	2.61
CR007131	63.43	3.81	62.83	3.78	76.47	1.87	63.20	2.31
CR007160	32.73	4.95	43.13	12.38	53.33	1.16	37.93	4.57
CR007117	42.30	3.91	45.17	8.43	50.27	3.07	45.63	1.66
CR007123	29.97	1.82	31.87	3.16	44.97	1.86	34.87	1.90
CR007168	34.37	3.04	29.30	2.84	50.80	3.05	35.93	2.68
CR007148	31.30	2.88	36.50	6.05	44.53	0.50	35.10	3.15
CR007104	28.20	2.69	29.73	1.65	32.67	2.40	31.40	1.59

	Donor 018		Donor 100		Donor 315		Donor 797	
Guide ID	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CR007164	44.90	2.13	53.77	6.24	57.77	2.12	44.90	0.70
CR007154	28.57	3.25	27.70	4.17	36.13	0.81	31.00	0.76
CR007102	21.20	3.44	29.03	3.49	35.23	2.20	24.10	1.93
CR007087	26.20	2.60	26.33	3.04	29.93	2.54	28.87	2.60
CR007134	No data		No data		37.77	3.71	25.40	1.59
CR007093	25.40	1.22	27.20	2.10	35.13	1.38	28.43	0.81
CR007124	31.63	3.59	36.37	5.73	49.37	1.45	42.90	3.57
CR007140	28.57	1.70	29.97	3.81	36.43	3.07	30.47	0.32
CR007089	13.60	1.31	13.13	4.74	32.03	0.50	20.53	2.37
CR007097	14.73	2.72	21.00	2.35	27.33	1.16	22.43	1.27
CR007167	11.33	0.38	10.50	0.87	14.93	1.10	12.23	1.37
CR007138	No data		No data		No data		No data	
CR007115	7.10	0.96	8.10	1.97	11.30	0.82	7.40	0.61
CR007095	6.47	0.57	6.80	0.80	13.03	0.70	10.53	0.40
CR003187 -VEGFA	15.60	4.90	17.80	14.71	16.33	1.78	13.40	1.15

[00227] Seven days following electroporation, cells were restimulated using a 1:1 ratio of cells to CD3/CD28 beads (Dynabeads, Life Technologies). On the eleventh day post electroporation, T cells were assayed by flow cytometry to assess LAG3 surface protein expression. T cells were incubated with antibodies recognizing LAG3 (Biolegend, Cat. 369314) and stained with fixable live dead dye (Thermo Fisher, Cat. L34975). Cells were subsequently processed on a Cytotflex LX instrument (Beckman Coulter) and data analyzed using the FlowJo software package. The percentage of cells expressing LAG3 cell surface proteins are shown in Table 4B and Figs. 2A-B.

Table 4B – Percentage of LAG3 positive human CD3+ T Cells after dual guide editing

	Donor 018		Donor 100		Donor 315		Donor 797	
crRNA	Mean % LAG3+	SD	Mean % LAG3+	SD	Mean % LAG3+	SD	Mean % LAG3+	SD
CR007106	5.6	0.3	7.4	1.1	6.0	0.5	5.5	0.2
CR007131	8.2	0.5	6.9	0.4	7.6	0.7	5.1	0.6

	Donor 018		Donor 100		Donor 315		Donor 797	
crRNA	Mean % LAG3+	SD	Mean % LAG3+	SD	Mean % LAG3+	SD	Mean % LAG3+	SD
CR007160	35.8	2.1	34.0	1.0	20.9	0.9	22.4	0.5
CR007117	38.1	2.4	31.2	0.7	33.3	3.1	23.8	1.8
CR007123	54.6	1.0	52.6	2.5	43.4	1.2	31.2	2.3
CR007168	55.2	0.4	54.6	2.0	35.4	1.7	34.5	3.0
CR007148	56.6	1.8	56.7	3.0	43.7	0.6	33.5	1.2
CR007104	58.5	1.7	62.1	3.1	61.0	1.7	44.8	1.3
CR007164	63.8	1.8	56.5	5.6	43.0	2.7	41.0	1.7
CR007154	64.8	0.3	69.0	2.1	59.2	2.0	49.5	0.8
CR007102	65.3	1.7	71.8	3.1	54.7	2.5	52.1	2.6
CR007087	66.7	1.1	64.9	4.8	64.2	3.0	48.1	2.0
CR007134	69.8	2.2	68.3	3.4	41.6	3.4	41.2	0.8
CR007093	70.0	1.2	67.9	1.6	53.7	1.9	46.0	0.9
CR007124	74.2	1.6	71.0	0.6	52.8	2.9	46.8	1.2
CR007140	77.9	0.4	74.9	1.5	63.2	2.3	57.2	0.7
CR007089	80.4	2.7	78.8	0.4	55.3	4.1	56.8	5.5
CR007097	83.8	2.2	82.5	2.5	76.4	0.9	69.2	0.8
CR007167	86.9	1.7	88.6	0.6	76.7	4.6	70.0	1.0
CR007138	87.9	1.4	89.5	1.6	80.5	2.1	74.8	1.7
CR007115	88.0	0.6	90.4	1.1	77.5	4.6	73.5	2.2
CR007095	88.7	1.0	87.1	3.2	78.2	2.0	67.5	1.4
CR003187 -VEGFA	88.7	1.0	85.6	6.0	81.3	0.9	74.1	0.7
CR003187	88.1	1.4	90.1	2.4	81.3	0.9	74.1	0.7

Example 4 - Off-target analysis of LAG3 guides

[00228] A biochemical method (See, e.g., Cameron et al., *Nature Methods*. 6, 600-606; 2017) was used to determine potential off-target genomic sites cleaved by Cas9 using guides targeting *LAG3*. Guides showing the most LAG3 negative cells in Example 3 were tested for potential off-target genomic cleavage sites with this assay. In this experiment, 15 dgRNAs targeting human *LAG3* were screened in triplicate using purified human genomic DNA from

pooled male human peripheral blood mononuclear cells (PBMCs) alongside a positive control guide, G000645 with known off-target profile. The number of potential off-target sites detected using a guide concentration of 64 nM in the biochemical assay are shown in Table 4C.

Table 4C – Potential off-target sites for LAG3 guides predicted by biochemical assay

Guide	Target	Total Sites
CR007087	LAG3	43
CR007093	LAG3	45
CR007102	LAG3	100
CR007104	LAG3	126
CR007106	LAG3	272
CR007117	LAG3	224
CR007123	LAG3	51
CR007124	LAG3	223
CR007131	LAG3	93
CR007134	LAG3	78
CR007148	LAG3	233
CR007154	LAG3	504
CR007160	LAG3	442
CR007164	LAG3	232
CR007168	LAG3	770
G000645	VEGFA	6071

Example 4.1. Targeted sequencing for validating potential off-target sites

[00229] In known off-target detection assays such as the biochemical method used above, a large number of potential off-target sites are typically recovered, by design, so as to “cast a wide net” for potential sites that can be validated in other contexts, e.g., in a primary cell of interest. For example, the biochemical method typically overrepresents the number of potential off-target sites as the assay utilizes purified high molecular weight genomic DNA free of the cell environment and is dependent on the dose of Cas9 RNP used. Accordingly, potential off-target sites identified by these methods may be validated using targeted sequencing of the identified potential off-target sites.

[00230] In one approach, primary T cells are treated with LNPs comprising Cas9 mRNA and a sgRNA of interest (e.g., a sgRNA having potential off-target sites for evaluation). The primary T cells are then lysed and primers flanking the potential off-target site(s) are used to generate an amplicon for NGS analysis. Identification of indels at a certain level may validate potential off-target site, whereas the lack of indels found at the potential off-target site may indicate a false positive in the off-target assay.

Example 5- Single Guide Analysis in CD3⁺ T cells

Example 5.1.- Delivery of RNPs to T cells

[00231] T cells were prepared as outlined in Example 3. Single guide (sgRNA) was incubated at 95°C for 2 min and cooled to room temperature. Then the sgRNA was incubated with Spy Cas9 protein to form a ribonucleoprotein (RNP) complex. CD3⁺ T cells were transfected with an RNP containing Spy Cas9 (10 nM) and individual sgRNA (10 nM) nucleofected using the P3 Primary Cell 96-well Nucleofector™ Kit (Lonza, Cat. V4SP-3960) using the manufacturer's Amaxa™ 96-well Shuttle™ Protocol for Stimulated Human T Cells. T cell media was added to cells immediately post-nucleofection and cultured for 10 more days before harvesting and performing NGS as in Example 1.

[00232] On day seven post electroporation, media was prepared with IL-2 and CD3/CD28 beads (Dnyabeads). The cell to bead ratio was 1:1 for restimulation. Restimulated editing levels were measured by NGS as in Example 1 and shown in Fig. 3A and Table 5. Restimulated protein levels were measured by flow cytometry as in Example 3.1 and shown in Fig. 3B and Table 6.

Table 5: Mean percent editing at the LAG3 locus in primary T Cells after sgRNA editing

Guide	Donor 1162		Donor 907	
	Mean % Editing	SD	Mean % Editing	SD
G015069	88.37	1.40	87.77	3.07
G015068	53.80	3.10	66.83	2.85
G015070	57.43	1.90	71.90	6.82
G015071	37.17	0.84	50.90	5.28
VEGFA (Control) G000294	81.70	1.42	66.85	6.45

Table 6: Mean percentage of LAG3 positive human CD3⁺ T Cells after sgRNA editing (n=3)

Guide	Donor 1162		Donor 907	
	Mean	SEM	Mean	SEM
G015069	3.86	0.36	2.43	0.16
G015068	26.40	2.97	14.17	2.47
G15070	32.23	2.51	34.33	3.18
G015071	43.23	3.86	37.93	1.92
VEGFA (Control) G000294	60.77	3.90	77.53	3.00

Example 6 – LAG3 editing with various doses of RNA

[00233] T cells were edited with increasing amounts of lipid nanoparticles co-formulated with mRNA encoding Cas9 and a sgRNA targeting LAG3 or control loci. Cryopreserved T-cells were thawed in a water bath. T-cells were resuspended at a density of 15×10^6 per 10 mL of cytokine media. TransAct (Miltenyi) was added at a 1:100 dilution to each flask, and was incubated at 37°C overnight.

Example 6.1. LNP Incubation

[00234] T-cells were harvested and resuspended in Media (X-VIVO™ base media without serum) prepared with cytokines (IL-2 (200U/mL), IL-7 (5ng/mL), and IL-15 (5ng/mL)). ApoE3 was added to a final concentration of 1ug/mL in X-VIVO™ 5% HS media. LNPs formulated with guides shown in Table 7 were prepared to a 2x final concentration in the ApoE media, and were incubated at 37°C for 15 minutes. 50 µL of the LNP-ApoE and 50 µL of T-cells were mixed and incubated for 24 hours. NGS analysis was performed as in Example 1. NGS data was shown in Table 7 and Fig. 4.

Table 7: Percent indels for T cells editing with various doses of LNPs

Dose (µg/ml)	G018433		G018434		G018438 (Control)		G000739 (Control)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
10	97.03	0.40	98.33	0.15	94.63	1.36	0.17	0.06
5	94.73	0.64	98.13	0.15	91.80	1.11	0.10	0.00
2.5	87.27	0.97	95.93	0.59	76.30	3.38	0.17	0.06
1.25	61.70	4.92	84.57	3.00	28.47	3.58	0.13	0.06
0.625	31.57	5.15	62.67	6.12	6.53	1.23	0.10	0.00

Dose ($\mu\text{g/ml}$)	G018433		G018434		G018438 (Control)		G000739 (Control)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0.3125	12.10	1.92	33.10	8.12	1.70	0.10	0.10	0.00
0.15	3.87	0.72	14.77	3.61	0.87	0.29	0.10	0.00
0.07	1.53	0.32	6.17	0.21	0.47	0.21	0.17	0.06

Example 7 – Engineered T cells with inhibitor gene knockouts

[00235] T cells were engineered with a series of gene disruptions and insertions. Healthy donor cells were treated sequentially with three LNPs, each LNP co-formulated with mRNA encoding Cas9 and a sgRNA targeting. Cells were first edited to knockout TRBC. A transgenic T cell receptor targeting Wilm's tumor antigen (WT1 TCR) (SEQ ID NO: 1001) was then integrated into the TRAC cut site by delivering a homology directed repair template using AAV. Lastly, T cells were edited to knock out LAG3.

7.1. T cell Preparation

[00236] Healthy human donor apheresis was obtained commercially (HemaCare), washed and re-suspended in CliniMACS PBS/EDTA buffer (Miltenyi cat. 130-070-525). T cells from three donors were isolated via positive selection using CD4 and CD8 magnetic beads (Miltenyi BioTec, Cat.130-030-401, 130-030-801) using the CliniMACS Plus and CliniMACS LS disposable kit. T cells were aliquoted into vials and cryopreserved in a 1:1 formulation of Cryostor CS10 (StemCell Technologies cat. 07930) and Plasmalyte A (Baxter cat. 2B2522X) for future use. The day before initiating T cell editing, cells were thawed and rested overnight in T cell activation media (TCAM): CTS OpTmizer (ThermoFisher, Cat. A3705001) supplemented with 2.5% human AB serum (Gemini, Cat. 100-512), 1X GlutaMAX (ThermoFisher, Cat.35050061), 10 mM HEPES (ThermoFisher, Cat. 15630080), 200 U/mL IL-2 (Peprotech, Cat. 200-02), IL-7 (Peprotech, Cat. 200-07), IL-15 (Peprotech, Cat. 200-15).

7.2. LNP Treatment and Expansion of T cells

[00237] On day 1, LNPs containing Cas9 mRNA and sgRNA targeting TRBC (G016239) were incubated at a concentration of 5 $\mu\text{g/mL}$ in TCAM containing 1 $\mu\text{g/mL}$ rhApoE3 (Peprotech, Cat. 350-02). Meanwhile, T cells were harvested, washed, and resuspended at a density of 2×10^6 cells/mL in TCAM with a 1:50 dilution of T Cell TransAct, human reagent

(Miltenyi, Cat. 130-111-160). T cells and LNP-ApoE media were mixed at a 1:1 ratio and T cells plated in culture flasks overnight.

[00238] On day 3, T cells were harvested, washed, and resuspended at a density of 1×10^6 cells/mL in TCAM. LNPs containing Cas9 mRNA and sgRNA targeting TRAC (G013006) were incubated at a concentration of 5 ug/mL in TCAM containing 5 ug/mL rhApoE3 (Peprtech, Cat. 350-02). T cells and LNP-ApoE media were mixed at a 1:1 ratio and T cells plated in culture flasks. WT1 TCR-containing AAV was then added to each group at a MOI of 3×10^5 genome copies/cell. Cells with these edits are designated “WT1 T cells” in the tables and figures.

[00239] On day 4, T cells were harvested, washed, and resuspended at a density of 1×10^6 cells/mL in TCAM. LNPs containing Cas9 mRNA and one of the gRNAs listed in Table 9. LNPs were incubated at a concentration of 5 ug/mL in TCAM containing 5 ug/mL rhApoE3 (Peprtech, Cat. 350-02). LNP-ApoE solution was then added to the appropriate culture at a 1:1 ratio.

[00240] On days 5-11, T cells were transferred to a 24-well GREX plate (Wilson Wolf, Cat. 80192) in T cell expansion media (TCM): CTS OpTmizer (Thermofisher, Cat. A3705001) supplemented with 5% CTS Immune Cell Serum Replacement (Thermofisher, Cat. A2596101), 1X GlutaMAX (Thermofisher, Cat. 35050061), 10 mM HEPES (Thermofisher, Cat. 15630080), 200 U/mL IL-2 (Peprtech, Cat. 200-02), IL-7 (Peprtech, Cat. 200-07), and IL-15 (Peprtech, Cat. 200-15)). Cells were expanded per manufacturers protocols. T-cells were expanded for 6-days, with media exchanges every other day. Cells were counted using a Vi-CELL cell counter (Beckman Coulter) and all samples showed similar fold-expansion.

7.3. Quantification of T cell editing by flow cytometry and NGS

[00241] Post expansion, edited T cells were assayed by flow cytometry to determine TCR insertion and memory cell phenotype. T cells were incubated with an antibody cocktail targeting the following molecules: CD4 (Biolegend, Cat. 300524), CD8 (Biolegend, Cat. 301045), Vb8 (Biolegend, Cat. 348106), CD3 (Biolegend, Cat. 300327), CD62L (Biolegend, Cat. 304844), CD45RO (Biolegend, Cat. 304230), CCR7 (Biolegend, Cat. 353214), and CD45RA (Biolegend, Cat. 304106). Cells were subsequently processed on a Cytotflex LX instrument (Beckman Coulter) and data analyzed using the FlowJo software package.

[00242] The percentage of cells expressing relevant cell surface proteins following sequential T cell engineering are shown in Tables 8A-8C and Figs. 5A-5C. Table 8A shows

the total percentage of CD8⁺ cells following T cell engineering and the proportion of CD8⁺ or CD4⁺ cells expressing the engineered TCR as detected with the Vb8 antibody. Table 8B and Fig. 5A shows the percentage of CD8⁺Vb8⁺ cells with the stem cell memory phenotype (Tscm; CD45RA⁺ CD62L⁺). Table 8C and Fig. 5B shows the percentage of CD8⁺Vb8⁺ cells with the central memory cell phenotype (Tcm; CD45RO⁺ CD62L⁺). Table 8C and Fig. 5C show the percentage of total cells with the effector memory phenotype (Tem; CD45RO⁺ CD62L⁻ CCR7⁻). In addition to flow cytometry analysis, genomic DNA was prepared and NGS analysis performed as described in Example 1 to determine editing rates at each target site. Table 9 and Figs. 6A-6B show results for indel frequency at loci engineered in the third sequential edit.

Table 8A – Percentage of cells expressing designated surface proteins.

	%CD8 ⁺ of total		%Vb8 ⁺ of CD8 ⁺		%Vb8 ⁺ of CD4 ⁺	
Sample	Mean	SD	Mean	SD	Mean	SD
WT1 T cells	57.77	7.95	57.87	5.02	62.63	5.17
G018434	57.67	8.66	58.27	5.65	64.50	5.81

Table 8B – Percentage of Vb8⁺CD8⁺ cells with stem cell memory phenotype

	% CD45RA ⁺ CD62L ⁺ CCR7 ⁺		% CD45RA ⁺ CD62L ⁺ CCR7 ⁻	
Sample	Mean	SD	Mean	SD
WT1 T cells	13.64	12.95	15.88	12.61
G018434	12.20	11.12	18.45	14.10

Table 8C – Percentage of Vb8⁺CD8⁺ cells with central memory cell phenotype or with effector memory cell phenotype.

	% CD45RO ⁺ CD62L ⁺ CCR7 ⁺		%CD45RO ⁺ CD62L ⁺ CCR7 ⁻		% CD45RO ⁺ CD62L ⁻ CCR7 ⁻	
Sample	Mean	SD	Mean	SD	Mean	SD
WT1 T cells	3.48	1.70	17.73	7.12	36.67	24.49
G018434	3.00	1.37	17.30	6.61	35.10	23.14

Table 9 – Indel frequency for genes engineered in third sequential edit

	Primer Set 1			Primer Set 2		
Sample	Mean	SD	n	Mean	SD	n
G018434 [LAG3]	0.99	0.00	2	0.99	0.00	3
G018436 [TIM3]	0.83	0.06	2	0.85	0.05	3
G020845 [TIM3]	0.92	0.01	2	0.88	0.05	3

Sample	Primer Set 1			Primer Set 2		
	Mean	SD	n	Mean	SD	n
G021215 [2B4]	no data			0.58	0.06	3
G021216 [2B4]	0.61	0.06	2	0.63	0.05	3

Example 8 – Inhibition of Proliferation of AML Cells Using Engineered T-cells

[00243] Checkpoint inhibitors are associated with immune exhaustion which can arise in proliferative disorders such as cancer. Proliferative disorders associated with WT1 include a number of hematological malignancies including acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). Cells prepared by the methods of Example 7 to reduce expression of checkpoint inhibitors and induce expression of the WT1 TCR are tested using known models of AML both in vitro and in vivo (see, e.g., Zhou et al., Blood (2009) 114:3793-3802).

[00244] *In vitro* cell killing assays can be used to detect the activity of T cells against cells with abnormal proliferation. The ability of T-cells prepared by the method of Example 7 to eliminate target cells is assessed by co-culturing the engineered T-cells with primary leukemic blasts (CD33+ cells) from an acute myeloid leukemia (AML) with high expression of the WT1 antigen. Leukemic blasts can be assayed as in, e.g., Example 9.

[00245] A human WT1 expression AML cell line are injected into mice via an intravenous route at a lethal dose on day 0. Cells prepared by the methods of Example 7 are administered intravenously at day 14. Mice are monitored for survival. Mice treated with T-cells engineered to express the WT1 TCR are viable longer than mice treated with T cells not expressing the WT1 TCR. Mice treated with T-cells engineered to inhibit expression of a checkpoint inhibitor in addition to expression the WT1 TCR are viable longer than mice treated with T cells expressing the WT1 TCR and all of the endogenous checkpoint inhibitors.

Example 9 – Target cell killing by engineered T cells

[00246] T cells engineered in Example 7 were assessed for the ability to kill primary leukemic blasts using the Incucyte Live Imaging system. Briefly, T cells were engineered to insert a WT1 TCR into the TRAC locus and knockout the TRBC locus in two T cell donor samples (WT1 T cells). At the third engineering step, some WT1 T cells were treated to knockout LAG3 using G018434. WT1-expressing primary leukemic blasts harvested from 3 HLA-A*02:01 patients were labeled with the NucLight Rapid Red reagent (Essen Biosciences) for live-cell nuclear labeling and co-cultured with engineered lymphocytes at

different (5:1, 1:1 and 1:5) effector to target (E:T) ratios in the presence of Caspase 3/7 green reagent. Twenty thousand blasts for the E:T ratio of 5:1 and 75,000 blasts for E:T ratios of 1:1 and 1:5 were used. Co-cultures were seeded in flat-bottom 96 well plates in X-VIVO supplemented with 5% FBS, 1% penicillin-streptomycin (BioWhittaker/Lonza), 2 mM glutamine (BioWhittaker/Lonza), 1 µg/mL CD28 monoclonal antibody (BD Biosciences), G-CSF and IL-3 (20 ng/mL; Bio-techne). Images were taken every 60 minutes and green fluorescent Caspase 3/7 signal in red target cells was quantified using the Incucyte Live-Cell Imaging and Analysis software (Essen Biosciences). Live AML cells fluoresce in red only, while dead AML cells fluoresce in both red and green in this assay. Table 10 and Figs 7A-I show mean $\pm$ SEM of the mean area of each image (um²/image) fluorescing in both green and red. For each effector population, engineered cells from 2 distinct T cell donors, as above, were used.

Table 10 – Mean area of each image (um²/image) fluorescing in both green and red following exposure of WT1 expressing AML cells to engineered T cells

			AML only		WT1 T cells		G018434	
Cell	E:T	Time (h)	Mean	SD	Mean	SD	Mean	SD
pAML1	1:5	1	3354	425	3558	1253	3865	1433
pAML1	1:5	2	4950	59	5246	986	5631	880
pAML1	1:5	3	6025	567	6879	69	7789	1046
pAML1	1:5	4	6558	1074	8320	644	9872	152
pAML1	1:5	5	7545	1341	9755	2081	11461	713
pAML1	1:5	6	7666	2215	10902	2883	12593	1372
pAML1	1:5	7	7752	2651	11272	3548	12916	1983
pAML1	1:5	8	8092	2428	11439	2987	12541	1507
pAML1	1:5	9	8082	2776	11135	3449	12153	1907
pAML1	1:5	10	7993	2486	10709	3038	11675	1259
pAML1	1:5	11	8056	2822	10507	2363	11459	774
pAML1	1:5	12	8169	3029	9784	2530	10380	1020
pAML1	1:5	13	8012	3644	9293	2710	9557	1061
pAML1	1:5	14	7859	3600	8941	2398	9211	762
pAML1	1:5	15	7449	4138	8363	2053	8529	455
pAML1	1:5	16	7051	3838	7641	2231	7875	320
pAML1	1:5	17	6789	3482	7049	2066	7246	255
pAML1	1:5	18	6541	3407	6760	1893	6805	88
pAML1	1:5	19	6298	3571	6229	2005	6467	13
pAML1	1:5	20	5860	3227	5748	1623	5932	89
pAML1	1:5	21	5739	3232	5509	1603	5476	113
pAML1	1:5	22	5486	3336	4638	130	5580	370

			AML only		WT1 T cells		G018434	
Cell	E:T	Time (h)	Mean	SD	Mean	SD	Mean	SD
pAML1	1:5	23	5048	3561	5171	1804	5201	40
pAML1	1:5	24	4875	3090	4682	1375	4641	45
pAML1	1:1	0	2827	509	13236	792	15194	6678
pAML1	1:1	1	3354	425	13804	5477	19700	10903
pAML1	1:1	2	4950	59	19052	5728	27535	13626
pAML1	1:1	3	6025	567	26223	6816	38197	15826
pAML1	1:1	4	6558	1074	35499	4617	54205	17599
pAML1	1:1	5	7545	1341	45746	2096	66773	13027
pAML1	1:1	6	7666	2215	53641	2027	74720	8108
pAML1	1:1	7	7752	2651	56628	3269	80989	6238
pAML1	1:1	8	8092	2428	61273	4878	83580	7844
pAML1	1:1	9	8082	2776	60981	3635	83207	6463
pAML1	1:1	10	7993	2486	61917	4229	84022	8033
pAML1	1:1	11	8056	2822	61609	2905	82642	9299
pAML1	1:1	12	8169	3029	61417	3408	80962	8914
pAML1	1:1	13	8012	3644	59798	1717	77608	9537
pAML1	1:1	14	7859	3600	59052	2513	76300	11155
pAML1	1:1	15	7449	4138	57879	1056	74709	11978
pAML1	1:1	16	7051	3838	54344	223	71536	10860
pAML1	1:1	17	6789	3482	53236	871	68877	11648
pAML1	1:1	18	6541	3407	51299	1296	67389	12000
pAML1	1:1	19	6298	3571	50863	1123	65897	12281
pAML1	1:1	20	5860	3227	49140	509	63785	12535
pAML1	1:1	21	5739	3232	49144	560	61051	12487
pAML1	1:1	22	5486	3336	48020	1809	58750	9309
pAML1	1:1	23	5048	3561	45640	2347	55942	4114
pAML1	1:1	24	4875	3090	44944	1257	57469	8462
pAML1	5:1	0	260	94	11330	5133	10383	7093
pAML1	5:1	1	429	220	13196	4743	13169	9428
pAML1	5:1	2	627	209	19065	4442	18910	11413
pAML1	5:1	3	776	151	27606	4557	27727	14947
pAML1	5:1	4	908	160	39114	1808	37391	14496
pAML1	5:1	5	915	198	50163	2145	46082	13297
pAML1	5:1	6	952	211	57449	4329	52019	12750
pAML1	5:1	7	911	254	61267	6398	54150	11088
pAML1	5:1	8	1029	293	63554	4397	54533	13450
pAML1	5:1	9	1029	387	63260	3866	53782	13379
pAML1	5:1	10	1037	420	61830	3055	50826	12276
pAML1	5:1	11	1132	485	61700	1135	50799	16311
pAML1	5:1	12	1180	540	60149	442	48119	14262

			AML only		WT1 T cells		G018434	
Cell	E:T	Time (h)	Mean	SD	Mean	SD	Mean	SD
pAML1	5:1	13	1140	562	57421	409	45843	14799
pAML1	5:1	14	1166	592	56596	2191	43050	15285
pAML1	5:1	15	1119	613	54439	3881	42112	16153
pAML1	5:1	16	985	492	52113	4265	37991	13233
pAML1	5:1	17	984	510	50843	6004	37983	14986
pAML1	5:1	18	874	487	49954	6454	37026	15740
pAML1	5:1	19	816	422	47822	6412	35339	15240
pAML1	5:1	20	775	463	47665	7717	33865	14839
pAML1	5:1	21	780	474	46969	7606	33299	15107
pAML1	5:1	22	768	523	46262	11319	32617	16130
pAML1	5:1	23	661	352	41513	4150	29345	11086
pAML1	5:1	24	639	353	42152	6450	30030	13727
pAML2	1:5	1	5874	3593	-128	7179	-1144	8250
pAML2	1:5	2	8990	2303	4735	8794	4066	7288
pAML2	1:5	3	10952	2796	8464	9292	9197	6863
pAML2	1:5	4	10432	5484	12167	7231	13739	5140
pAML2	1:5	5	10817	4334	16482	4777	18762	3923
pAML2	1:5	6	11265	6212	21199	2227	22715	847
pAML2	1:5	7	10492	7822	22442	1160	24862	1272
pAML2	1:5	8	10232	6164	23501	1059	25094	1193
pAML2	1:5	9	10518	7563	24885	2627	25575	3193
pAML2	1:5	10	9472	7470	24114	3122	25168	3787
pAML2	1:5	11	9351	8653	23935	5093	24267	3978
pAML2	1:5	12	8614	8981	23349	4417	23047	4748
pAML2	1:5	13	8045	8457	21814	5360	21830	5412
pAML2	1:5	14	6364	8590	20406	4731	20024	4979
pAML2	1:5	15	5270	9421	18965	4726	18167	4633
pAML2	1:5	16	3744	9415	17229	5532	15951	6157
pAML2	1:5	17	1725	8950	15487	5228	14246	5223
pAML2	1:5	18	763	9149	13494	5668	12139	4973
pAML2	1:5	19	-606	8876	11518	5824	10427	5286
pAML2	1:5	20	-1906	8549	9623	4578	8578	4849
pAML2	1:5	21	-3578	8225	8117	5170	6905	4630
pAML2	1:5	22	-3438	6448	6284	4824	4313	5875
pAML2	1:5	23	-3948	9503	4222	8373	1348	8945
pAML2	1:5	24	-5862	8226	1826	6660	-498	6927
pAML2	1:1	0	2827	509	13236	792	15194	6678
pAML2	1:1	1	3354	425	13804	5477	19700	10903
pAML2	1:1	2	4950	59	19052	5728	27535	13626
pAML2	1:1	3	6025	567	26223	6816	38197	15826

			AML only		WT1 T cells		G018434	
Cell	E:T	Time (h)	Mean	SD	Mean	SD	Mean	SD
pAML2	1:1	4	6558	1074	35499	4617	54205	17599
pAML2	1:1	5	7545	1341	45746	2096	66773	13027
pAML2	1:1	6	7666	2215	53641	2027	74720	8108
pAML2	1:1	7	7752	2651	56628	3269	80989	6238
pAML2	1:1	8	8092	2428	61273	4878	83580	7844
pAML2	1:1	9	8082	2776	60981	3635	83207	6463
pAML2	1:1	10	7993	2486	61917	4229	84022	8033
pAML2	1:1	11	8056	2822	61609	2905	82642	9299
pAML2	1:1	12	8169	3029	61417	3408	80962	8914
pAML2	1:1	13	8012	3644	59798	1717	77608	9537
pAML2	1:1	14	7859	3600	59052	2513	76300	11155
pAML2	1:1	15	7449	4138	57879	1056	74709	11978
pAML2	1:1	16	7051	3838	54344	223	71536	10860
pAML2	1:1	17	6789	3482	53236	871	68877	11648
pAML2	1:1	18	6541	3407	51299	1296	67389	12000
pAML2	1:1	19	6298	3571	50863	1123	65897	12281
pAML2	1:1	20	5860	3227	49140	509	63785	12535
pAML2	1:1	21	5739	3232	49144	560	61051	12487
pAML2	1:1	22	5486	3336	48020	1809	58750	9309
pAML2	1:1	23	5048	3561	45640	2347	55942	4114
pAML2	1:1	24	4875	3090	44944	1257	57469	8462
pAML2	5:1	0	8544	6060	28453	4417	30682	6113
pAML2	5:1	1	5486	2264	25864	6247	28717	17226
pAML2	5:1	2	5389	2108	34805	5246	37294	19089
pAML2	5:1	3	5464	1824	45856	4647	52389	26964
pAML2	5:1	4	5618	1740	63955	154	71604	32338
pAML2	5:1	5	5707	1704	81405	8675	90561	33792
pAML2	5:1	6	5933	1616	96371	19045	107698	36546
pAML2	5:1	7	5794	1747	104357	24148	114853	32752
pAML2	5:1	8	5951	1493	110958	27899	121794	36525
pAML2	5:1	9	5951	1635	112764	28875	124586	37679
pAML2	5:1	10	5812	1582	114032	27647	126614	39621
pAML2	5:1	11	5923	1592	114965	26691	127047	42637
pAML2	5:1	12	5652	1846	115372	26562	124228	40799
pAML2	5:1	13	5699	1742	115277	23959	123311	41800
pAML2	5:1	14	5540	1738	112945	21372	121853	47137
pAML2	5:1	15	5410	1741	112218	22840	119511	45965
pAML2	5:1	16	5246	1920	110570	23432	116067	44018
pAML2	5:1	17	4937	1814	108018	20391	114444	46302
pAML2	5:1	18	4867	1720	107372	19439	111696	45180

			AML only		WT1 T cells		G018434	
Cell	E:T	Time (h)	Mean	SD	Mean	SD	Mean	SD
pAML2	5:1	19	4613	1713	105140	19053	108696	42307
pAML2	5:1	20	4545	1686	103490	15295	106840	45485
pAML2	5:1	21	4424	1608	101914	15531	103516	42626
pAML2	5:1	22	4503	1393	97216	3580	104422	46692
pAML2	5:1	23	4421	1496	102070	16516	100893	38756
pAML2	5:1	24	4147	1398	97400	12875	95829	39164
pAML3	1:5	1	12582	3249	10361	2988	13450	1776
pAML3	1:5	2	15298	4803	13869	4097	17261	952
pAML3	1:5	3	18963	6429	18221	5604	21985	2753
pAML3	1:5	4	22457	6780	23222	5874	27755	2390
pAML3	1:5	5	24776	6067	27676	5023	32400	1324
pAML3	1:5	6	25600	4957	30200	3609	35033	213
pAML3	1:5	7	24996	4617	30785	2581	34896	160
pAML3	1:5	8	24152	3733	31237	943	35432	1377
pAML3	1:5	9	23057	3264	30090	757	33099	725
pAML3	1:5	10	21695	3120	29159	79	31556	864
pAML3	1:5	11	20472	2724	27871	360	30172	1208
pAML3	1:5	12	19238	2457	25938	12	28323	1825
pAML3	1:5	13	17694	2026	24060	494	26137	2181
pAML3	1:5	14	16470	2080	22555	726	24557	2139
pAML3	1:5	15	15310	1591	21151	24	22643	1869
pAML3	1:5	16	14109	1249	19708	143	21277	2248
pAML3	1:5	17	12846	1490	18351	61	19737	1842
pAML3	1:5	18	11779	1441	16742	130	18294	2102
pAML3	1:5	19	10918	885	15463	357	16843	1716
pAML3	1:5	20	10100	1021	14204	233	15522	1714
pAML3	1:5	21	9347	760	13434	171	14620	2192
pAML3	1:5	22	8605	960	11888	589	12919	3069
pAML3	1:5	23	7917	111	10922	1673	11988	3916
pAML3	1:5	24	7298	494	9859	1286	10974	3994
pAML3	1:1	0	68259	25727	97207	18214	99268	11650
pAML3	1:1	1	55874	3593	86234	13603	84335	21749
pAML3	1:1	2	58990	2303	100750	10127	101768	22106
pAML3	1:1	3	60952	2796	121403	6229	121378	24160
pAML3	1:1	4	60432	5484	139119	1211	146801	22198
pAML3	1:1	5	60817	4334	165467	14640	177736	15958
pAML3	1:1	6	61265	6212	189110	28702	202164	2650
pAML3	1:1	7	60492	7822	203695	40458	218625	5759
pAML3	1:1	8	60232	6164	216221	47755	233022	14611
pAML3	1:1	9	60518	7563	225326	55164	239473	21050

			AML only		WT1 T cells		G018434	
Cell	E:T	Time (h)	Mean	SD	Mean	SD	Mean	SD
pAML3	1:1	10	59472	7470	229487	63218	245910	23578
pAML3	1:1	11	59351	8653	231348	60991	246588	24195
pAML3	1:1	12	58614	8981	233469	62597	244765	30089
pAML3	1:1	13	58045	8457	232452	63694	244950	29428
pAML3	1:1	14	56364	8590	230905	58826	242654	27265
pAML3	1:1	15	55270	9421	227313	59089	240076	30689
pAML3	1:1	16	53744	9415	224262	58529	234441	30991
pAML3	1:1	17	51725	8950	219496	54219	230176	26605
pAML3	1:1	18	50763	9149	214232	55788	223835	27128
pAML3	1:1	19	49394	8876	210735	51467	219440	25391
pAML3	1:1	20	48094	8549	208073	50046	214170	23696
pAML3	1:1	21	46422	8225	203897	48794	209062	21152
pAML3	1:1	22	46562	6448	204648	40380	208991	14888
pAML3	1:1	23	46052	9503	200231	49006	204352	25984
pAML3	1:1	24	44138	8226	193355	41211	196535	21111
pAML3	5:1	0	1497	181	16645	4286	18192	9755
pAML3	5:1	1	1057	557	17905	8072	19206	14762
pAML3	5:1	2	1365	689	23199	9299	24441	16289
pAML3	5:1	3	1787	743	31499	12103	33240	20906
pAML3	5:1	4	2038	587	42510	11975	45169	24094
pAML3	5:1	5	2242	301	51711	11057	54997	23214
pAML3	5:1	6	2197	121	58555	7821	60579	21166
pAML3	5:1	7	2117	38	61037	5875	62009	18896
pAML3	5:1	8	1914	40	60639	5195	61627	18609
pAML3	5:1	9	1780	67	60299	6339	59008	18087
pAML3	5:1	10	1591	112	58519	7213	57478	18803
pAML3	5:1	11	1470	121	56218	7214	54658	17888
pAML3	5:1	12	1327	83	53737	7027	51337	17928
pAML3	5:1	13	1217	153	52654	7676	48883	18902
pAML3	5:1	14	1093	140	50252	8369	47392	20334
pAML3	5:1	15	1025	139	47335	7062	44515	18821
pAML3	5:1	16	940	165	45286	7436	42214	17649
pAML3	5:1	17	867	151	43601	8013	40924	18384
pAML3	5:1	18	796	137	42304	7789	38065	16036
pAML3	5:1	19	743	157	41231	7661	37443	16330
pAML3	5:1	20	678	128	38692	6746	36358	17251
pAML3	5:1	21	641	85	37339	6557	34832	17097
pAML3	5:1	22	578	83	36893	7383	33911	16892
pAML3	5:1	23	513	112	34432	3912	31524	11745
pAML3	5:1	24	485	93	33681	5254	30508	12417

Example 9 – Additional Embodiments

[00247] Embodiment 1 is an engineered cell comprising a genetic modification in a human LAG3 sequence, within genomic coordinates of chr12: 6772483-6778455.

[00248] Embodiment 2 is the engineered cell of embodiment 1, wherein the genetic modification is selected from an insertion, a deletion, and a substitution.

[00249] Embodiment 3 is the engineered cell of embodiment 1 or 2, wherein the genetic modification inhibits expression of the LAG3 gene.

[00250] Embodiment 4 is the engineered cell of any one of embodiments 1-3, wherein the genetic modification comprises a modification of at least one nucleotide within the genomic coordinates selected from:

LAG 3 NO	Genomic Coordinates (hg38)
LAG3-1	chr12:6773938-6773958
LAG3-2	chr12:6774678-6774698
LAG3-3	chr12:6772894-6772914
LAG3-4	chr12:6774816-6774836
LAG3-5	chr12:6774742-6774762
LAG3-6	chr12:6775380-6775400
LAG3-7	chr12:6774727-6774747
LAG3-8	chr12:6774732-6774752
LAG3- 9	chr12:6777435-6777455
LAG3- 10	chr12:6774771-6774791
LAG3- 11	chr12:6772909-6772929
LAG3- 12	chr12:6774735-6774755
LAG3- 13	chr12:6773783-6773803
LAG3- 14	chr12:6775292-6775312
LAG3- 15	chr12:6777433-6777453
LAG3- 16	chr12:6778268-6778288
LAG3- 17	chr12:6775444-6775464
LAG3-24	chr12:6777783-6777803
LAG3-26	chr12:6777784-6777804
LAG3-41	chr12:6778252-6778272

LAG 3 NO	Genomic Coordinates (hg38)
LAG3-59	chr12:6777325-6777345
LAG3-83	chr12:6777329-6777349

optionally the genomic coordinates selected from those targeted by LAG3-1 through LAG3-15; LAG3-1 through LAG3-11; LAG3-1 through LAG3-4; or LAG3-1, LAG3-4, LAG3-5, and LAG3-9.

[00251] Embodiment 5 is the engineered cell of any one of embodiments 1-4, wherein the engineered cell comprises a genetic modification within the genomic coordinates of an endogenous T cell receptor (TCR) sequence, wherein the genetic modification inhibits expression of the TCR gene.

[00252] Embodiment 6 is the engineered cell of embodiment 5, wherein the TCR gene is TRAC or TRBC.

[00253] Embodiment 7 is the engineered cell of embodiment 6, comprising a genetic modification of TRBC within genomic coordinates selected from:

TRBC NO:	Genomic Coordinates (hg38)
TRBC-1	chr7:142791996-142792016
TRBC-2	chr7:142792047-142792067
TRBC-3	chr7:142792008-142792028
TRBC-4	chr7:142791931-142791951
TRBC-5	chr7:142791930-142791950
TRBC-6	chr7:142791748-142791768
TRBC-7	chr7:142791720-142791740
TRBC-8	chr7:142792041-142792061
TRBC-9	chr7:142802114-142802134
TRBC-10	chr7:142792009-142792029
TRBC-11	chr7:142792697-142792717
TRBC-12	chr7:142791963-142791983
TRBC-13	chr7:142791976-142791996
TRBC-14	chr7:142791974-142791994
TRBC-15	chr7:142791970-142791990
TRBC-16	chr7:142791948-142791968
TRBC-17	chr7:142791913-142791933

TRBC NO:	Genomic Coordinates (hg38)
TRBC-18	chr7:142791961-142791981
TRBC-19	chr7:142792068-142792088
TRBC-20	chr7:142791975-142791995
TRBC-21	chr7:142791773-142791793
TRBC-22	chr7:142791919-142791939
TRBC-23	chr7:142791834-142791854
TRBC-24	chr7:142791878-142791898
TRBC-25	chr7:142802141-142802161
TRBC-26	chr7:142791844-142791864
TRBC-27	chr7:142801154-142801174
TRBC-28	chr7:142791961-142791981
TRBC-29	chr7:142792001-142792021
TRBC-30	chr7:142791979-142791999
TRBC-31	chr7:142792041-142792061
TRBC-32	chr7:142792003-142792023
TRBC-33	chr7:142791984-142792004
TRBC-34	chr7:142792002-142792022
TRBC-35	chr7:142791966-142791986
TRBC-36	chr7:142792007-142792027
TRBC-37	chr7:142791993-142792013
TRBC-38	chr7:142791902-142791922
TRBC-39	chr7:142791724-142791744
TRBC-40	chr7:142791973-142791993
TRBC-41	chr7:142791920-142791940
TRBC-42	chr7:142791994-142792014
TRBC-43	chr7:142791887-142791907
TRBC-44	chr7:142791907-142791927
TRBC-45	chr7:142791952-142791972
TRBC-46	chr7:142791721-142791741
TRBC-47	chr7:142792718-142792738
TRBC-48	chr7:142791729-142791749

TRBC NO:	Genomic Coordinates (hg38)
TRBC-49	chr7:142791911-142791931
TRBC-50	chr7:142791867-142791887
TRBC-51	chr7:142791899-142791919
TRBC-52	chr7:142791727-142791747
TRBC-53	chr7:142791949-142791969
TRBC-54	chr7:142791933-142791953
TRBC-55	chr7:142791932-142791952
TRBC-56	chr7:142792057-142792077
TRBC-57	chr7:142791940-142791960
TRBC-58	chr7:142791747-142791767
TRBC-59	chr7:142791881-142791901
TRBC-60	chr7:142791779-142791799
TRBC-61	chr7:142792054-142792074
TRBC-62	chr7:142792069-142792089
TRBC-63	chr7:142792712-142792732
TRBC-64	chr7:142791729-142791749
TRBC-65	chr7:142791821-142791841
TRBC-66	chr7:142792052-142792072
TRBC-67	chr7:142791916-142791936
TRBC-68	chr7:142791899-142791919
TRBC-69	chr7:142791772-142791792
TRBC-70	chr7:142792714-142792734
TRBC-71	chr7:142792042-142792062
TRBC-72	chr7:142791962-142791982
TRBC-73	chr7:142791988-142792008
TRBC-74	chr7:142791982-142792002
TRBC-75	chr7:142792049-142792069
TRBC-76	chr7:142791839-142791859
TRBC-77	chr7:142791893-142791913
TRBC-78	chr7:142791945-142791965
TRBC-79	chr7:142791964-142791984

TRBC NO:	Genomic Coordinates (hg38)
TRBC-80	chr7:142791757-142791777
TRBC-81	chr7:142792048-142792068
TRBC-82	chr7:142791774-142791794
TRBC-83	chr7:142792048-142792068
TRBC-84	chr7:142791830-142791850
TRBC-85	chr7:142791909-142791929
TRBC-86	chr7:142791912-142791932
TRBC-87	chr7:142791766-142791786
TRBC-88	chr7:142791880-142791900
TRBC-89	chr7:142791919-142791939

[00254] Embodiment 8 is the engineered cell of any one of embodiments 5-7, comprising a genetic modification of TRAC within genomic coordinates selected from:

TRAC NO:	Genomic Coordinates (hg38)
TRAC-90	chr14:22547524-22547544
TRAC-91	chr14:22550581-22550601
TRAC-92	chr14:22550608-22550628
TRAC-93	chr14:22550611-22550631
TRAC-94	chr14:22550622-22550642
TRAC-95	chr14:22547529-22547549
TRAC-96	chr14:22547512-22547532
TRAC-97	chr14:22547525-22547545
TRAC-98	chr14:22547536-22547556
TRAC-99	chr14:22547575-22547595
TRAC-100	chr14:22547640-22547660
TRAC-101	chr14:22547647-22547667
TRAC-102	chr14:22547777-22547797
TRAC-103	chr14:22549638-22549658
TRAC-104	chr14:22549646-22549666
TRAC-105	chr14:22550600-22550620
TRAC-106	chr14:22550605-22550625

TRAC NO:	Genomic Coordinates (hg38)
TRAC-107	chr14:22550625-22550645
TRAC-108	chr14:22539116-22539136
TRAC-109	chr14:22539120-22539140
TRAC-110	chr14:22547518-22547538
TRAC-111	chr14:22539082-22539102
TRAC-112	chr14:22539061-22539081
TRAC-113	chr14:22539097-22539117
TRAC-114	chr14:22547697-22547717
TRAC-115	chr14:22550571-22550591
TRAC-116	chr14:22550631-22550651
TRAC-117	chr14:22550658-22550678
TRAC-118	chr14:22547712-22547732
TRAC-119	chr14:22550636-22550656
TRAC-120	chr14:22550636-22550656
TRAC-121	chr14:22550582-22550602
TRAC-122	chr14:22550606-22550626
TRAC-123	chr14:22550609-22550629
TRAC-124	chr14:22547691-22547711
TRAC-125	chr14:22547576-22547596
TRAC-126	chr14:22549648-22549668
TRAC-127	chr14:22549660-22549680
TRAC-128	chr14:22547716-22547736
TRAC-129	chr14:22547514-22547534
TRAC-130	chr14:22550662-22550682
TRAC-131	chr14:22550593-22550613
TRAC-132	chr14:22550612-22550632
TRAC-133	chr14:22547521-22547541
TRAC-134	chr14:22547540-22547560
TRAC-135	chr14:22539121-22539141
TRAC-136	chr14:22547632-22547652
TRAC-137	chr14:22547674-22547694

TRAC NO:	Genomic Coordinates (hg38)
TRAC-138	chr14:22549643-22549663
TRAC-139	chr14:22547655-22547675
TRAC-140	chr14:22547667-22547687
TRAC-141	chr14:22539085-22539105
TRAC-142	chr14:22549634-22549654
TRAC-143	chr14:22539064-22539084
TRAC-144	chr14:22547639-22547659
TRAC-145	chr14:22547731-22547751
TRAC-146	chr14:22547734-22547754
TRAC-147	chr14:22547591-22547611
TRAC-148	chr14:22547657-22547677
TRAC-149	chr14:22547519-22547539
TRAC-150	chr14:22549674-22549694
TRAC-151	chr14:22547678-22547698
TRAC-152	chr14:22539087-22539107
TRAC-153	chr14:22547595-22547615
TRAC-154	chr14:22547633-22547653
TRAC-155	chr14:22547732-22547752
TRAC-156	chr14:22547656-22547676
TRAC-157	chr14:22539086-22539106
TRAC-158	chr14:22547491-22547511
TRAC-159	chr14:22547618-22547638
TRAC-160	chr14:22549644-22549664
TRAC-161	chr14:22547522-22547542
TRAC-162	chr14:22539089-22539109
TRAC-163	chr14:22539062-22539082
TRAC-164	chr14:22547597-22547617
TRAC-165	chr14:22547677-22547697
TRAC-166	chr14:22549645-22549665
TRAC-167	chr14:22550610-22550630
TRAC-168	chr14:22547511-22547531

TRAC NO:	Genomic Coordinates (hg38)
TRAC-169	chr14:22550607-22550627
TRAC-170	chr14:22550657-22550677
TRAC-171	chr14:22550604-22550624
TRAC-172	chr14:22539132-22539152
TRAC-173	chr14:22550632-22550652
TRAC-174	chr14:22547571-22547591
TRAC-175	chr14:22547711-22547731
TRAC-176	chr14:22547666-22547686
TRAC-177	chr14:22547567-22547587
TRAC-178	chr14:22547624-22547644
TRAC-185	chr14:22547501-22547521
TRAC-213	chr14:22547519-22547539
TRAC-214	chr14:22547556-22547576
TRAC-215	chr14:22547486-22547506
TRAC-216	chr14:22547487-22547507
TRAC-217	chr14:22547493-22547513
TRAC-218	chr14:22547502-22547522

optionally the genetic modification is within genomic coordinates selected from chr14:22547524-22547544, chr14:22547529-22547549, chr14:22547525-22547545, chr14:22547536-22547556, chr14:22547501-22547521, chr14:22547556-22547576, and chr14:22547502-22547522.

[00255] Embodiment 9 is the engineered cell of any one of embodiments 1-8, wherein the cell comprises a genetic modification, wherein the genetic modification inhibits expression of one or more MHC class I proteins.

[00256] Embodiment 10 is the engineered cell of embodiment 9, wherein the genetic modification that inhibits expression of one or more MHC class I proteins is a genetic modification in a B2M sequence, wherein the genetic modification is within genomic coordinates selected from:

Genomic Location (hg38)	Guide Sequence	SEQ ID NO	B2M-#
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Genomic Location (hg38)	Guide Sequence	SEQ ID NO	B2M-#
chr15:44711469-44711494	UGGCUGGGCA CGCGUUUAAU AUAAG		B2M-1
chr15:44711472-44711497	CUGGGCACGC GUUUAUAUA AGUGG	115	B2M-2
chr15:44711483-44711508	UUUAAUAUAA GUGGAGGCGU CGCGC	116	B2M-3
chr15:44711486-44711511	AAUAUAAGUG GAGGCGUCGC GCUGG	117	B2M-4
chr15:44711487-44711512	AUAUAAGUGG AGGCGUCGCG CUGGC	118	B2M-5
chr15:44711512-44711537	GGGCAUUCU GAAGCUGACA GCAUU	119	B2M-6
chr15:44711513-44711538	GGCAUUCUG AAGCUGACAG CAUUC	120	B2M-7
chr15:44711534-44711559	AUUCGGGCCG AGAUGUCUCG CUCCG	121	B2M-8
chr15:44711568-44711593	CUGUGCUCGC GCUACUCUCU CUUUC	122	B2M-9
chr15:44711573-44711598	CUCGCGCUAC UCUCUCUUUC UGGCC	123	B2M-10
chr15:44711576-44711601	GCGCUACUCU CUCUUUCUGG CCUGG	124	B2M-11
chr15:44711466-44711491	AUAUUAACG CGUGCCCAGC CAAUC	125	B2M-12
chr15:44711522-44711547	UCUCGGCCCG AAUGCUGUCA GCUUC	126	B2M-13
chr15:44711544-44711569	GCUAAGGCCA CGGAGCGAGA CAUCU	127	B2M-14
chr15:44711559-44711584	AGUAGCGCGA GCACAGCUAA GGCCA	128	B2M-15
chr15:44711565-44711590	AGAGAGAGUA GCGCGAGCAC AGCUA	129	B2M-16
chr15:44711599-44711624	GAGAGACUCA CGCUGGAUAG CCUCC	130	B2M-17
chr15:44711611-44711636	GCGGGAGGGU AGGAGAGACU CACGC	131	B2M-18
chr15:44715412-44715437	UAUUCCUCAG GUACUCCAAA GAUUC	132	B2M-19
chr15:44715440-44715465	UUUACUCACG UCAUCCAGCA GAGAA	133	B2M-20
chr15:44715473-44715498	CAAAUUUCCU GAAUUGCUAU GUGUC	134	B2M-21
chr15:44715474-44715499	AAAUUCCUG AAUUGCUAUG UGUCU	135	B2M-22
chr15:44715515-44715540	ACAUUGAAGU UGACUUACUG AAGAA	136	B2M-23
chr15:44715535-	AAGAAUGGAG	137	B2M-24

Genomic Location (hg38)	Guide Sequence	SEQ ID NO	B2M-#
44715560	AGAGAAUUGA AAAAG		
chr15:44715562- 44715587	GAGCAUUCAG ACUUGUCUUU CAGCA	138	B2M-25
chr15:44715567- 44715592	UUCAGACUUG UCUUUCAGCA AGGAC	139	B2M-26
chr15:44715672- 44715697	UUUGUCACAG CCCAAGAUAG UUAAG	140	B2M-27
chr15:44715673- 44715698	UUGUCACAGC CCAAGAUAGU UAAGU	141	B2M-28
chr15:44715674- 44715699	UGUCACAGCC CAAGAUAGUU AAGUG	142	B2M-29
chr15:44715410- 44715435	AUCUUUGGAG UACCUGAGGA AUAUC	143	B2M-30
chr15:44715411- 44715436	AAUCUUUGGA GUACCUGAGG AAUAU	144	B2M-31
chr15:44715419- 44715444	UAAACCUGAA UCUUUGGAGU ACCUG	145	B2M-32
chr15:44715430- 44715455	GAUGACGUGA GUAAACCUGA AUCUU	146	B2M-33
chr15:44715457- 44715482	GGAAAUUUGA CUUCCAUUC UCUGC	147	B2M-34
chr15:44715483- 44715508	AUGAAACCCA GACACAUAGC AAUUC	148	B2M-35
chr15:44715511- 44715536	UCAGUAAGUC AACUCAAUG UCGGA	149	B2M-36
chr15:44715515- 44715540	UUCUUCAGUA AGUCAACUUC AAUGU	150	B2M-37
chr15:44715629- 44715654	CAGGCAUACU CAUCUUUUUC AGUGG	151	B2M-38
chr15:44715630- 44715655	GCAGGCAUAC UCAUCUUUUU CAGUG	152	B2M-39
chr15:44715631- 44715656	GGCAGGCAUA CUCAUCUUUU UCAGU	153	B2M-40
chr15:44715632- 44715657	CGGCAGGCAU ACUCAUCUUU UUCAG	154	B2M-41
chr15:44715653- 44715678	GACAAAGUCA CAUGGUUCAC ACGGC	155	B2M-42
chr15:44715657- 44715682	CUGUGACAAA GUCACAUGGU UCACA	156	B2M-43
chr15:44715666- 44715691	UAUCUUGGGC UGUGACAAAG UCACA	157	B2M-44
chr15:44715685- 44715710	AAGACUUACC CCACUUAACU AUCUU	158	B2M-45
chr15:44715686- 44715711	UAAGACUUAC CCCACUUAAC UAUCU	159	B2M-46
chr15:44716326- 44716351	AGAUCGAGAC AUGUAAGCAG CAUCA	160	B2M-47

Genomic Location (hg38)	Guide Sequence	SEQ ID NO	B2M-#
chr15:44716329-44716354	UCGAGACAUG UAAGCAGCAU CAUGG	161	B2M-48
chr15:44716313-44716338	AUGUCUCGAU CUAUGAAAAA GACAG	162	B2M-49
chr15:44717599-44717624	UUUUCAGGUU UGAAGAUGCC GCAUU	163	B2M-50
chr15:44717604-44717629	AGGUUUGAAG AUGCCGCAUU UGGAU	164	B2M-51
chr15:44717681-44717706	CACUUACACU UUAUGCACAA AAUGU	165	B2M-52
chr15:44717682-44717707	ACUUACACUU UAUGCACAAA AUGUA	166	B2M-53
chr15:44717702-44717727	AUGUAGGGUU AUAUAAUGU UAACA	167	B2M-54
chr15:44717764-44717789	GUCUCCAUGU UUGAUGUAUC UGAGC	168	B2M-55
chr15:44717776-44717801	GAUGUAUCUG AGCAGGUUGC UCCAC	169	B2M-56
chr15:44717786-44717811	AGCAGGUUGC UCCACAGGUA GCUCU	170	B2M-57
chr15:44717789-44717814	AGGUUGCUCU ACAGGUAGCU CUAGG	171	B2M-58
chr15:44717790-44717815	GGUUGCUCU CAGGUAGCUC UAGGA	172	B2M-59
chr15:44717794-44717819	GCUCCACAGG UAGCUCUAGG AGGGC	173	B2M-60
chr15:44717805-44717830	AGCUCUAGGA GGGCUGGCAA CUUAG	174	B2M-61
chr15:44717808-44717833	UCUAGGAGGG CUGGCAACUU AGAGG	175	B2M-62
chr15:44717809-44717834	CUAGGAGGGC UGGCAACUUA GAGGU	176	B2M-63
chr15:44717810-44717835	UAGGAGGGCU GGCAACUUAG AGGUG	177	B2M-64
chr15:44717846-44717871	AUUCUCUUAU CCAACAUCAA CAUCU	178	B2M-65
chr15:44717945-44717970	CAAUUUACAU ACUCUGCUUA GAAUU	179	B2M-66
chr15:44717946-44717971	AAUUUACAU CUCUGCUUAG AAUUU	180	B2M-67
chr15:44717947-44717972	AUUUACAUAC UCUGCUUAGA AUUUG	181	B2M-68
chr15:44717948-44717973	UUUACAUACU CUGCUUAGAA UUUGG	182	B2M-69
chr15:44717973-44717998	GGGAAAAUUU AGAAAUUAAA UUGAC	183	B2M-70
chr15:44717981-	UUAGAAAUAU	184	B2M-71

Genomic Location (hg38)	Guide Sequence	SEQ ID NO	B2M-#
44718006	AAUUGACAGG AUUAU		
chr15:44718056- 44718081	UACUUCUUAU ACAUUUGAUA AAGUA	185	B2M-72
chr15:44718061- 44718086	CUUAUACAUA UGAUAAAGUA AGGCA	186	B2M-73
chr15:44718067- 44718092	CAUUUGAUAA AGUAAGGCAU GGUUG	187	B2M-74
chr15:44718076- 44718101	AAGUAAGGCA UGGUUGUGGU UAAUC	188	B2M-75
chr15:44717589- 44717614	CUUCAAAACCU GAAAAGAAAA GAAAA	189	B2M-76
chr15:44717620- 44717645	AUUUGGAAUU CAUCCAAUCC AAAUG	190	B2M-77
chr15:44717642- 44717667	UAUUAAAAAG CAAGCAAGCA GAAUU	191	B2M-78
chr15:44717771- 44717796	GCAACCUGCU CAGAUACAUC AAACA	192	B2M-79
chr15:44717800- 44717825	UUGCCAGCCC UCCUAGAGCU ACCUG	193	B2M-80
chr15:44717859- 44717884	UCAAUCUGA CCAAGAUGUU GAUGU	194	B2M-81
chr15:44717947- 44717972	CAAAUUCUAA GCAGAGUAUG UAAAU	195	B2M-82
chr15:44718119- 44718144	CAAGUUUUUAU GAUUUAUUUA ACUUG	196	B2M-83

[00257] Embodiment 11 is the engineered cell of embodiment 9, wherein the genetic modification that inhibits expression of one or more MHC class I proteins is a genetic modification in an HLA-A sequence and optionally wherein the genetic modification is within genomic coordinates chosen from chr6:29942854 to chr6:29942913 and chr6:29943518 to chr6: 29943619, optionally genomic coordinates chosen from: chr6:29942864-29942884; chr6:29942868-29942888; chr6:29942876-29942896; chr6:29942877-29942897; chr6:29942883-29942903; chr6:29943126-29943146; chr6:29943528-29943548; chr6:29943529-29943549; chr6:29943530-29943550; chr6:29943537-29943557; chr6:29943549-29943569; chr6:29943589-29943609; and chr6:29944026-29944046.

[00258] Embodiment 12 is the engineered cell of any one of the previous embodiments, wherein the cell comprises a genetic modification, wherein the genetic modification inhibits expression of one or more MHC class II proteins.

[00259] Embodiment 13 is the engineered cell of embodiment 12, wherein the genetic modification that inhibits expression of one or more MHC class II proteins is a genetic modification in a CIITA sequence, wherein the genetic modification is within the genomic coordinates selected from chr:16:10902171-10923242, optionally, chr16:10902662-10923285, chr16:10906542-10923285, or chr16:10906542-10908121, optionally chr16:10908132-10908152, chr16:10908131-10908151, chr16:10916456-10916476, chr16:10918504-10918524, chr16:10909022-10909042, chr16:10918512-10918532, chr16:10918511-10918531, chr16:10895742-10895762, chr16:10916362-10916382, chr16:10916455-10916475, chr16:10909172-10909192, chr16:10906492-10906512, chr16:10909006-10909026, chr16:10922478-10922498, chr16:10895747-10895767, chr16:10916348-10916368, chr16:10910186-10910206, chr16:10906481-10906501, chr16:10909007-10909027, chr16:10895410-10895430, and chr16:10908130-10908150; optionally chr16:10918504-10918524, chr16:10923218-10923238, chr16:10923219-10923239, chr16:10923221-10923241, chr16:10906486-10906506, chr16:10906485-10906505, chr16:10903873-10903893, chr16:10909172-10909192, chr16:10918423-10918443, chr16:10916362-10916382, chr16:10916450-10916470, chr16:10922153-10922173, chr16:10923222-10923242, chr16:10910176-10910196, chr16:10895742-10895762, chr16:10916449-10916469, chr16:10923214-10923234, chr16:10906492-10906512, and chr16:10906487-1090650; or optionally chr16:10916432-10916452, chr16:10922444-10922464, chr16:10907924-10907944, chr16:10906985-10907005, chr16:10908073-10908093, chr16:10907433-10907453, chr16:10907979-10907999, chr16:10907139-10907159, chr16:10922435-10922455, chr16:10907384-10907404, chr16:10907434-10907454, chr16:10907119-10907139, chr16:10907539-10907559, chr16:10907810-10907830, chr16:10907315-10907335, chr16:10916426-10916446, chr16:10909138-10909158, chr16:10908101-10908121, chr16:10907790-10907810, chr16:10907787-10907807, chr16:10907454-10907474, chr16:10895702-10895722, chr16:10902729-10902749, chr16:10918492-10918512, chr16:10907932-10907952, chr16:10907623-10907643, chr16:10907461-10907481, chr16:10902723-10902743, chr16:10907622-10907642, chr16:10922441-10922461, chr16:10902662-10902682, chr16:10915626-10915646, chr16:10915592-10915612, chr16:10907385-10907405, chr16:10907030-10907050, chr16:10907935-10907955, chr16:10906853-10906873, chr16:10906757-10906777, chr16:10907730-10907750, and chr16:10895302-10895322.

[00260] Embodiment 14 is the engineered cell of embodiment 12 or 13, wherein the genetic modification that inhibits expression of one or more MHC class II proteins a modification of at least one nucleotide of a CIITA splice site, optionally

- a) a modification of at least one nucleotide of a CIITA splice donor site; and/or
- b) a modification of a CIITA splice site boundary nucleotide.

[00261] Embodiment 15 is the engineered cell of any one of embodiments 1-14, wherein the cell has reduced cell surface expression of LAG3 protein.

[00262] Embodiment 16 is the engineered cell of any one of embodiments 1-15, wherein the cell has reduced cell surface expression of LAG3 protein and reduced cell surface expression of TRAC protein.

[00263] Embodiment 17 is the engineered cell of embodiment 15 or 16 further comprising reduced cell surface expression of a TRBC protein.

[00264] Embodiment 18 is the engineered cell of any one of embodiments 15-17, wherein cell surface expression of LAG3 is below the level of detection.

[00265] Embodiment 19 is the engineered cell of any one of embodiments 15-18, wherein cell surface expression of at least one of TRAC and TRBC is below the level of detection.

[00266] Embodiment 20 is the engineered cell of embodiment 19, wherein cell surface expression of each of LAG3, TRAC, and TRBC is below the level of detection.

[00267] Embodiment 21 is the engineered cell of any one of the previous embodiments, comprising a genetic modification in a human 2B4/ CD244 sequence, within genomic coordinates of chr1:5016-37743.

[00268] Embodiment 22 is the engineered cell of embodiment 21, wherein the genetic modification in 2B4/CD244 is within genomic coordinates selected from:

2B4 NO	Genomic Coordinates (hg38)
2B4-1	chr1:160841611-160841631
2B4-2	chr1:160841865-160841885
2B4-3	chr1:160862624-160862644
2B4-4	chr1:160862671-160862691
2B4-5	chr1:160841622-160841642
2B4-6	chr1:160841819-160841839
2B4-7	chr1:160841823-160841843
2B4-8	chr1:160841717-160841737
2B4-9	chr1:160841859-160841879

2B4-10	chr1:160841806-160841826
2B4-11	chr1:160841834-160841854
2B4-12	chr1:160841780-160841800
2B4-13	chr1:160841713-160841733
2B4-14	chr1:160841631-160841651
2B4-15	chr1:160841704-160841724
2B4-16	chr1:160841584-160841604
2B4-17	chr1:160841679-160841699
2B4-18	chr1:160841874-160841894
2B4-19	chr1:160841750-160841770
2B4-20	chr1:160841577-160841597
2B4-21	chr1:160841459-160841479
2B4-22	chr1:160841466-160841486
2B4-23	chr1:160841461-160841481
2B4-24	chr1:160841460-160841480
2B4-25	chr1:160841360-160841380
2B4-26	chr1:160841304-160841324
2B4-27	chr1:160841195-160841215
2B4-28	chr1:160841305-160841325

optionally the genomic coordinates selected from those targeted by 2B4-1 through 2B4-5; 2B4-1 and 2B4-2; or 2B4-3, 2B4-4, 2B4-10, and 2B4-17.

[00269] Embodiment 23 is the engineered cell of any one of the previous embodiments, comprising a genetic modification in a human TIM3 sequence, within the genomic coordinates of chr5:157085832-157109044.

[00270] Embodiment 24 is the engineered cell of embodiment 23, wherein the genetic modification in TIM3 is within genomic coordinates selected from:

TIM 3 NO	Genomic Coordinates (hg38)
TIM3 - 1	chr5:157106867-157106887
TIM3 - 2	chr5:157106862-157106882
TIM3 - 3	chr5:157106803-157106823
TIM3 - 4	chr5:157106850-157106870

TIM3 – 5	chr5:157104726-157104746
TIM3 – 6	chr5:157106668-157106688
TIM3 – 7	chr5:157104681-157104701
TIM3 – 8	chr5:157104681-157104701
TIM3 – 9	chr5:157104680-157104700
TIM3 – 10	chr5:157106676-157106696
TIM3 – 11	chr5:157087271-157087291
TIM3 – 12	chr5:157095432-157095452
TIM3 – 13	chr5:157095361-157095381
TIM3 – 14	chr5:157095360-157095380
TIM3 – 15	chr5:157108945-157108965
TIM3 - 18	chr5:157106751-157106771
TIM3 – 19	chr5:157095419-157095439
TIM3 – 22	chr5:157104679-157104699
TIM3 – 23	chr5:157106824-157106844
TIM3 – 26	chr5:157087117-157087137
TIM3 – 29	chr5:157095379-157095399
TIM3 – 32	chr5:157106864-157106884
TIM3 – 42	chr5:157095405-157095425
TIM3 – 44	chr5:157095404-157095424
TIM3 – 56	chr5:157106888-157106908
TIM3 – 58	chr5:157087126-157087146
TIM3 – 59	chr5:157087253-157087273
TIM3 – 62	chr5:157106889-157106909
TIM3 – 63	chr5:157106935-157106955
TIM3 – 66	chr5:157106641-157106661
TIM3 – 69	chr5:157087084-157087104
TIM3 – 75	chr5:157104663-157104683
TIM3 – 82	chr5:157106875-157106895
TIM3 - 86	chr5:157087184-157087204
TIM3 – 87	chr5:157106936-157106956
TIM3 - 88	chr5:157104696-157104716

optionally the genomic coordinates selected from those targeted by TIM3-1 through TIM3-4, TIM3-6 through TIM3-15, TIM3-18, TIM3-19, TIM3-22, TIM3-29, TIM3-42, TIM3-44, TIM3-58, TIM3-62, TIM3-69, TIM3-82, TIM3-86, and TIM3-88; TIM3-1 through TIM3-5, TIM3-7, TIM3-8, TIM3-12 through TIM3-15, TIM3-23, TIM3-26, TIM3-32, TIM3-56, TIM3-59, TIM3-63, TIM3-66, TIM3-75, and TIM3-87; TIM3-2, TIM3-4, TIM3-15, TIM3-23, TIM3-56, TIM3-59, TIM3-63, TIM3-75, and TIM3-87; TIM3-1 through TIM3-4; TIM3-2, TIM3-4, and TIM3-15; TIM3-2, TIM3-4, TIM3-15, TIM3-63, and TIM3-87; TIM3-2 and TIM3-15; TIM3-63 and TIM3-87; or TIM3-15.

[00271] Embodiment 25 is the engineered cell of any one of the previous embodiments, comprising a genetic modification in a human PD-1 sequence, within the genomic coordinates of chr2: 241849881-241858908,.

[00272] Embodiment 26 is the engineered cell of any one of embodiments 21-25, wherein the genetic modification in the indicated genomic coordinates is selected from an insertion, a deletion, and a substitution.

[00273] Embodiment 27 is the engineered cell of any one of embodiments 21-26, wherein the genetic modification inhibits expression of the gene in which the genetic modification is present.

[00274] Embodiment 28 is the engineered cell of any one of the previous embodiments, wherein the genetic modification comprises an indel.

[00275] Embodiment 29 is the engineered cell of any one of the previous embodiments, wherein the genetic modification comprises an insertion of a heterologous coding sequence.

[00276] Embodiment 30 is the engineered cell of any one of the previous embodiments, wherein the genetic modification comprises a substitution.

[00277] Embodiment 31 is the engineered cell of embodiment 30, wherein the substitution comprises a C to T substitution or an A to G substitution.

[00278] Embodiment 32 is the engineered cell of any one of the previous embodiments, wherein the genetic modification results in a change in the nucleic acid sequence that prevents translation of a full-length protein having an amino acid sequence of the full-length protein prior to genetic modification.

[00279] Embodiment 33 is the engineered cell of embodiment 32, wherein the genetic modification results in a change in the nucleic acid sequence that results in a premature stop codon in a coding sequence of the full-length protein.

[00280] Embodiment 34 is the engineered cell of embodiment 32, wherein the genetic modification results in a change in the nucleic acid sequence that results in a change in splicing of a pre-mRNA from the genomic locus.

[00281] Embodiment 35 is the engineered cell of any one of the previous embodiments, wherein the inhibition results in reduced cell surface expression of a protein from the gene comprising a genetic modification.

[00282] Embodiment 36 is the engineered cell of any one of the previous embodiments, wherein the inhibition results in reduced cell surface expression of a protein regulated by the gene comprising a genetic modification.

[00283] Embodiment 37 is the engineered cell of any one of the previous embodiments, wherein the cell comprises an exogenous nucleic acid encoding a targeting receptor that is expressed on the surface of the engineered cell.

[00284] Embodiment 38 is the engineered cell of embodiment 37, wherein the targeting receptor is a CAR.

[00285] Embodiment 39 is the engineered cell of embodiment 37, wherein the targeting receptor is a TCR.

[00286] Embodiment 40 is the engineered cell of embodiment 39, wherein the targeting receptor is a WT1 TCR.

[00287] Embodiment 41 is the engineered cell of any one of the previous embodiments, wherein the engineered cell is an immune cell.

[00288] Embodiment 42 is the engineered cell of embodiment 41, wherein the engineered cell is a monocyte, macrophage, mast cell, dendritic cell, or granulocyte.

[00289] Embodiment 43 is the engineered cell of embodiment 41, wherein the engineered cell is a lymphocyte.

[00290] Embodiment 44 is the engineered cell of embodiment 43, wherein the engineered cell is a T cell.

[00291] Embodiment 45 is a pharmaceutical composition comprising the engineered cell of any one of embodiments 1-44.

[00292] Embodiment 46 is a population of cells comprising the engineered cell of any one of embodiments 1-44.

[00293] Embodiment 47 is a pharmaceutical composition comprising a population of cells, wherein the population of cells comprises engineered cell of any one of embodiments 1-44.

[00294] Embodiment 48 is a method of administering the engineered cell, population of cells, or pharmaceutical composition of any one of the preceding embodiments to a subject in need thereof.

[00295] Embodiment 49 is a method of administering the engineered cell, population of cells, or pharmaceutical composition of any one of the preceding embodiments to a subject as an adoptive cell transfer (ACT) therapy.

[00296] Embodiment 50 is an engineered cell, population of cells, or pharmaceutical composition of any one of the preceding embodiments, for use as an ACT therapy.

[00297] Embodiment 51 is a LAG3 guide RNA that specifically hybridizes to a LAG3 sequence comprising a nucleotide sequence selected from:

- a. a guide sequence comprising a nucleotide sequence selected from SEQ ID NOs: 1-17, 24, 26, 41, 59, and 83;
- b. a guide sequence comprising a nucleotide sequence of at least 17, 18, 19, or 20 contiguous nucleotides of a nucleotide sequence selected from the sequence of SEQ ID NOs: 1-17, 24, 26, 41, 59, and 83;
- c. a guide sequence comprising a nucleotide sequence at least 95% identical or at least 90% identical to a nucleotide sequence selected from SEQ ID Nos: 1-17, 24, 26, 41, 59, and 83;
- d. a guide sequence comprising a nucleotide sequence selected from SEQ ID NOs: 1-15;
- e. a guide sequence comprising a nucleotide sequence selected from SEQ ID NOs: 1-11;
- f. a guide sequence comprising a nucleotide sequence selected from SEQ ID NOs: 1-4; and
- g. a guide sequence comprising a nucleotide sequence selected from SEQ ID NOs: 1, 4, 5, and 9.

[00298] Embodiment 52 is a LAG3 guide RNA comprising a guide sequence that directs an RNA-guided DNA binding agent to a chromosomal location within the genomic coordinates selected from those targeted by SEQ ID NO: 1-17, 24, 26, 41, 59, and 83, optionally genomic coordinates selected from the genomic coordinates targeted by SEQ ID NOs: 1-15, optionally selected from the genomic coordinates targeted by SEQ ID NOs: 1-11, optionally selected from the genomic coordinates targeted by SEQ ID NOs: 1-4, or optionally selected from genomic coordinates targeted by SEQ ID NOs. 1, 4, 5, or 9.

[00299] Embodiment 53 is the guide RNA of embodiment 51 or 52, wherein the guide RNA is a dual guide RNA (dgRNA).

[00300] Embodiment 54 is the guide RNA of embodiment 51 or 52, wherein the guide RNA is a single guide RNA (sgRNA).

[00301] Embodiment 55 is the guide RNA of embodiment 54, further comprising the nucleotide sequence of SEQ ID NO: 400 3' to the guide sequence, wherein the guide RNA comprises a 5' end modification or a 3' end modification.

[00302] Embodiment 56 is the guide RNA of embodiment 54, further comprising 5' end modification or a 3' end modification and a conserved portion of an gRNA comprising one or more of:

A. a shortened hairpin 1 region or a substituted and optionally shortened hairpin 1 region relative to SEQ ID NO: 400, wherein

1. at least one of the following pairs of nucleotides are substituted in the substituted and optionally shortened hairpin 1 with Watson-Crick pairing nucleotides: H1-1 and H1-12, H1-2 and H1-11, H1-3 and H1-10, or H1-4 and H1-9, and the hairpin 1 region optionally lacks

a. any one or two of H1-5 through H1-8,

b. one, two, or three of the following pairs of nucleotides: H1-1 and H1-12, H1-2 and H1-11, H1-3 and H1-10, and H1-4 and H1-9, or

c. 1-8 nucleotides of hairpin 1 region; or

2. the shortened hairpin 1 region lacks 4-8 nucleotides, preferably 4-6 nucleotides; and

a. one or more of positions H1-1, H1-2, or H1-3 is deleted or substituted relative to SEQ ID NO: 400; or

b. one or more of positions H1-6 through H1-10 is substituted relative to SEQ ID NO: 400; or

3. the shortened hairpin 1 region lacks 5-10 nucleotides, preferably 5-6 nucleotides, and one or more of positions N18, H1-12, or n is substituted relative to SEQ ID NO: 400; or

B. a shortened upper stem region, wherein the shortened upper stem region lacks 1-6 nucleotides and wherein the 6, 7, 8, 9, 10, or 11 nucleotides of the shortened upper stem region include less than or equal to 4 substitutions relative to SEQ ID NO: 400; or

C. a substitution relative to SEQ ID NO: 400 at any one or more of LS6, LS7, US3, US10, B3, N7, N15, N17, H2-2 and H2-14, wherein the substituent nucleotide is neither a pyrimidine that is followed by an adenine, nor an adenine that is preceded by a pyrimidine; or

D. an upper stem region, wherein the upper stem modification comprises a modification to any one or more of US1-US12 in the upper stem region.

[00303] Embodiment 57 is the guide RNA of embodiment 54, further comprising the nucleotide sequence of GUUUUAGAGCUAUGCUGUUUUG (SEQ ID NO: 200) 3' to the guide sequence.

[00304] Embodiment 58 is the guide RNA of embodiment 54, further comprising the nucleotide sequence of

GUUUUAGAGCUAGAAAUAGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUU
GAAAAAGUGGCACCGAGUCGGUGC (SEQ ID NO: 201) 3' to the guide sequence,

optionally

GUUUUAGAGCUAGAAAUAGCAAGUUAUUAAAGGCUAGUCCGUUAUCAACUU
GAAAAAGUGGCACCGAGUCGGUGCUUUU (SEQ ID NO: 202) 3' to the guide

sequence.

[00305] Embodiment 59 is the guide RNA of embodiment 57 or 58, wherein the guide RNA is modified according to the pattern

mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUAGAmGmCmUmAmGmAmAmAmU
mAmGmCAAGUUAUUAAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAm
AmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU

(SEQ ID NO: 300), where "N" may be any natural or non-natural nucleotide, m is a 2'-O-methyl modified nucleotide, and * is a phosphorothioate linkage between nucleotide residues; and wherein the N's are collectively the nucleotide sequence of a guide sequence of any preceding embodiment.

[00306] Embodiment 60 is the guide RNA of embodiment 59, wherein each N is independently any natural or non-natural nucleotide and the guide sequence targets Cas9 to the LAG3 gene.

[00307] Embodiment 61 is the guide RNA of any one of embodiments 54-60, wherein the guide RNA comprises a modification.

[00308] Embodiment 62 is the guide RNA of embodiment 61, wherein the modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide or a 2'-F modified nucleotide.

[00309] Embodiment 63 is the guide RNA of embodiment 61-63, wherein the modification comprises a phosphorothioate (PS) bond between nucleotides.

[00310] Embodiment 64 is the guide RNA of any one of embodiments 61-63, wherein the guide RNA is a sgRNA and the modification, comprises a modification at one or more of the five nucleotides at the 5' end of the guide RNA.

[00311] Embodiment 65 is the guide RNA of any one of embodiments 61-64, wherein the guide RNA is a sgRNA and the modification, comprises a modification at one or more of the five nucleotides at the 3' end of the guide RNA.

[00312] Embodiment 66 is the guide RNA of any one of embodiments 61-65, wherein the guide RNA is a sgRNA and the modification, comprises a PS bond between each of the four nucleotides at the 5' end of the guide RNA.

[00313] Embodiment 67 is the guide RNA of any one of embodiments 61-66, wherein the guide RNA is a sgRNA and the modification, comprises a PS bond between each of the four nucleotides at the 3' end of the guide RNA.

[00314] Embodiment 68 is the guide RNA of any one of embodiments 61-66, wherein the guide RNA is a sgRNA and the modification, comprises a 2'-O-Me modified nucleotide at each of the first three nucleotides at the 5' end of the guide RNA.

[00315] Embodiment 69 is the guide RNA of any one of embodiments 61-68, wherein the guide RNA is a sgRNA and the modification, comprises a 2'-O-Me modified nucleotide at each of the last three nucleotides at the 3' end of the guide RNA.

[00316] Embodiment 70 is a composition comprising a guide RNA of any one of embodiments 53-69 and an RNA guided DNA binding agent wherein the RNA guided DNA binding agent is a polypeptide RNA guided DNA binding agent or a nucleic acid encoding an RNA guided DNA binding agent polypeptide, optionally the RNA guided DNA-binding agent is a Cas9 nuclease.

[00317] Embodiment 71 is the composition of embodiment 70, wherein the RNA guided DNA binding agent is a polypeptide capable of making a modification within a DNA sequence.

[00318] Embodiment 72 is the composition of embodiment 71, wherein the RNA guided DNA binding agent is a *S. pyogenes* Cas9 nuclease.

[00319] Embodiment 73 is the composition of any one of embodiments 70-72, wherein the nuclease is selected from the group of cleavase, nickase, and dead nuclease.

[00320] Embodiment 74 is the composition of embodiment 70, wherein the nucleic acid encoding an RNA guided DNA binding agent is selected from:

- a. a DNA coding sequence;
- b. an mRNA with an open reading frame (ORF);
- c. a coding sequence in an expression vector;
- d. a coding sequence in a viral vector.

[00321] Embodiment 75 is the composition of any one of embodiments 70-74 further comprising a guide RNA that specifically hybridizes to genomic coordinates chosen from:

TRAC NO:	Genomic Coordinates (hg38)
TRAC-90	chr14:22547524-22547544
TRAC-91	chr14:22550581-22550601
TRAC-92	chr14:22550608-22550628
TRAC-93	chr14:22550611-22550631
TRAC-94	chr14:22550622-22550642
TRAC-95	chr14:22547529-22547549
TRAC-96	chr14:22547512-22547532
TRAC-97	chr14:22547525-22547545
TRAC-98	chr14:22547536-22547556
TRAC-99	chr14:22547575-22547595
TRAC-100	chr14:22547640-22547660
TRAC-101	chr14:22547647-22547667
TRAC-102	chr14:22547777-22547797
TRAC-103	chr14:22549638-22549658
TRAC-104	chr14:22549646-22549666
TRAC-105	chr14:22550600-22550620
TRAC-106	chr14:22550605-22550625
TRAC-107	chr14:22550625-22550645
TRAC-108	chr14:22539116-22539136
TRAC-109	chr14:22539120-22539140
TRAC-110	chr14:22547518-22547538
TRAC-111	chr14:22539082-22539102
TRAC-112	chr14:22539061-22539081
TRAC-113	chr14:22539097-22539117
TRAC-114	chr14:22547697-22547717

TRAC NO:	Genomic Coordinates (hg38)
TRAC-115	chr14:22550571-22550591
TRAC-116	chr14:22550631-22550651
TRAC-117	chr14:22550658-22550678
TRAC-118	chr14:22547712-22547732
TRAC-119	chr14:22550636-22550656
TRAC-120	chr14:22550636-22550656
TRAC-121	chr14:22550582-22550602
TRAC-122	chr14:22550606-22550626
TRAC-123	chr14:22550609-22550629
TRAC-124	chr14:22547691-22547711
TRAC-125	chr14:22547576-22547596
TRAC-126	chr14:22549648-22549668
TRAC-127	chr14:22549660-22549680
TRAC-128	chr14:22547716-22547736
TRAC-129	chr14:22547514-22547534
TRAC-130	chr14:22550662-22550682
TRAC-131	chr14:22550593-22550613
TRAC-132	chr14:22550612-22550632
TRAC-133	chr14:22547521-22547541
TRAC-134	chr14:22547540-22547560
TRAC-135	chr14:22539121-22539141
TRAC-136	chr14:22547632-22547652
TRAC-137	chr14:22547674-22547694
TRAC-138	chr14:22549643-22549663
TRAC-139	chr14:22547655-22547675
TRAC-140	chr14:22547667-22547687
TRAC-141	chr14:22539085-22539105
TRAC-142	chr14:22549634-22549654
TRAC-143	chr14:22539064-22539084
TRAC-144	chr14:22547639-22547659
TRAC-145	chr14:22547731-22547751

TRAC NO:	Genomic Coordinates (hg38)
TRAC-146	chr14:22547734-22547754
TRAC-147	chr14:22547591-22547611
TRAC-148	chr14:22547657-22547677
TRAC-149	chr14:22547519-22547539
TRAC-150	chr14:22549674-22549694
TRAC-151	chr14:22547678-22547698
TRAC-152	chr14:22539087-22539107
TRAC-153	chr14:22547595-22547615
TRAC-154	chr14:22547633-22547653
TRAC-155	chr14:22547732-22547752
TRAC-156	chr14:22547656-22547676
TRAC-157	chr14:22539086-22539106
TRAC-158	chr14:22547491-22547511
TRAC-159	chr14:22547618-22547638
TRAC-160	chr14:22549644-22549664
TRAC-161	chr14:22547522-22547542
TRAC-162	chr14:22539089-22539109
TRAC-163	chr14:22539062-22539082
TRAC-164	chr14:22547597-22547617
TRAC-165	chr14:22547677-22547697
TRAC-166	chr14:22549645-22549665
TRAC-167	chr14:22550610-22550630
TRAC-168	chr14:22547511-22547531
TRAC-169	chr14:22550607-22550627
TRAC-170	chr14:22550657-22550677
TRAC-171	chr14:22550604-22550624
TRAC-172	chr14:22539132-22539152
TRAC-173	chr14:22550632-22550652
TRAC-174	chr14:22547571-22547591
TRAC-175	chr14:22547711-22547731
TRAC-176	chr14:22547666-22547686

TRAC NO:	Genomic Coordinates (hg38)
TRAC-177	chr14:22547567-22547587
TRAC-178	chr14:22547624-22547644
TRAC-185	chr14:22547501-22547521
TRAC-213	chr14:22547519-22547539
TRAC-214	chr14:22547556-22547576
TRAC-215	chr14:22547486-22547506
TRAC-216	chr14:22547487-22547507
TRAC-217	chr14:22547493-22547513
TRAC-218	chr14:22547502-22547522

optionally the genetic modification is within genomic coordinates selected from chr14:22547524-22547544, chr14:22547529-22547549, chr14:22547525-22547545, chr14:22547536-22547556, chr14:22547501-22547521, chr14:22547556-22547576, and chr14:22547502-22547522.

[00322] Embodiment 76 is the composition of any one of embodiments 70-75 further comprising a guide RNA that specifically hybridizes to genomic coordinates chosen from:

TRBC NO:	Genomic Coordinates (hg38)
TRBC-1	chr7:142791996-142792016
TRBC-2	chr7:142792047-142792067
TRBC-3	chr7:142792008-142792028
TRBC-4	chr7:142791931-142791951
TRBC-5	chr7:142791930-142791950
TRBC-6	chr7:142791748-142791768
TRBC-7	chr7:142791720-142791740
TRBC-8	chr7:142792041-142792061
TRBC-9	chr7:142802114-142802134
TRBC-10	chr7:142792009-142792029
TRBC-11	chr7:142792697-142792717
TRBC-12	chr7:142791963-142791983
TRBC-13	chr7:142791976-142791996
TRBC-14	chr7:142791974-142791994

TRBC NO:	Genomic Coordinates (hg38)
TRBC-15	chr7:142791970-142791990
TRBC-16	chr7:142791948-142791968
TRBC-17	chr7:142791913-142791933
TRBC-18	chr7:142791961-142791981
TRBC-19	chr7:142792068-142792088
TRBC-20	chr7:142791975-142791995
TRBC-21	chr7:142791773-142791793
TRBC-22	chr7:142791919-142791939
TRBC-23	chr7:142791834-142791854
TRBC-24	chr7:142791878-142791898
TRBC-25	chr7:142802141-142802161
TRBC-26	chr7:142791844-142791864
TRBC-27	chr7:142801154-142801174
TRBC-28	chr7:142791961-142791981
TRBC-29	chr7:142792001-142792021
TRBC-30	chr7:142791979-142791999
TRBC-31	chr7:142792041-142792061
TRBC-32	chr7:142792003-142792023
TRBC-33	chr7:142791984-142792004
TRBC-34	chr7:142792002-142792022
TRBC-35	chr7:142791966-142791986
TRBC-36	chr7:142792007-142792027
TRBC-37	chr7:142791993-142792013
TRBC-38	chr7:142791902-142791922
TRBC-39	chr7:142791724-142791744
TRBC-40	chr7:142791973-142791993
TRBC-41	chr7:142791920-142791940
TRBC-42	chr7:142791994-142792014
TRBC-43	chr7:142791887-142791907
TRBC-44	chr7:142791907-142791927
TRBC-45	chr7:142791952-142791972

TRBC NO:	Genomic Coordinates (hg38)
TRBC-46	chr7:142791721-142791741
TRBC-47	chr7:142792718-142792738
TRBC-48	chr7:142791729-142791749
TRBC-49	chr7:142791911-142791931
TRBC-50	chr7:142791867-142791887
TRBC-51	chr7:142791899-142791919
TRBC-52	chr7:142791727-142791747
TRBC-53	chr7:142791949-142791969
TRBC-54	chr7:142791933-142791953
TRBC-55	chr7:142791932-142791952
TRBC-56	chr7:142792057-142792077
TRBC-57	chr7:142791940-142791960
TRBC-58	chr7:142791747-142791767
TRBC-59	chr7:142791881-142791901
TRBC-60	chr7:142791779-142791799
TRBC-61	chr7:142792054-142792074
TRBC-62	chr7:142792069-142792089
TRBC-63	chr7:142792712-142792732
TRBC-64	chr7:142791729-142791749
TRBC-65	chr7:142791821-142791841
TRBC-66	chr7:142792052-142792072
TRBC-67	chr7:142791916-142791936
TRBC-68	chr7:142791899-142791919
TRBC-69	chr7:142791772-142791792
TRBC-70	chr7:142792714-142792734
TRBC-71	chr7:142792042-142792062
TRBC-72	chr7:142791962-142791982
TRBC-73	chr7:142791988-142792008
TRBC-74	chr7:142791982-142792002
TRBC-75	chr7:142792049-142792069
TRBC-76	chr7:142791839-142791859

TRBC NO:	Genomic Coordinates (hg38)
TRBC-77	chr7:142791893-142791913
TRBC-78	chr7:142791945-142791965
TRBC-79	chr7:142791964-142791984
TRBC-80	chr7:142791757-142791777
TRBC-81	chr7:142792048-142792068
TRBC-82	chr7:142791774-142791794
TRBC-83	chr7:142792048-142792068
TRBC-84	chr7:142791830-142791850
TRBC-85	chr7:142791909-142791929
TRBC-86	chr7:142791912-142791932
TRBC-87	chr7:142791766-142791786
TRBC-88	chr7:142791880-142791900
TRBC-89	chr7:142791919-142791939

[00323] Embodiment 77 is the composition of any one of embodiments 70-76 further comprising a guide RNA that specifically hybridizes to genomic coordinates chosen from chr16:10902171-10923242, optionally, chr16:10902662- chr16:10923285, chr16:10906542- chr16:10923285, or chr16:10906542- chr16:10908121, optionally chr16:10908132-10908152, chr16:10908131-10908151, chr16:10916456-10916476, chr16:10918504-10918524, chr16:10909022-10909042, chr16:10918512-10918532, chr16:10918511-10918531, chr16:10895742-10895762, chr16:10916362-10916382, chr16:10916455-10916475, chr16:10909172-10909192, chr16:10906492-10906512, chr16:10909006-10909026, chr16:10922478-10922498, chr16:10895747-10895767, chr16:10916348-10916368, chr16:10910186-10910206, chr16:10906481-10906501, chr16:10909007-10909027, chr16:10895410-10895430, and chr16:10908130-10908150; optionally chr16:10918504-10918524, chr16:10923218-10923238, chr16:10923219-10923239, chr16:10923221-10923241, chr16:10906486-10906506, chr16:10906485-10906505, chr16:10903873-10903893, chr16:10909172-10909192, chr16:10918423-10918443, chr16:10916362-10916382, chr16:10916450-10916470, chr16:10922153-10922173, chr16:10923222-10923242, chr16:10910176-10910196, chr16:10895742-10895762, chr16:10916449-10916469, chr16:10923214-10923234, chr16:10906492-10906512, and chr16:10906487-1090650; or optionally chr16:10916432-10916452, chr16:10922444-10922464, chr16:10907924-10907944, chr16:10906985-10907005, chr16:10908073-

10908093, chr16:10907433-10907453, chr16:10907979-10907999, chr16:10907139-10907159, chr16:10922435-10922455, chr16:10907384-10907404, chr16:10907434-10907454, chr16:10907119-10907139, chr16:10907539-10907559, chr16:10907810-10907830, chr16:10907315-10907335, chr16:10916426-10916446, chr16:10909138-10909158, chr16:10908101-10908121, chr16:10907790-10907810, chr16:10907787-10907807, chr16:10907454-10907474, chr16:10895702-10895722, chr16:10902729-10902749, chr16:10918492-10918512, chr16:10907932-10907952, chr16:10907623-10907643, chr16:10907461-10907481, chr16:10902723-10902743, chr16:10907622-10907642, chr16:10922441-10922461, chr16:10902662-10902682, chr16:10915626-10915646, chr16:10915592-10915612, chr16:10907385-10907405, chr16:10907030-10907050, chr16:10907935-10907955, chr16:10906853-10906873, chr16:10906757-10906777, chr16:10907730-10907750, and chr16:10895302-10895322.

[00324] Embodiment 78 is the composition of any one of embodiments 70-77 further comprising a guide RNA that specifically hybridizes to genomic coordinates chosen from chr6:29942854-29942913 and chr6:29943518-29943619, optionally genomic coordinates chosen from: chr6:29942864-29942884; chr6:29942868-29942888; chr6:29942876-29942896; chr6:29942877-29942897; chr6:29942883-29942903; chr6:29943126-29943146; chr6:29943528-29943548; chr6:29943529-29943549; chr6:29943530-29943550; chr6:29943537-29943557; chr6:29943549-29943569; chr6:29943589-29943609; and chr6:29944026-29944046

[00325] Embodiment 79 is the guide RNA of any one of embodiments 51-69 or the composition of any one of any one of embodiments 70-78, wherein the composition further comprises a pharmaceutically acceptable excipient.

[00326] Embodiment 80 is the guide or composition of embodiment 79, wherein the composition is non-pyrogenic.

[00327] Embodiment 81 is the guide RNA of any one of embodiments 51-69 or composition of any one of embodiments 70-80, wherein the guide RNA is associated with a lipid nanoparticle (LNP).

[00328] Embodiment 82 is a method of making a genetic modification in a LAG3 sequence within a cell, comprising contacting the cell with the guide RNA or composition of any one of embodiments 51-81.

[00329] Embodiment 83 is the method of embodiment 82, further comprising making a genetic modification in a TCR sequence to inhibit expression of a TCR gene.

[00330] Embodiment 84 is a method of preparing a population of cells for immunotherapy comprising:

- a. making a genetic modification in a LAG3 sequence in the cells in the population with a LAG3 guide RNA or composition of any one of embodiments 51-83;
- b. making a genetic modification in a TCR sequence in the cells of the population to reduce expression of the TCR protein on the surface of the cells in the population;
- c. expanding the population of cells in culture.

[00331] Embodiment 85 is the method of embodiment 84, wherein expression of the TCR protein on the surface of the cells is reduced to below the level of detection in at least 40%, 45%, 50%, 55%, 60%, 65%, preferably at least 70%, 75%, 80%, 85%, 90%, or 95% of the cells in the population.

[00332] Embodiment 86 is the method of embodiment 84 or 85, wherein the genetic modification of a TCR sequence in the cells of the population comprises modification of two or more TCR sequences.

[00333] Embodiment 87 is the method of embodiment 86 wherein the two or more TCR sequences comprise TRAC and TRBC.

[00334] Embodiment 88 is the method of any of embodiments 84-87, comprising insertion of an exogenous nucleic acid encoding a targeting receptor that is expressed on the surface of the engineered cell, e.g. a TCR or a CAR, optionally at a TRAC locus.

[00335] Embodiment 89 is the method of any one of embodiments 84-88, further comprising contacting the cells with an LNP composition comprising the LAG3 guide RNA.

[00336] Embodiment 90 is the method of embodiment 89 comprising contacting the cells with a second LNP composition comprising a guide RNA.

[00337] Embodiment 91 is a population of cells made by the method of any one of embodiments 82-90.

[00338] Embodiment 92 is the population of cells of embodiment 91, wherein the population of cells is altered *ex vivo*.

[00339] Embodiment 93 is a pharmaceutical composition comprising a population of cells of embodiment 91 or 92.

[00340] Embodiment 94 is a method of administering the population of cells of embodiment 91 or 92, or pharmaceutical composition of embodiment 93 to a subject in need thereof.

[00341] Embodiment 95 is a method of administering the population of cells of embodiment 91 or 92, or pharmaceutical composition of embodiment 93 to a subject as an adoptive cell transfer (ACT) therapy.

[00342] Embodiment 96 is a population of cells of embodiment 90 or 91, or pharmaceutical composition of embodiment 91, for use as an ACT therapy.

[00343] Embodiment 97 is a population of cells comprising a genetic modification of a LAG3 gene, wherein at least 40%, 45%, 50%, 55%, 60%, 65%, preferably at least 70%, 75%, 80%, 85%, 90%, or 95% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence.

[00344] Embodiment 98 is the populations of cells of embodiment 97, wherein the genetic modification is as defined in any of embodiments 1-4.

[00345] Embodiment 99 is the population of cells of embodiment 97 or 98, wherein expression of LAG3 is decreased by at least 40%, 45%, 50%, 55%, 60%, 65%, preferably at least 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 LAG3 gene has not been modified.

[00346] Embodiment 100 is a population of cells comprising a genetic modification of a TCR gene, wherein at least 40%, 45%, 50%, 55%, 60%, 65%, preferably at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of cells comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous TCR gene sequence.

[00347] Embodiment 101 is the populations of cells of embodiment 100, wherein the genetic modification is as defined in any of embodiments 5-8.

[00348] Embodiment 102 is the population of cells of embodiment 100 or 101, wherein expression of TCR is decreased by at least 40%, 45%, 50%, 55%, 60%, 65%, preferably at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the TCR gene has not been modified.

[00349] Embodiment 103 is the population of cells of any of embodiments 97-102, wherein the population comprises at least 10^3 , 10^4 , 10^5 or 10^6 cells, preferably 10^7 , 2×10^7 , 5×10^7 , or 10^8 cells.

[00350] Embodiment 104 is the population of cells of any one of embodiments 97-103, wherein at least 70% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence.

[00351] Embodiment 105 is the population of cells of any one of embodiments 97-104, wherein at least 80% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence.

[00352] Embodiment 106 is the population of cells of any one of embodiments 97-105, wherein at least 90% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence.

[00353] Embodiment 107 is the population of cells of any one of embodiments 97-106, wherein at least 95% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence.

[00354] Embodiment 108 is the population of cells of any one of embodiments 97-107, wherein expression of LAG3 is decreased by at least 70%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified.

[00355] Embodiment 109 is the population of cells of any one of embodiments 97-108, wherein expression of LAG3 is decreased by at least 80%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified.

[00356] Embodiment 110 is the population of cells of any one of embodiments 97-109, wherein expression of LAG3 is decreased by at least 90%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified.

[00357] Embodiment 111 is the population of cells of any one of embodiments 97-110, wherein expression of LAG3 is decreased by at least 95%, or to below the limit of detection of the assay as compared to a suitable control, e.g., wherein the LAG3 gene has not been modified.

[00358] Embodiment 112 is a pharmaceutical composition comprising the population of cells of any of embodiments 97-111.

[00359] Embodiment 113 is the population of cells of any of embodiments 97-111 or the pharmaceutical composition of embodiment 112, for use as an ACT therapy.

[00360] Embodiment 114 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6773938-6773958.

[00361] Embodiment 115 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6774678-6774698.

[00362] Embodiment 116 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6772894-6772914.

[00363] Embodiment 117 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6774816-6774836.

[00364] Embodiment 118 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6774742-6774762.

[00365] Embodiment 119 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6775380-6775400.

[00366] Embodiment 120 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6774727-6774747.

[00367] Embodiment 121 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6774732-6774752.

[00368] Embodiment 122 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6774435-6774455.

[00369] Embodiment 123 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6774771-6774791.

[00370] Embodiment 124 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6772909-6772929.

[00371] Embodiment 125 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6774735-6774755.

[00372] Embodiment 126 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6773783-6773803.

[00373] Embodiment 127 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6775292-6775312.

[00374] Embodiment 128 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6777433-6777453.

[00375] Embodiment 129 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6778268-6778288.

[00376] Embodiment 130 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6775444-6775464.

[00377] Embodiment 131 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6777783-6777803.

[00378] Embodiment 132 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6777784-6777804.

[00379] Embodiment 133 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6778252-6778272.

[00380] Embodiment 134 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6777325-6777345.

[00381] Embodiment 135 is the engineered cell, guide RNA, composition, pharmaceutical composition, or method of any one of the preceding embodiments, wherein the genetic modification is within the genomic coordinates of chr12:6777329-6777349.

[00382] Embodiment 136 is the engineered cell of embodiment 25, wherein the genetic modification comprises a modification of at least one nucleotide within the genomic coordinates selected from:

PD1 NO.	Genomic Coordinates (hg38)
PD1-29	chr2:241852703-241852723
PD1-43	chr2:241858807-241858827
PD1-5	chr2:241858789-241858809
PD1-6	chr2:241858788-241858808
PD1-8	chr2:241858755-241858775
PD1-11	chr2:241852919-241852939
PD1-12	chr2:241852915-241852935
PD1-22	chr2:241852755-241852775
PD1-23	chr2:241852751-241852771
PD1-24	chr2:241852750-241852770
PD1-36	chr2:241852264-241852284
PD1-57	chr2:241852201-241852221
PD1-58	chr2:241852749-241852769
PD1-17	chr2:241852821-241852841
PD1-38	chr2:241852265-241852285
PD1-56	chr2:241851221-241851241
PD1-41	chr2:241852188-241852208

; or

the genomic coordinates selected from chr2:241852919-241852939, chr2:241852915-241852935, chr2:241852750-241852770, chr2:241852264-241852284, chr2:241852265-241852285, chr2:241858807-241858827, chr2:241852201-241852221, chr2:241858789-241858809, chr2:241858788-241858808, chr2:241858755-241858775, chr2:241852755-241852775, chr2:241852751-241852771, and chr2:241852703-241852723, respectively; or the genomic coordinates selected from chr2:241858788-241858808, chr2:241858755-241858775, chr2:241852919-241852939, chr2:241852915-241852935, chr2:241852751-241852771, chr2:241858807-241858827, and chr2:241852703-241852723, respectively; or the genomic coordinates selected from chr2: 241858789-241858809, chr2:241852919-241852939, chr2:241852915-241852935, chr2:241852755-241852775, chr2:241852751-241852771, and chr2:241858807-241858827, respectively; or the genomic coordinates selected from chr2:241858788-241858808, chr2:241858755-241858775, chr2:241852751-241852771, and chr2:241852703-241852723, respectively; or

the genomic coordinates selected from chr2:241858788-241858808 and chr2:241852703-241852723, respectively; or
the genomic coordinates selected from chr2:241858788-241858808, chr2:241852751-241852771, chr2:241852703-241852723, chr2:241852188-241852208, and chr2:241852201-241852221, respectively; or
the genomic coordinates selected from chr2:241858788-241858808, chr2:241852703-241852723, and chr2:241852201-241852221, respectively; or
the genomic coordinates of chr2:241858807-241858827.

TABLE 11. Additional Sequences

Description	SEQ ID NO:	SEQUENCE
CR003187	100	GACCCCCUCCACCCCGCCUCGUUUUAGAGCUAUGCUGUUUUG
G013006	101	mC*mU*mC*UCAGCUGGUACACGGCAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
G016239	102	mG*mG*mC*CUCGGCGCUGACGAUCUGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
G015068	90	GACGUUGAAGCCAUCUCUGUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
G015069	91	GCGGUCCCUGAGGUGCACCGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
G015070	92	GCAAUGCCAGCUGUACCAGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
G015071	105	AGAGGAAGCUUUCGCUAAGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
G018433	106	mG*mU*mU*CCGGAACCAUAGCACAGGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
G018434	107	mG*mC*mG*GUCCCUGAGGUGCACCGGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
G018436	108	mA*mG*mC*AGCAGGACACAGUCAAGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
G020845	109	mA*mA*mC*CUCGUGCCCGUCUGCUGGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
G021215	110	mC*mU*mG*AACUUUUCAGAUUAACGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
G021216	111	mU*mG*mA*CCAUGUGGUUAGCAUCUGUUUUAGAmGmCmUmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
G000294	112	GACCCCCUCCACCCCGCCUCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAG

		UGGCACCGAGUCGGUGCUUUU
G000739	113	mG*mA*mU*CACGUCGGCCGUUGGCGUUUUAGAmGmCm UmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGU CCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGm CmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
G018438	114	mA*mG*mU*UGGGCAGAUAAACACUUGGUUUUAGAmGmCm UmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGU CCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGm CmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
Guide scaffold	200	GUUUUAGAGCUAUGCUGUUUUG
Guide scaffold	201	GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGU CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC
Guide scaffold	202	GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGU CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUU UU
Guide scaffold	300	mN*mN*mN*NNNNNNNNNNNNNNNNNNNGUUUUAGAmGmCm UmAmGmAmAmAmUmAmGmCAAGUUAAAAUAAGGCUAGU CCGUUAUCAmAmCmUmUmGmAmAmAmAmAmGmUmGmGm CmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
Guide scaffold	400	GUUUUAGAGC UAGAAAUAGC AAGUUAAAAU AAGGCUAGUC CGUUAUCAAC UUGAAAAAGU GGCACCGAGU CGGUGC
Guide scaffold 81	401	(N)20GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGC UAGUCCGUUAUCACGAAAGGGCACCGAGUCGGUGC
Guide scaffold 181	402	mN*mN*mN*(N)17GUUUUAGAmGmCmUmAmGmAmAmAmU mAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCACGAAA GGGCACCGAGUCGG*mU*mG*mC
Guide scaffold 94	403	(N)20GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGC UAGUCCGUUAUCAACUUGGCACCGAGUCGGUGC
Guide scaffold 194	404	mN*mN*mN*(N)17GUUUUAGAmGmCmUmAmGmAmAmAmU mAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUG GCACCGAGUCGG*mU*mG*mC
Guide scaffold 95	405	(N)20GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGC UAGUCCGUUAUCAACUUGGCACCGAGUCGGUGC
Guide scaffold 195	406	mN*mN*mN*(N)17GUUUUAGAmGmCmUmAmGmAmAmAmU mAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUG GCACCGAGUCGG*mU*mG*mC
Guide scaffold 871	407	(N)20GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGC UAGUCCGUUAUCACGAAAGGGCACCGAGUCGGUGC
Guide scaffold 971	408	mN*mN*mN*(N)17mGUUUfUAGmAmGmCmUmAmGmAmAmA mUmAmGmCmAmAGUfUmAfAmAfAmUAmAmGmGmCmUmA GUmCmCGUfUAmUmCmAmGmAmAmAmGmGmGmCmAmC mCmGmAmGmUmCmGmG*mU*mG*mC
Guide scaffold 872	409	(N)20GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGC UAGUCCGUUAUCACGAAAGGGCACCGAGUCGGUGC
Guide scaffold 972	410	mN*mN*mN*(N)17GUUUUAGAmGmCmUmAmGmAmAmAmU mAmGmCAAGUUAAAAUAAGGCUAGUCCGUUAUCACGAAA GGGCACCGAGUCGG*mU*mG*mC
tracrRNA	411	AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCA ACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUUU
Recombinant	800	MDKKYSIGLDIGTNSVGWAVITDEYKVPSSKFKVLGNTDRHS

Cas9-NLS amino acid sequence		IKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIF SNEMAKVDDSFHRLSEESFLVEEDKKHERHPIFGNIVDEVAYH EKYPTIYHLRKKLVDSTDKADLRILIYALAHMIKFRGHFLIEG DLNPDNSDVDFKLFQLVQTYNQLFEENPINASGVDAKAILSAR LSKSRLENLIAQLPGEKKNGLFGNLIALLSLGLTPNFKSNFDLA EDAKLQLSKDTYDDDLNLLAQIGDQYADLFLAAKNLSDAIL LSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLP EKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTE ELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYF FLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITP WNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEY FTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKV TVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIDK KDFLDNEENEDILEDIVLTLTLFEDREMIEERLKYAHLFDDKV MKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDFGA NRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPA IKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQ KNSRERMKRIEEGKELGSQILKEHPVENTQLQNEKLYLYLQ NGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTR SDKNRGKSDNVPSEEVVKMKMKNYWRQLLNAKLITQRKFDNL TKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKY DENDKLIREVKVITLKSCLVSDFRKDFQFYKVREINNYHHAHD AYLNAVVGTAIIKKYPKLESEFVYGDYKVYDVRKMIKSEQE IGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIV WDKGRDFATVRKVLSPQVNVKKTEVQTGGFSKESILPKRN SDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKEKGKSKK LKSVKELLGITIMERSSEFEKNPIDFLEAKGYKEVKKDLIIKLPK YSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHY EKLKGSPEDEQKQLFVEQHKHYLDEIIEQISEFSKRVLADAN LDKVLSAYNKHDKPIREQAENIIHLFTLTNLGAPAAFKYFDT TIDRKRYTSTKEVLDTLHQSITGLYETRIDLSQLGGDGGGSP KKKRKV
ORF encoding Sp. Cas9	801	ATGGACAAGAAGTACAGCATCGGACTGGACATCGGAACAA ACAGCGTCGGATGGGCAGTCATCACAGACGAATACAAGGT CCCGAGCAAGAAGTTCAAGGTCTGGGAAACACAGACAGA CACAGCATCAAGAAGAACCTGATCGGAGCACTGCTGTTTCG ACAGCGGAGAAACAGCAGAAGCAACAAGACTGAAGAGAA CAGCAAGAAGAAGATACACAAGAAGAAAGAACAGAATCT GCTACCTGCAGGAAATCTTCAGCAACGAAATGGCAAAGGT CGACGACAGCTTCTTCCACAGACTGGAAGAAAGCTTCTCTGG TCGAAGAAGACAAGAAGCACGAAAGACACCCGATCTTCGG AACATCGTCGACGAAGTCGCATACCACGAAAAGTACCCG ACAATCTACCACCTGAGAAAGAAGCTGGTCGACAGCACAG ACAAGGCAGACCTGAGACTGATCTACCTGGCACTGGCACA CATGATCAAGTTCAGAGGACACTTCTGATCGAAGGAGAC CTGAACCCGGACAACAGCGACGTCGACAAGCTGTTTCATCC AGCTGGTCCAGACATACAACCAGCTGTTCGAAGAAAACCC GATCAACGCAAGCGGAGTCGACGCAAAGGCAATCCTGAGC GCAAGACTGAGCAAGAGCAGAAGACTGGAAAACCTGATCG CACAGCTGCCGGGAGAAAAGAAGAACGGACTGTTCGGAAA CCTGATCGCACTGAGCCTGGGACTGACACCGAACTTCAAGA GCAACTTCGACCTGGCAGAAGACGCAAAGCTGCAGCTGAG

	CAAGGACACATACGACGACGACCTGGACAACCTGCTGGCA CAGATCGGAGACCAGTACGCAGACCTGTTCTGGCAGCAA AGAACCTGAGCGACGCAATCCTGCTGAGCGACATCCTGAG AGTCAACACAGAAATCACAAAGGCACCGCTGAGCGCAAGC ATGATCAAGAGATACGACGAACACCACCAGGACCTGACAC TGCTGAAGGCACTGGTCAGACAGCAGCTGCCGAAAAGTA CAAGGAAATCTTCTTCGACCAGAGCAAGAACGGATACGCA GGATACATCGACGGAGGAGCAAGCCAGGAAGAATTCTACA AGTTCATCAAGCCGATCCTGGAAAAGATGGACGGAACAGA AGAACTGCTGGTCAAGCTGAACAGAGAAGACCTGCTGAGA AAGCAGAGAACATTTCGACAACGGAAGCATCCCGCACCAGA TCCACCTGGGAGAACTGCACGCAATCCTGAGAAGACAGGA AGACTTCTACCCGTTCTGAAGGACAACAGAGAAAAGATC GAAAAGATCCTGACATTCAGAATCCCGTACTACGTCGGACC GCTGGCAAGAGGAAACAGCAGATTCGCATGGATGACAAGA AAGAGCGAAGAAACAATCACACCGTGGAACCTCGAAGAAG TCGTCGACAAGGGAGCAAGCGCACAGAGCTTCATCGAAAG AATGACAACTTCGACAAGAACCTGCCGAACGAAAAGGTC CTGCCGAAGCACAGCCTGCTGTACGAATACTTCACAGTCTA CAACGAACTGACAAAGGTCAAGTACGTCACAGAAGGAATG AGAAAGCCGGCATTCTGAGCGGAGAACAGAAGAAGGCAA TCGTCGACCTGCTGTTCAAGACAAACAGAAAGGTCACAGTC AAGCAGCTGAAGGAAGACTACTTCAAGAAGATCGAATGCT TCGACAGCGTCGAAATCAGCGGAGTCGAAGACAGATTCAA CGCAAGCCTGGGAACATACCACGACCTGCTGAAGATCATC AAGGACAAGGACTTCTGGACAACGAAGAAAACGAAGACA TCCTGGAAGACATCGTCTGACACTGACACTGTTTGAAGAC AGAGAAATGATCGAAGAAAGACTGAAGACATACGCACACC TGTTTCGACGACAAGGTCATGAAGCAGCTGAAGAGAAGAAG ATACACAGGATGGGGAAGACTGAGCAGAAAGCTGATCAAC GGAATCAGAGACAAGCAGAGCGGAAAGACAATCCTGGACT TCCTGAAGAGCGACGGATTTCGCAAACAGAACTTCATGCA GCTGATCCACGACGACAGCCTGACATTCAAGGAAGACATC CAGAAGGCACAGGTCAGCGGACAGGGAGACAGCCTGCACG AACACATCGCAAACCTGGCAGGAAGCCCGCAATCAAGAA GGGAATCCTGCAGACAGTCAAGGTCGTCGACGAACTGGTC AAGGTCATGGGAAGACACAAGCCGGAACATCGTCATCG AAATGGCAAGAGAAAACAGACAACACAGAAGGGACAGA AGAACAGCAGAGAAAAGAAATGAAGAGAATCGAAGAAGGAA TCAAGGAACTGGGAAGCCAGATCCTGAAGGAACACCCGGT CGAAAACACACAGCTGCAGAACGAAAAGCTGTACCTGTAC TACCTGCAGAACGGAAGAGACATGTACGTCGACCAGGAAC TGGACATCAACAGACTGAGCGACTACGACGTCGACCACAT CGTCCCGCAGAGCTTCTGAAGGACGACAGCATCGACAAC AAGGTCCTGACAAGAAGCGACAAGAACAGAGGAAAGAGC GACAACGTCCCGAGCGAAGAAGTCGTCAAGAAGATGAAGA ACTACTGGAGACAGCTGCTGAACGCAAAGCTGATCACACA GAGAAAGTTTCGACAACCTGACAAAGGCAGAGAGAGGAGG ACTGAGCGAACTGGACAAGGCAGGATTCATCAAGAGACAG CTGGTCGAAACAAGACAGATCACAAAGCACGTCGCACAGA TCCTGGACAGCAGAATGAACACAAAGTACGACGAAAACGA CAAGCTGATCAGAGAAGTCAAGGTCATCACACTGAAGAGC
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		AAGCTGGTCAGCGACTTCAGAAAGGACTTCCAGTTCTACAA GGTCAGAGAAATCAACAACTACCACCACGCACACGACGCA TACCTGAACGCAGTCGTGGAACAGCACTGATCAAGAAGT ACCCGAAGCTGGAAGCGAATTCGTCTACGGAGACTACAA GGTCTACGACGTCAGAAAGATGATCGCAAAGAGCGAACAG GAAATCGGAAAGGCAACAGCAAAGTACTTCTTCTACAGCA ACATCATGAACTTCTTCAAGACAGAAATCACACTGGCAAAC GGAGAAATCAGAAAGAGACCGCTGATCGAAACAAACGGA GAAACAGGAGAAATCGTCTGGGACAAGGGAAGAGACTTCG CAACAGTCAGAAAGGTCTGAGCATGCCGCAGGTCAACAT CGTCAAGAAGACAGAAGTCCAGACAGGAGGATTAGCAAG GAAAGCATCCTGCCGAAGAGAAACAGCGACAAGCTGATCG CAAGAAAGAAGGACTGGGACCCGAAGAAGTACGGAGGATT CGACAGCCCGACAGTCGCATACAGCGTCCTGGTCGTCGCAA AGGTCGAAAAGGGAAAGAGCAAGAAGCTGAAGAGCGTCA AGGAAGTGTGGGAATCACAATCATGGAAAGAAGCAGCTT CGAAAAGAACCCGATCGACTTCCTGGAAGCAAAGGGATAC AAGGAAGTCAAGAAGGACCTGATCATCAAGCTGCCGAAGT ACAGCCTGTTTCAACTGGAAAACGGAAGAAAGAGAATGCT GGCAAGCGCAGGAGAACTGCAGAAGGGAAACGAACTGGC ACTGCCGAGCAAGTACGTCAACTTCCTGTACCTGGCAAGCC ACTACGAAAAGCTGAAGGGAAGCCCGGAAGACAACGAAC AGAAGCAGCTGTTCTGTCGAACAGCACAAAGCACTACCTGGA CGAAATCATCGAACAGATCAGCGAATTCAGCAAGAGAGTC ATCCTGGCAGACGCAAACCTGGACAAGGTCTGAGCGCAT ACAACAAGCACAGAGACAAGCCGATCAGAGAACAGGCAG AAAACATCATCCACCTGTTTCACTGACAAACCTGGGAGCA CCGGCAGCATTCAAGTACTTCGACACAACAATCGACAGAA AGAGATACACAAGCACAAAGGAAGTCCTGGACGCAACACT GATCCACCAGAGCATCACAGGACTGTACGAAACAAGAATC GACCTGAGCCAGCTGGGAGGAGACGGAGGAGGAAGCCCG AAGAAGAAGAGAAAGGTCTAG
ORF encoding Sp. Cas9	802	ATGGACAAGAAGTACTCCATCGGCCTGGACATCGGCACCAACTCC GTGGGCTGGGCCGTGATCACCGACGAGTACAAGGTGCCCTCCAA GAAGTTCAAGGTGCTGGGCAACACCGACCGGCACTCCATCAAGA AGAACCTGATCGGCGCCCTGCTGTTCTGACTCCGGCGAGACCGCCG AGGCCACCCGGCTGAAGCGGACCGCCCGGCGGCGGTACACCCGG CGGAAGAACCGGATCTGCTACCTGCAGGAGATCTTCTCCAACGAG ATGGCCAAGGTGGACGACTCCTTCTTCCACCGGCTGGAGGAGTCC TTCCTGGTGGAGGAGGACAAGAAGCACGAGCGGCACCCCATCTT CGGCAACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCCA CCATCTACCACCTGCGGAAGAAGCTGGTGGACTCCACCGACAAG GCCGACCTGCGGCTGATCTACCTGGCCCTGGCCACATGATCAAG TTCCGGGGCCACTTCTGATCGAGGGCGACCTGAACCCCGACAAC TCCGACGTGGACAAGCTGTTTATCCAGCTGGTGCAGACCTACAAC CAGCTGTTTCGAGGAGAACCCCATCAACGCCTCCGGCGTGGACGCC AAGGCCATCCTGTCCGCCCGGCTGTCCAAGTCCCGGCGGCTGGAG AACCTGATCGCCAGCTGCCCGGCGAGAAGAAGAACGGCCTGTT CGGCAACCTGATCGCCCTGTCCCTGGGCCTGACCCCAACTTCAA GTCCAACCTCGACCTGGCCGAGGACGCAAGCTGCAGCTGTCCAA GGACACCTACGACGACGACCTGGACAACCTGTGGCCAGATCG GCGACCACTACGCCGACCTGTTCTGGCCGCCAAGAACCTGTCCG ACGCCATCCTGCTGTCCGACATCCTGCGGGTGAACACCGAGATCA CCAAGGCCCCCTGTCCGCCCTCCATGATCAAGCGGTACGACGAGC

	ACCACCAGGACCTGACCCTGCTGAAGGCCCTGGTGCGGCAGCAG CTGCCCCGAGAAGTACAAGGAGATCTTCTTCGACCAGTCCAAGAAC GGCTACGCCGGCTACATCGACGGCGGCCTCCCAGGAGGAGTTC TACAAGTTCATCAAGCCCATCCTGGAGAAGATGGACGGCACCGA GGAGCTGCTGGTGAAGCTGAACCGGGAGGACCTGCTGCGGAAGC AGCGGACCTTCGACAACGGCTCCATCCCCACCAGATCCACCTGG GCGAGCTGCACGCCATCCTGCGGCGGCAGGAGGACTTCTACCCCT TCCTGAAGGACAACCGGGAGAAGATCGAGAAGATCCTGACCTTC CGGATCCCCTACTACGTGGGCCCCCTGGCCCCGGGGCAACTCCCGG TTCGCCTGGATGACCCGGAAGTCCGAGGAGACCATACCCCTTG AACTTCGAGGAGGTGGTGGACAAGGGCGCCTCCGCCCAGTCTTC ATCGAGCGGATGACCAACTTCGACAAGAACCTGCCAACAGAGAA GGTGCTGCCCAAGCACTCCCTGCTGTACGAGTACTTCACCGTGTA CAACGAGCTGACCAAGGTGAAGTACGTGACCGAGGGCATGCGGA AGCCCCGCTTCCTGTCCGGCGAGCAGAAGAAGGCCATCGTGACCC TGCTGTTCAAGACCAACCGGAAGGTGACCGTGAAGCAGCTGAAG GAGGACTACTTCAAGAAGATCGAGTGCTTCGACTCCGTGGAGATC TCCGGCGTGGAGGACCGGTTCAACGCCTCCCTGGGCACCTACCAC GACCTGCTGAAGATCATCAAGGACAAGGACTTCCTGGACAACGA GGAGAACGAGGACATCCTGGAGGACATCGTGCTGACCTGACCC TGTTTCGAGGACCGGGAGATGATCGAGGAGCGGCTGAAGACCTAC GCCCACCTGTTTCGACGACAAGGTGATGAAGCAGCTGAAGCGGCG GCGGTACACCGGCTGGGGCCGGCTGTCCCGGAAGCTGATCAACG GCATCCGGGACAAGCAGTCCGGCAAGACCATCCTGGACTTCCTGA AGTCCGACGGCTTCGCCAACCGGAACCTTCATGCAGCTGATCCACG ACGACTCCCTGACCTTCAAGGAGGACATCCAGAAGGCCCAGGTGT CCGGCCAGGGCGACTCCCTGCACGAGCACATCGCCAACCTGGCCG GCTCCCCCGCCATCAAGAAGGGCATCCTGCAGACCGTGAAGGTG GTGGACGAGCTGGTGAAGGTGATGGGCCGGCACAAGCCCCGAGAA CATCGTGATCGAGATGGCCCCGGGAGAACCAGACCACCCAGAAGG GCCAGAAGAAGTCCCGGGAGCGGATGAAGCGGATCGAGGAGGGC ATCAAGGAGCTGGGCTCCCAGATCCTGAAGGAGCACCCCGTGGA GAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCA GAACGGCCGGGACATGTACGTGGACCAGGAGCTGGACATCAACC GGCTGTCCGACTACGACGTGGACCACATCGTGCCCCAGTCTTCC TGAAGGACGACTCCATCGACAACAAGGTGCTGACCCGGTCCGAC AAGAACCGGGGCAAGTCCGACAACGTGCCCTCCGAGGAGGTGGT GAAGAAGATGAAGAAGTACTGGCGGCAGCTGCTGAACGCCAAGC TGATCACCCAGCGGAAGTTCGACAACCTGACCAAGGCCGAGCGG GGCGGCTGTCCGAGCTGGACAAGGCCGGCTTCATCAAGCGGCA GCTGGTGGAGACCCGGCAGATCACCAAGCACGTGGCCCCAGATCC TGGACTCCCGGATGAACACCAAGTACGACGAGAACGACAAGCTG ATCCGGGAGGTGAAGGTGATCACCTGAAGTCCAAGCTGGTGTCC GACTTCCGGAAGGACTTCCAGTTCTACAAGGTGCGGGAGATCAAC AACTACCACCACGCCCACGACGCCTACCTGAACGCCGTGGTGGGC ACCGCCCTGATCAAGAAGTACCCCAAGCTGGAGTCCGAGTTCGTG TACGGCGACTACAAGGTGTACGACGTGCGGAAGATGATCGCCAA GTCCGAGCAGGAGATCGGCAAGGCCACCGCCAAGTACTTCTTCTA CTCCAACATCATGAAGTCTTCAAGACCGAGATCACCTGGCCAA CGGCGAGATCCGGAAGCGGCCCCCTGATCGAGACCAACGGCGAGA CCGGCGAGATCGTGTGGGACAAGGGCCGGGACTTCGCCACCGTG CGGAAGGTGCTGTCCATGCCCCAGGTGAACATCGTGAAGAAGAC CGAGGTGCAGACCGGCGGCTTCTCAAGGAGTCCATCCTGCCCAA GCGGAAGTCCGACAAGCTGATCGCCCGGAAGAAGGACTGGGACC CCAAGAAGTACGGCGGCTTCGACTCCCCACCGTGGCCTACTCCG TGCTGGTGGTGGCCAAGGTGGAGAAGGGCAAGTCCAAGAAGCTG
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		AAGTCCGTGAAGGAGCTGCTGGGCATCACCATCATGGAGCGGTCC TCCTTCGAGAAGAACCCCATCGACTTCCTGGAGGCCAAGGGCTAC AAGGAGGTGAAGAAGGACCTGATCATCAAGCTGCCCAAGTACTC CCTGTTCGAGCTGGAGAACGGCCGGAAGCGGATGCTGGCCTCCGC CGGCGAGCTGCAGAAGGGCAACGAGCTGGCCCTGCCCTCCAAGT ACGTGAACTTCCTGTACCTGGCCTCCCACTACGAGAAGCTGAAGG GCTCCCCGAGGACAACGAGCAGAAGCAGCTGTTCTGTGGAGCAG CACAAGCACTACCTGGACGAGATCATCGAGCAGATCTCCGAGTTC TCCAAGCGGGTGATCCTGGCCGACGCCAACCTGGACAAGGTGCTG TCCGCCTACAACAAGCACCGGGACAAGCCCATCCGGGAGCAGGC CGAGAACATCATCCACCTGTTACCCCTGACCAACCTGGGGCGCCCC CGCCGCCTTCAAGTACTTCGACACCACCATCGACCGGAAGCGGTA CACCTCCACCAAGGAGGTGCTGGACGCCACCCTGATCCACCAATC CATCACCGGCCTGTACGAGACCCGGATCGACCTGTCCAGCTGGG CGGCGACGGCGGGCGGCTCCCCAAGAAGAAGCGGAAGGTGTGA
Open reading frame for Cas9 with Hibit tag	803	AUGGACAAGAAGUACUCCAUCGGCCUGGACAUCCGACCAACU CCGUGGGCUGGGCCGUGAUCACCGACGAGUACAAGGUGCCCUCC AAGAAGUUAAGGUGCUGGGCAACACCGACCGGCACUCCAUA AGAAGAACCUGAUCGGCGCCUGCUGUUCGACUCCGGCGAGACC GCCGAGGCCACCCGGCUGAAGCGGACCGCCGGCGGGUACAC CCGGCGGAAGAACCUGAUCUGCUACCUGCAGGAGAUUUUCC AACGAGAUGGCCAAGGUGGACGACUCCUUCUCCACCGGCGUGG AGGAGUCCUUCUGGUGGAGGAGGACAAGAAGCACGAGCGGCA CCCCAUCUUCGGCAACAUCGUGGACGAGGUGGCCUACCACGAGA AGUACCCACCAUCUACCACCUGCGGAAGAAGCUGGUGGACUCC ACCGACAAGGCCGACCGUGCGGCUGAUCUACCUGGCCUUGGCCA CAUGAUCAGUUCGGGGCCACUUCUGAUCGAGGGCGACCGUG AACCCCGACAACUCCGACGUGGACAAGCUGUUAUCCAGCUGGU GCAGACCUACAACCAGCUGUUCGAGGAGAACCCCAUCAACGCCU CCGGCGUGGACGCCAAGGCCAUCCUGUCCGCCCGGCGUGUCCAAG UCCCGGCGGCGUGGAGAACCUGAUCGCCCAGCUGCCCGGCGAGAA GAAGAACGGCCUGUUCGGCAACCUGAUCGCCCUGUCCUGGGCC UGACCCCAACUUAAGUCCAACUUCGACCUGGCCGAGGACGCC AAGCUGCAGCUGUCCAAGGACACCUACGACGACGACCUUGGACA ACCUGCUGGCCCGAGUCCGGCGACCAUACGCCGACCUUGUCCUG GCCGCCAAGAACCUGUCCGACGCCAUCCUGCUGUCCGACAUCUCC GCGGGUGAACACCGAGAUACCAAGGCCCCCCUGUCCGCCUCCA UGAUCAGCGGUACGACGAGCACCACCAGGACCUGACCCUGCUG AAGGCCUGGUGCGGCAGCAGCUGCCCGAGAAGUACAAGGAGA UCUUCUUCGACCAAGUCCAAGAACGGCUACGCCGGCUACAUCGAC GGCGGCGCCUCCAGGAGGAGUUCUACAAGUUAUCAAGCCCA UCCUGGAGAAGAUGGACGGCACCAGGAGCUGCUGGUGAAGCU GAACCGGGAGGACCUGCUGCGGAAGCAGCGGACCUUCGACAAC GGCUCCAUCCCCACCAGAUCCACCUGGGCGAGCUGCACGCCAU CCUGCGGCGGCAGGAGGACUUCUACCCCUUCCUGAAGGACAACC GGGAGAAGAUAGAGAAGAUCCUGACCUUCCGGAUCCCCUACUA CGUGGGCCCCUGGCCCGGGGCAACUCCCGGUUCGCCUGGAUGA CCCGGAAGUCCGAGGAGACCAUACCCCUUGGAACUUCGAGGAG GUGGUGGACAAGGGCGCCUCCGCCAGUCCUUAUCGAGCGGA UGACCAACUUCGACAAGAACCUGCCCAACGAGAAGGUGCUGCCC AAGCACUCCUGCUGUACGAGUACUUCACCGUGUACAACGAGC UGACCAAGGUGAAGUACGUGACCGAGGGCAUGCGGAAGCCCGC CUUCCUGUCCGGCGAGCAGAAGAAGGCCAUUCGUGGACCUGCUG UUAAGACCAACCGGAAGGUGACCGUGAAGCAGCUGAAGGAGG ACUACUUAAGAAGAUUCGAGUGCUUCGACUCCGUGGAGUUC CGGCGUGGAGGACCGGUUAACGCCUCCUGGGCACCUACCACG ACCUGCUGAAGAUCAUCAAGGACAAGGACUUCUGGACAACGA GGAGAACGAGGACAUCCUGGAGGACAUCGUGCUGACCCUGACC CUGUUCGAGGACCGGGAGAUGAUCGAGGAGCGGCUGAAGACCU

		ACGCCCACCUGUUCGACGACAAGGUGAUGAAGCAGCUGAAGCG GCGGCGGUACACCGGCUGGGGCCGGCUGUCCCGGAAGCUGAUC AACGGCAUCCGGGACAAGCAGUCCGGCAAGACCAUCCUGGACU UCCUGAAGUCCGACGGCUUCGCCAACCGGAACUUCAGCAGCUG AUCCACGACGACUCCUGACCUUCAAGGAGGACAUCAGAAAGGC CCAGGUGUCCGGCCAGGGCGACUCCUGCAGGACACAUCGCCA ACCUGGCCGGCUCCCCGCCAUCAAGAAGGGCAUCCUGCAGACC GUGAAGGUGGUGGACGAGCUGGUGAAGGUGAUGGGCCGGCACA AGCCCGAGAACAUCGUGAUCGAGAUUGGCCCGGGAGAACAGAC CACCCAGAAGGGCCAGAAGAACUCCCGGGAGCGGAUGAAGCGG AUCGAGGAGGGCAUCAAGGAGCUGGGCUCCAGAUCCUGAAGG AGCACCCCGUGGAGAACACCCAGCUGCAGAACGAGAAGCUGUA CCUGUACUACCUGCAGAACGGCCGGGACAUGUACGUGGACCAG GAGCUGGACAUCAACCGGCUGUCCGACUACGACGUGGACCACA UCGUGCCCCAGUCCUCCUGAAGGACGACUCCAUCCGACAACAG GUGCUGACCCGGUCCGACAAGAACCAGGGCAAGUCCGACAACG UGCCCUCCGAGGAGGUGGUGAAGAAGAUGAAGAACUACUGGCG GCAGCUGCUGAACGCCAAGCUGAUCACCCAGCGGAAGUUCGAC AACCUGACCAAGGCCGAGCGGGGCGGCCUGUCCGAGCUGGACA AGGCCGGCUUCAUCAAGCGGCAGCUGGUGGAGACCCGGCAGAU CACCAAGCACGUGGCCCAGAUCCUGGACUCCCGGAUGAACACCA AGUACGACGAGAACGACAAGCUGAUCGGGAGGUGAAGGUGAU CACCCUGAAGUCCAAGCUGGUGUCCGACUUCGGGAAGGACUUC AGUUCUACAAGGUGCGGGAGAUCAACAACUACCACCGCCAC GACGCCUACCUGAACGCCGUGGUGGGCACCGCCUGAUCAGAA GUACCCCAAGCUGGAGUCCGAGUUCGUGUACGGCGACUACAAG GUGUACGACGUGCGGAAGAUGAUCGCCAAGUCCGAGCAGGAGA UCGGCAAGGCCACCGCCAAGUACUUCUUCUACUCCAACAUCUG AACUUCUUAAGACCGAGAUACCCUGGCCAACGGCGAGAUCCG GAAGCGGCCCCUGAUCGAGACCAACGGCGAGACCGGCGAGAU GUGUGGGACAAGGGCCGGGACUUCGCCACCGUGCGGAAGGUGC UGUCCAUGCCCCAGGUGAACAUUCGUGAAGAAGACCGAGGUGCA GACCGGCGGCUUCUCCAAGGAGUCCAUCCUGCCCCAAGCGGAACU CCGACAAGCUGAUCGCCCCGGAAGAAGGACUGGGACCCCCAAGAA GUACGGCGGCUUCGACUCCCCACCGUGGCCUACUCCUGGUG UGGUGGGCAAGGUGGAGAAGGGCAAGUCCAAGAAGCUGAAGUC CGUGAAGGAGCUGCUGGGCAUCACCAUCAUGGAGCGGUCCUCC UUCGAGAAGAACCCCAUCGACUUCUGGAGGCCAAGGGCUACA AGGAGGUGAAGAAGGACCUGAUCAUCAAGCUGCCCCAAGUACUC CCUGUUCGAGCUGGAGAACGGCCGGAAGCGGAUGCUGGCCUCC GCCGGCGAGCUGCAGAAGGGCAACGAGCUGGCCCUCCUCCAA GUACGUGAACUUCUGUACCUUGGCCUCCACUACGAGAAGCUG AAGGGCUCCCCCGAGGACAACGAGCAGAAGCAGCUGUUCGUGG AGCAGCACAAAGCACUACCUGGACGAGAUCAUCGAGCAGAUUC CGAGUUCUCCAAGCGGGUGAUCUUGGCCGACGCCAACCUGGACA AGGUGCUGUCCGCCUACAACAAGCACCGGGACAAGCCAUCCGG GAGCAGGCCGAGAACAUCAUCCACCUGUUCACCCUGACCAACCU GGGCGCCCCCGCCGCCUUAAGUACUUCGACACCACCAUCGACC GGAAGCGGUACACCUCCACCAAGGAGGUGCUGGACGCCACCCUG AUCCACCAGUCCAUCACCGGCCUGUACGAGACCCGGAUCGACCU GUCCCAGCUGGGCGGCGACGGCGGCGGCUCUCCCCAAGAAGAAGC GGAAGGUGUCCGAGUCCGCCACCCCCGAGUCCGUGUCCGGCUGG CGGCUGUUCAAGAAGAUCCUGA
HD1 TCR insertion including ITRs	1001	TTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTACTGAGGCCGGG CGACCAAAGGTCGCCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCA GTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCAC TAGGGGTTCTTAGATCTTGCCAACATACCATAAACCTCCCATTCT GCTAATGCCAGCCTAAGTTGGGGAGACCACTCCAGATTCCAAGA TGTACAGTTTGCTTTGCTGGGCCTTTTCCCATGCCTGCTTTACTC TGCCAGAGTTATATTGCTGGGGTTTTGAAGAAGATCCTATTAAAT

	AAAAGAATAAGCAGTATTATTAAGTAGCCCTGCATTTTCAGGTTTC CTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCA TGGCCTCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCC AGTCCATCACGAGCAGCTGGTTTCTAAGATGCTATTTCCCGTATA AAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTG TCCATCACTGGCATCTGGACTCCAGCCTGGGTTGGGGCAAAGAGG GAAATGAGATCATGTCCTAACCCCTGATCCTCTTGTCCACAGATA TCCAGAACCCTGACCCTGCGGCTCCGGTGCCCGTCAGTGGGCAGA GCGCACATCGCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTGCG GCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGG GAAAGTGATGTCGTGTAAGTGGCTCCGCCTTTTTCCCGAGGGTGGG GGAGAACCCTATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTT CGCAACGGGTTTGGCCGAGAACACAGGTAAGTGCCGTGTGTGGT TCCCGCGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTGCCTT GAATTACTTCCACGCCCTGGCTGCAGTACGTGATTCCTTGATCCCCG AGCTTCGGGTTTGAAGTGGGTGGGAGAGTTCGAGGCCTTGCGCTT AAGGAGCCCCCTTCGCTCGTGCTTGAGTTGAGGCCTGGCTTGGGC GCTGGGGCCGCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGTC TCGCTGCTTTGATAAGTCTCTAGCCATTTAAAATTTTTGATGACC TGCTGCGACGCTTTTTTTCTGGCAAGATAGTCTTGTAATGCGGGC CAAGATGTGCACACTGGTATTTTCGGTTTTTGGGGCCGCGGGCGGC GACGGGGCCCGTGCGTCCCAGCGCACATGTTCCGGCAGGCGGGG CCTGCGAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAG CTGGCCGGCCTGCTCTGGTGCCTGGCCTCGCGCCGCCGTGTATCG CCCC GCCCTGGGCGGCAAGGCTGGCCCGGTGCGCCACCAATGCGGT GAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTGCAGGGAGCTCA AAATGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAGTCACC CACACAAAGGAAAAGGGCCTTTCCGTCCTCAGCCGTGCTTCATG TGACTCCACGAGTACCGGGCGCCGTCCAGGCACCTCGATTAGTT CTCGAGCTTTTGGAGTACGTCGTCTTAGGTTGGGGGGAGGGGTT TTATGCGATGGAGTTTCCCCACACTGAGTGGGTGGAGACTGAAGT TAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCCTT TTTGAGTTTGGATCTTGGTTCAATTCTCAAGCCTCAGACAGTGGTTC AAAGTTTTTTTCTTCCATTTACAGGTGTCGTGATGCGGCCGCCACCA TGGGATCTTGGACACTGTGTGCGTGTCCTGTGCATCTGCTGGC CAAGCACACAGATGCCGGCGTGATCCAGTCTCCTAGACACGAAGT GACCGAGATGGGCCAAGAAGTGACCCTGCGCTGCAAGCCTATCA GCGGCCACGATTACCTGTTCTGGTACAGACAGACCATGATGAGAG GCCTGGAAGTCTGATCTACTTCAACAACAACGTGCCCATCGACG ACAGCGGCATGCCCCAGGATAGATTACGCGCCAAGATGCCAAC GCCAGCTTCAGCACCTGAAGATCCAGCCTAGCGAGCCAGAGAT AGCGCCGTGTACTTCTGCGCCAGCAGAAAGACAGGCGGCTACAG CAATCAGCCCCAGCACTTTGGAGATGGCACCCGGCTGAGCATCCT GGAAGATCTGAAGAACGTGTTCCACCTGAGGTGGCCGTGTTCTGA GCCTTCTGAGGCCGAGATCAGCCACACACAGAAAGCCACACTCGT GTGTCTGGCCACCGGCTTCTATCCCGATCACGTGGAAGTGTCTTG GTGGGTCAACGGCAAAGAGGTGCACAGCGGCGTCAGCACCGATC CTCAGCCTCTGAAAGAGCAGCCCGCTCTGAACGACAGCAGATACT GCCTGAGCAGCAGACTGAGAGTGTCCGCCACCTTCTGGCAGAACC CCAGAAACCACTTCAGATGCCAGGTGCAGTTCTACGGCCTGAGCG AGAACGATGAGTGGACCCAGGATAGAGCCAAGCCTGTGACACAG ATCGTGTCTGCCGAAGCCTGGGGCAGAGCCGATTGTGGCTTTACC AGCGAGAGCTACCAGCAGGGCGTGCTGTCTGCCACAATCCTGTAC GAGATCCTGCTGGGCAAAGCCACTCTGTACGCCGTGCTGGTGTCT GCCCTGGTGTGATGGCCATGGTCAAGCGGAAGGATAGCAGGGG CGGCTCCGGTGCCACAAACTTCTCCCTGCTCAAGCAGGCCGGAGA TGTGGAAGAGAACCCTGGCCCTATGGAAACCCTGCTGAAGGTGCT GAGCGGCACACTGCTGTGGCAGCTGACATGGGTCCGATCTCAGCA GCCTGTGCAGTCTCCTCAGGCCGTGATTCTGAGAGAAGGCGAGGA CGCCGTGATCAACTGCAGCAGCTCTAAGGCCCTGTACAGCGTGCA
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		CTGGTACAGACAGAAGCACGGCGAGGCCCTGTGTTCTGATGAT CCTGCTGAAAGGCGGCGAGCAGAAAGGGCCACGAGAAGATCAGCG CCAGCTTCAACGAGAAGAAGCAGCAGTCCAGCCTGTACCTGACA GCCAGCCAGCTGAGCTACAGCGGCACCTACTTTTGTGGCACCGCC TGGATCAACGACTACAAGCTGTCTTTCGGAGCCGGCACCACAGTG ACAGTGCGGGCCAATATTCAGAACCCCGATCCTGCCGTGTACCAG CTGAGAGACAGCAAGAGCAGCGACAAGAGCGTGTGCCTGTTTAC CGACTTCGACAGCCAGACCAACGTGTCCCAGAGCAAGGACAGCG ACGTGTACATCACCGATAAGACTGTGCTGGACATGCGGAGCATGG ACTTCAAGAGCAACAGCGCCGTGGCCTGGTCCAACAAGAGCGAT TTCGCCTGCGCCAACGCCTTCAACAACAGCATTATCCCCGAGGAC ACATTCTTCCCAAGTCCTGAGAGCAGCTGCGACGTGAAGCTGGTG GAAAAGAGCTTCGAGACAGACACCAACCTGAACTTCCAGAACCT GAGCGTGATCGGCTTCAGAATCCTGCTGCTCAAGGTGGCCGGCTT CAACCTGCTGATGACCCTGAGACTGTGGTCCAGCTAACCTCGACT GTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCCTCCCCCGTGC CTTCCTTGACCCTGGAAGGTGCCACTCCCCTGTCTTTTCTAATA AAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCTATTCTAT TCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGG AAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTT CTGAGGCGGAAAGAACCAGCTGGGGCTCTAGGGGGTATCCCCAC TAGTCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTG TCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTACAAAG TAAGGATTCTGATGTGTATATCACAGACAAAACCTGTGCTAGACAT GAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCA ACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATT TTCCAGAAGACACCTTCTTCCCCAGCCAGGTAAGGGCAGCTTTG GTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAATGGCCAGGTTCTG CCCAGAGCTCTGGTCAATGATGTCTAAAACCTCTCTGATTGGTGG TCTCGGCCTTATCCATTGCCACCAAAACCTCTTTTTACTAAGAAA CAGTGAGCCTTGTCTGGCAGTCCAGAGAATGACACGGGAAAAA AGCAGATGAAGAGAAGGTGGCAGGAGAGGGCACGTGGCCAGCC TCAGTCTCTAGATCTAGGAACCCCTAGTGATGGAGTTGGCCACTC CCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCCGGGCAAAGC CCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGC GAGCGCGCAGAGAGGGAGTGGCCAA
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What is Claimed is:

1. An engineered cell comprising a genetic modification in a human LAG3 sequence, within genomic coordinates of chr12: 6772483-6778455.
2. The engineered cell of claim 1, wherein the genetic modification is selected from an insertion, a deletion, and a substitution.
3. The engineered cell of claim 1 or 2, wherein the genetic modification inhibits expression of the LAG3 gene.
4. The engineered cell of any one of claims 1-3, wherein the genetic modification comprises a modification of at least one nucleotide within the genomic coordinates selected from:

LAG 3 NO	Genomic Coordinates (hg38)
LAG3-1	chr12:6773938-6773958
LAG3-2	chr12:6774678-6774698
LAG3-3	chr12:6772894-6772914
LAG3-4	chr12:6774816-6774836
LAG3-5	chr12:6774742-6774762
LAG3-6	chr12:6775380-6775400
LAG3-7	chr12:6774727-6774747
LAG3-8	chr12:6774732-6774752
LAG3- 9	chr12:6777435-6777455
LAG3-10	chr12:6774771-6774791
LAG3-11	chr12:6772909-6772929
LAG3-12	chr12:6774735-6774755
LAG3-13	chr12:6773783-6773803
LAG3-14	chr12:6775292-6775312
LAG3-15	chr12:6777433-6777453
LAG3-16	chr12:6778268-6778288
LAG3-17	chr12:6775444-6775464
LAG3-24	chr12:6777783-6777803
LAG3-26	chr12:6777784-6777804
LAG3-41	chr12:6778252-6778272
LAG3-59	chr12:6777325-6777345

LAG 3 NO	Genomic Coordinates (hg38)
LAG3-83	chr12:6777329-6777349

;or

the genomic coordinates selected from those targeted by LAG3-1 through LAG3-15:

chr12:6773938-6773958, chr12:6774678-6774698, chr12:6772894-6772914, chr12:6774816-6774836, chr12:6774742-6774762, chr12:6775380-6775400, chr12:6774727-6774747, chr12:6774732-6774752, chr12:6777435-6777455, chr12:6774771-6774791, chr12:6772909-6772929, chr12:6774735-6774755, chr12:6773783-6773803, chr12:6775292-6775312, and chr12:6777433-6777453; or

the genomic coordinates selected from those targeted by LAG3-1 through LAG3-11:

chr12:6773938-6773958, chr12:6774678-6774698, chr12:6772894-6772914, and chr12:6774816-6774836, chr12:6774742-6774762, chr12:6775380-6775400, chr12:6774727-6774747, chr12:6774732-6774752, chr12:6777435-6777455, chr12:6774771-6774791, and chr12:6772909-6772929; or

the genomic coordinates selected from those targeted by LAG3-1 through LAG3-4:

chr12:6773938-6773958, chr12:6774678-6774698, chr12:6772894-6772914, and chr12:6774816-6774836; or

the genomic coordinates selected from those targeted by LAG3-1, LAG3-4, LAG3-5, and LAG3-9: chr12:6773938-6773958, chr12:6774816-6774836, chr12:6774742-6774762, and chr12:6777435-6777455.

5. The engineered cell of any one of claims 1-4, wherein the engineered cell comprises a genetic modification within the genomic coordinates of an endogenous T cell receptor (TCR) sequence, wherein the genetic modification inhibits expression of the TCR gene, optionally wherein the TCR gene is TRAC or TRBC.

6. The engineered cell of claim 5, comprising a genetic modification of TRBC within genomic coordinates selected from:

TRBC NO:	Genomic Coordinates (hg38)
TRBC-1	chr7:142791996-142792016
TRBC-2	chr7:142792047-142792067
TRBC-3	chr7:142792008-142792028
TRBC-4	chr7:142791931-142791951
TRBC-5	chr7:142791930-142791950
TRBC-6	chr7:142791748-142791768

TRBC-7	chr7:142791720-142791740
TRBC-8	chr7:142792041-142792061
TRBC-9	chr7:142802114-142802134
TRBC-10	chr7:142792009-142792029
TRBC-11	chr7:142792697-142792717
TRBC-12	chr7:142791963-142791983
TRBC-13	chr7:142791976-142791996
TRBC-14	chr7:142791974-142791994
TRBC-15	chr7:142791970-142791990
TRBC-16	chr7:142791948-142791968
TRBC-17	chr7:142791913-142791933
TRBC-18	chr7:142791961-142791981
TRBC-19	chr7:142792068-142792088
TRBC-20	chr7:142791975-142791995
TRBC-21	chr7:142791773-142791793
TRBC-22	chr7:142791919-142791939
TRBC-23	chr7:142791834-142791854
TRBC-24	chr7:142791878-142791898
TRBC-25	chr7:142802141-142802161
TRBC-26	chr7:142791844-142791864
TRBC-27	chr7:142801154-142801174
TRBC-28	chr7:142791961-142791981
TRBC-29	chr7:142792001-142792021
TRBC-30	chr7:142791979-142791999
TRBC-31	chr7:142792041-142792061
TRBC-32	chr7:142792003-142792023
TRBC-33	chr7:142791984-142792004
TRBC-34	chr7:142792002-142792022
TRBC-35	chr7:142791966-142791986
TRBC-36	chr7:142792007-142792027
TRBC-37	chr7:142791993-142792013
TRBC-38	chr7:142791902-142791922

TRBC-39	chr7:142791724-142791744
TRBC-40	chr7:142791973-142791993
TRBC-41	chr7:142791920-142791940
TRBC-42	chr7:142791994-142792014
TRBC-43	chr7:142791887-142791907
TRBC-44	chr7:142791907-142791927
TRBC-45	chr7:142791952-142791972
TRBC-46	chr7:142791721-142791741
TRBC-47	chr7:142792718-142792738
TRBC-48	chr7:142791729-142791749
TRBC-49	chr7:142791911-142791931
TRBC-50	chr7:142791867-142791887
TRBC-51	chr7:142791899-142791919
TRBC-52	chr7:142791727-142791747
TRBC-53	chr7:142791949-142791969
TRBC-54	chr7:142791933-142791953
TRBC-55	chr7:142791932-142791952
TRBC-56	chr7:142792057-142792077
TRBC-57	chr7:142791940-142791960
TRBC-58	chr7:142791747-142791767
TRBC-59	chr7:142791881-142791901
TRBC-60	chr7:142791779-142791799
TRBC-61	chr7:142792054-142792074
TRBC-62	chr7:142792069-142792089
TRBC-63	chr7:142792712-142792732
TRBC-64	chr7:142791729-142791749
TRBC-65	chr7:142791821-142791841
TRBC-66	chr7:142792052-142792072
TRBC-67	chr7:142791916-142791936
TRBC-68	chr7:142791899-142791919
TRBC-69	chr7:142791772-142791792
TRBC-70	chr7:142792714-142792734

TRBC-71	chr7:142792042-142792062
TRBC-72	chr7:142791962-142791982
TRBC-73	chr7:142791988-142792008
TRBC-74	chr7:142791982-142792002
TRBC-75	chr7:142792049-142792069
TRBC-76	chr7:142791839-142791859
TRBC-77	chr7:142791893-142791913
TRBC-78	chr7:142791945-142791965
TRBC-79	chr7:142791964-142791984
TRBC-80	chr7:142791757-142791777
TRBC-81	chr7:142792048-142792068
TRBC-82	chr7:142791774-142791794
TRBC-83	chr7:142792048-142792068
TRBC-84	chr7:142791830-142791850
TRBC-85	chr7:142791909-142791929
TRBC-86	chr7:142791912-142791932
TRBC-87	chr7:142791766-142791786
TRBC-88	chr7:142791880-142791900
TRBC-89	chr7:142791919-142791939

7. The engineered cell of any one of claims 4-6, comprising a genetic modification of TRAC within genomic coordinates selected from:

TRAC NO:	Genomic Coordinates (hg38)
TRAC-90	chr14:22547524-22547544
TRAC-91	chr14:22550581-22550601
TRAC-92	chr14:22550608-22550628
TRAC-93	chr14:22550611-22550631
TRAC-94	chr14:22550622-22550642
TRAC-95	chr14:22547529-22547549
TRAC-96	chr14:22547512-22547532
TRAC-97	chr14:22547525-22547545
TRAC-98	chr14:22547536-22547556

TRAC-99	chr14:22547575-22547595
TRAC-100	chr14:22547640-22547660
TRAC-101	chr14:22547647-22547667
TRAC-102	chr14:22547777-22547797
TRAC-103	chr14:22549638-22549658
TRAC-104	chr14:22549646-22549666
TRAC-105	chr14:22550600-22550620
TRAC-106	chr14:22550605-22550625
TRAC-107	chr14:22550625-22550645
TRAC-108	chr14:22539116-22539136
TRAC-109	chr14:22539120-22539140
TRAC-110	chr14:22547518-22547538
TRAC-111	chr14:22539082-22539102
TRAC-112	chr14:22539061-22539081
TRAC-113	chr14:22539097-22539117
TRAC-114	chr14:22547697-22547717
TRAC-115	chr14:22550571-22550591
TRAC-116	chr14:22550631-22550651
TRAC-117	chr14:22550658-22550678
TRAC-118	chr14:22547712-22547732
TRAC-119	chr14:22550636-22550656
TRAC-120	chr14:22550636-22550656
TRAC-121	chr14:22550582-22550602
TRAC-122	chr14:22550606-22550626
TRAC-123	chr14:22550609-22550629
TRAC-124	chr14:22547691-22547711
TRAC-125	chr14:22547576-22547596
TRAC-126	chr14:22549648-22549668
TRAC-127	chr14:22549660-22549680
TRAC-128	chr14:22547716-22547736
TRAC-129	chr14:22547514-22547534
TRAC-130	chr14:22550662-22550682

TRAC-131	chr14:22550593-22550613
TRAC-132	chr14:22550612-22550632
TRAC-133	chr14:22547521-22547541
TRAC-134	chr14:22547540-22547560
TRAC-135	chr14:22539121-22539141
TRAC-136	chr14:22547632-22547652
TRAC-137	chr14:22547674-22547694
TRAC-138	chr14:22549643-22549663
TRAC-139	chr14:22547655-22547675
TRAC-140	chr14:22547667-22547687
TRAC-141	chr14:22539085-22539105
TRAC-142	chr14:22549634-22549654
TRAC-143	chr14:22539064-22539084
TRAC-144	chr14:22547639-22547659
TRAC-145	chr14:22547731-22547751
TRAC-146	chr14:22547734-22547754
TRAC-147	chr14:22547591-22547611
TRAC-148	chr14:22547657-22547677
TRAC-149	chr14:22547519-22547539
TRAC-150	chr14:22549674-22549694
TRAC-151	chr14:22547678-22547698
TRAC-152	chr14:22539087-22539107
TRAC-153	chr14:22547595-22547615
TRAC-154	chr14:22547633-22547653
TRAC-155	chr14:22547732-22547752
TRAC-156	chr14:22547656-22547676
TRAC-157	chr14:22539086-22539106
TRAC-158	chr14:22547491-22547511
TRAC-159	chr14:22547618-22547638
TRAC-160	chr14:22549644-22549664
TRAC-161	chr14:22547522-22547542
TRAC-162	chr14:22539089-22539109

TRAC-163	chr14:22539062-22539082
TRAC-164	chr14:22547597-22547617
TRAC-165	chr14:22547677-22547697
TRAC-166	chr14:22549645-22549665
TRAC-167	chr14:22550610-22550630
TRAC-168	chr14:22547511-22547531
TRAC-169	chr14:22550607-22550627
TRAC-170	chr14:22550657-22550677
TRAC-171	chr14:22550604-22550624
TRAC-172	chr14:22539132-22539152
TRAC-173	chr14:22550632-22550652
TRAC-174	chr14:22547571-22547591
TRAC-175	chr14:22547711-22547731
TRAC-176	chr14:22547666-22547686
TRAC-177	chr14:22547567-22547587
TRAC-178	chr14:22547624-22547644
TRAC-185	chr14:22547501-22547521
TRAC-213	chr14:22547519-22547539
TRAC-214	chr14:22547556-22547576
TRAC-215	chr14:22547486-22547506
TRAC-216	chr14:22547487-22547507
TRAC-217	chr14:22547493-22547513
TRAC-218	chr14:22547502-22547522

or the genetic modification is within genomic coordinates selected from chr14:22547524-22547544, chr14:22547529-22547549, chr14:22547525-22547545, chr14:22547536-22547556, chr14:22547501-22547521, chr14:22547556-22547576, and chr14:22547502-22547522.

8. The engineered cell of any one of claims 1-7, wherein the cell comprises a genetic modification, wherein the genetic modification inhibits expression of one or more MHC class I proteins.

9. The engineered cell of claim 8, wherein the genetic modification that inhibits expression of one or more MHC class I proteins is a genetic modification in a B2M sequence, wherein the genetic modification is within genomic coordinates selected from:

B2M NO:	Genomic Location (hg38)
B2M-1	chr15:44711469-44711494
B2M-2	chr15:44711472-44711497
B2M-3	chr15:44711483-4471 1508
B2M-4	chr15:44711486-44711511
B2M-5	chr15:44711487-44711512
B2M-6	chr15:44711512-44711537
B2M-7	chr15:44711513-44711538
B2M-8	chr15:44711534-44711559
B2M-9	chr15:44711568-44711593
B2M-10	chr15:44711573-44711598
B2M-11	chr15:44711576-44711601
B2M-12	chr15:44711466-44711491
B2M-13	chr15:44711522-44711547
B2M-14	chr15:44711544-44711569
B2M-15	chr15:44711559-44711584
B2M-16	chr15:44711565-44711590
B2M-17	chr15:44711599-44711624
B2M-18	chr15:44711611-44711636
B2M-19	chr15:44715412-44715437
B2M-20	chr15:44715440-44715465
B2M-21	chr15:44715473-44715498
B2M-22	chr15:44715474-44715499
B2M-23	chr15:44715515-44715540
B2M-24	chr15:44715535-44715560
B2M-25	chr15:44715562-44715587
B2M-26	chr15:44715567-44715592
B2M-27	chr15:44715672-44715697
B2M-28	chr15:44715673-44715698
B2M-29	chr15:44715674-44715699
B2M-30	chr15:44715410-44715435
B2M-31	chr15:44715411-44715436

B2M NO:	Genomic Location (hg38)
B2M-32	chr15:44715419-44715444
B2M-33	chr15:44715430-44715455
B2M-34	chr15:44715457-44715482
B2M-35	chr15:44715483-44715508
B2M-36	chr15:44715511-44715536
B2M-37	chr15:44715515-44715540
B2M-38	chr15:44715629-44715654
B2M-39	chr15:44715630-44715655
B2M-40	chr15:44715631-44715656
B2M-41	chr15:44715632-44715657
B2M-42	chr15:44715653-44715678
B2M-43	chr15:44715657-44715682
B2M-44	chr15:44715666-44715691
B2M-45	chr15:44715685-44715710
B2M-46	chr15:44715686-44715711
B2M-47	chr15:44716326-44716351
B2M-48	chr15:44716329-44716354
B2M-49	chr15:44716313-44716338
B2M-50	chr15:44717599-44717624
B2M-51	chr15:44717604-44717629
B2M-52	chr15:44717681-44717706
B2M-53	chr15:44717682-44717707
B2M-54	chr15:44717702-44717727
B2M-55	chr15:44717764-44717789
B2M-56	chr15:44717776-44717801
B2M-57	chr15:44717786-44717811
B2M-58	chr15:44717789-44717814
B2M-59	chr15:44717790-44717815
B2M-60	chr15:44717794-44717819
B2M-61	chr15:44717805-44717830
B2M-62	chr15:44717808-44717833

B2M NO:	Genomic Location (hg38)
B2M-63	chr15:44717809-44717834
B2M-64	chr15:44717810-44717835
B2M-65	chr15:44717846-44717871
B2M-66	chr15:44717945-44717970
B2M-67	chr15:44717946-44717971
B2M-68	chr15:44717947-44717972
B2M-69	chr15:44717948-44717973
B2M-70	chr15:44717973-44717998
B2M-71	chr15:44717981-44718006
B2M-72	chr15:44718056-44718081
B2M-73	chr15:44718061-44718086
B2M-74	chr15:44718067-44718092
B2M-75	chr15:44718076-44718101
B2M-76	chr15:44717589-44717614
B2M-77	chr15:44717620-44717645
B2M-78	chr15:44717642-44717667
B2M-79	chr15:44717771-44717796
B2M-80	chr15:44717800-44717825
B2M-81	chr15:44717859-44717884
B2M-82	chr15:44717947-44717972
B2M-83	chr15:44718119-44718144

10. The engineered cell of claim 8, wherein the genetic modification that inhibits expression of one or more MHC class I proteins is a genetic modification in an HLA-A sequence and optionally wherein the genetic modification is within genomic coordinates chosen from chr6:29942854 to chr6:29942913 and chr6:29943518 to chr6: 29943619, optionally genomic coordinates chosen from: chr6:29942864-29942884; chr6:29942868-29942888; chr6:29942876-29942896; chr6:29942877-29942897; chr6:29942883-29942903; chr6:29943126-29943146; chr6:29943528-29943548; chr6:29943529-29943549; chr6:29943530-29943550; chr6:29943537-29943557; chr6:29943549-29943569; chr6:29943589-29943609; and chr6:29944026-29944046.

11. The engineered cell of any one of claims 1-10, wherein the cell comprises a genetic modification, wherein the genetic modification inhibits expression of one or more MHC class II proteins.

12. The engineered cell of claim 11, wherein the genetic modification that inhibits expression of one or more MHC class II proteins is a genetic modification in a CIITA sequence, wherein the genetic modification is within the genomic coordinates selected from chr16:10902171-10923242, optionally, chr16:10902662-10923285, chr16:10906542-10923285, or chr16:10906542-10908121, optionally chr16:10908132-10908152, chr16:10908131-10908151, chr16:10916456-10916476, chr16:10918504-10918524, chr16:10909022-10909042, chr16:10918512-10918532, chr16:10918511-10918531, chr16:10895742-10895762, chr16:10916362-10916382, chr16:10916455-10916475, chr16:10909172-10909192, chr16:10906492-10906512, chr16:10909006-10909026, chr16:10922478-10922498, chr16:10895747-10895767, chr16:10916348-10916368, chr16:10910186-10910206, chr16:10906481-10906501, chr16:10909007-10909027, chr16:10895410-10895430, and chr16:10908130-10908150; optionally chr16:10918504-10918524, chr16:10923218-10923238, chr16:10923219-10923239, chr16:10923221-10923241, chr16:10906486-10906506, chr16:10906485-10906505, chr16:10903873-10903893, chr16:10909172-10909192, chr16:10918423-10918443, chr16:10916362-10916382, chr16:10916450-10916470, chr16:10922153-10922173, chr16:10923222-10923242, chr16:10910176-10910196, chr16:10895742-10895762, chr16:10916449-10916469, chr16:10923214-10923234, chr16:10906492-10906512, and chr16:10906487-1090650; or optionally chr16:10916432-10916452, chr16:10922444-10922464, chr16:10907924-10907944, chr16:10906985-10907005, chr16:10908073-10908093, chr16:10907433-10907453, chr16:10907979-10907999, chr16:10907139-10907159, chr16:10922435-10922455, chr16:10907384-10907404, chr16:10907434-10907454,

chr16:10907119-10907139, chr16:10907539-10907559, chr16:10907810-10907830,
 chr16:10907315-10907335, chr16:10916426-10916446, chr16:10909138-10909158,
 chr16:10908101-10908121, chr16:10907790-10907810, chr16:10907787-10907807,
 chr16:10907454-10907474, chr16:10895702-10895722, chr16:10902729-10902749,
 chr16:10918492-10918512, chr16:10907932-10907952, chr16:10907623-10907643,
 chr16:10907461-10907481, chr16:10902723-10902743, chr16:10907622-10907642,
 chr16:10922441-10922461, chr16:10902662-10902682, chr16:10915626-10915646,
 chr16:10915592-10915612, chr16:10907385-10907405, chr16:10907030-10907050,
 chr16:10907935-10907955, chr16:10906853-10906873, chr16:10906757-10906777,
 chr16:10907730-10907750, and chr16:10895302-10895322.

13. The engineered cell of any one of claims 1-12, wherein the cell has reduced cell surface expression of LAG3 protein, or wherein the cell has reduced cell surface expression of LAG3 protein and the cell has reduced cell surface expression of TRAC protein or TRBC protein.

14. The engineered cell of any one of claims 1-13, comprising a genetic modification in a human 2B4/CD244 sequence, within genomic coordinates of chr1: 160830160-160862887.

15. The engineered cell of claim 14, wherein the genetic modification in 2B4/CD244 is within genomic coordinates selected from:

2B4 NO	Genomic Coordinates (hg38)
2B4-1	chr1:160841611-160841631
2B4-2	chr1:160841865-160841885
2B4-3	chr1:160862624-160862644
2B4-4	chr1:160862671-160862691
2B4-5	chr1:160841622-160841642
2B4-6	chr1:160841819-160841839
2B4-7	chr1:160841823-160841843
2B4-8	chr1:160841717-160841737
2B4-9	chr1:160841859-160841879
2B4-10	chr1:160841806-160841826
2B4-11	chr1:160841834-160841854
2B4-12	chr1:160841780-160841800
2B4-13	chr1:160841713-160841733

2B4 NO	Genomic Coordinates (hg38)
2B4-14	chr1:160841631-160841651
2B4-15	chr1:160841704-160841724
2B4-16	chr1:160841584-160841604
2B4-17	chr1:160841679-160841699
2B4-18	chr1:160841874-160841894
2B4-19	chr1:160841750-160841770
2B4-20	chr1:160841577-160841597
2B4-21	chr1:160841459-160841479
2B4-22	chr1:160841466-160841486
2B4-23	chr1:160841461-160841481
2B4-24	chr1:160841460-160841480
2B4-25	chr1:160841360-160841380
2B4-26	chr1:160841304-160841324
2B4-27	chr1:160841195-160841215
2B4-28	chr1:160841305-160841325

or

the genomic coordinates selected from those targeted by 2B4-1 through 2B4-5:

chr1:160841611-160841631, chr1:160841865-160841885, chr1:160862624-160862644, chr1:160862671-160862691, and chr1:160841622-160841642; or

the genomic coordinates selected from those targeted by 2B4-1 and 2B4-2: chr1:160841611-160841631 and chr1:160841865-160841885; or

the genomic coordinates selected from those targeted by 2B4-3, 2B4-4, 2B4-10, and 2B4-17: chr1:160862624-160862644, chr1:160862671-160862691, chr1:160841806-160841826, and chr1:160841679-160841699.

16. The engineered cell of any one of claims 1-15, comprising a genetic modification in a human TIM3 sequence, within the genomic coordinates of chr5:157085832-157109044.

17. The engineered cell of claim 16, wherein the genetic modification in TIM3 is within genomic coordinates selected from:

TIM 3 NO	Genomic Coordinates (hg38)
TIM3 - 1	chr5:157106867-157106887
TIM3 - 2	chr5:157106862-157106882
TIM3 - 3	chr5:157106803-157106823

TIM 3 NO	Genomic Coordinates (hg38)
TIM3 – 4	chr5:157106850-157106870
TIM3 – 5	chr5:157104726-157104746
TIM3 – 6	chr5:157106668-157106688
TIM3 – 7	chr5:157104681-157104701
TIM3 – 8	chr5:157104681-157104701
TIM3 – 9	chr5:157104680-157104700
TIM3 – 10	chr5:157106676-157106696
TIM3 – 11	chr5:157087271-157087291
TIM3 – 12	chr5:157095432-157095452
TIM3 – 13	chr5:157095361-157095381
TIM3 – 14	chr5:157095360-157095380
TIM3 – 15	chr5:157108945-157108965
TIM3 - 18	chr5:157106751-157106771
TIM3 – 19	chr5:157095419-157095439
TIM3 – 22	chr5:157104679-157104699
TIM3 – 23	chr5:157106824-157106844
TIM3 – 26	chr5:157087117-157087137
TIM3 – 29	chr5:157095379-157095399
TIM3 – 32	chr5:157106864-157106884
TIM3 – 42	chr5:157095405-157095425
TIM3 – 44	chr5:157095404-157095424
TIM3 – 56	chr5:157106888-157106908
TIM3 – 58	chr5:157087126-157087146
TIM3 – 59	chr5:157087253-157087273
TIM3 – 62	chr5:157106889-157106909
TIM3 – 63	chr5:157106935-157106955
TIM3 – 66	chr5:157106641-157106661
TIM3 – 69	chr5:157087084-157087104
TIM3 – 75	chr5:157104663-157104683
TIM3 – 82	chr5:157106875-157106895
TIM3 - 86	chr5:157087184-157087204

TIM 3 NO	Genomic Coordinates (hg38)
TIM3 – 87	chr5:157106936-157106956
TIM3 - 88	chr5:157104696-157104716

; or

the genomic coordinates selected from those targeted by TIM3-1 through TIM3-4, TIM3-6 through TIM3-15, TIM3-18, TIM3-19, TIM3-22, TIM3-29, TIM3-42, TIM3-44, TIM3-58, TIM3-62, TIM3-69, TIM3-82, TIM3-86, and TIM3-88: chr5:157106867-157106887, chr5:157106862-157106882, chr5:157106803-157106823, chr5:157106850-157106870, chr5:157106668-157106688, chr5:157104681-157104701, chr5:157104681-157104701, chr5:157104680-157104700, chr5:157106676-157106696, chr5:157087271-157087291, chr5:157095432-157095452, chr5:157095361-157095381, chr5:157095360-157095380, chr5:157108945-157108965, chr5:157106751-157106771, chr5:157095419-157095439, chr5:157104679-157104699, chr5:157095379-157095399, chr5:157095405-157095425, chr5:157095404-157095424, chr5:157087126-157087146, chr5:157106889-157106909, chr5:157087084-157087104, chr5:157106875-157106895, chr5:157087184-157087204, and chr5:157104696-157104716; or

the genomic coordinates selected from those targeted by TIM3-1 through TIM3-5, TIM3-7, TIM3-8, TIM3-12 through TIM3-15, TIM3-23, TIM3-26, TIM3-32, TIM3-56, TIM3-59, TIM3-63, TIM3-66, TIM3-75, and TIM3-87: chr5:157106867-157106887, chr5:157106862-157106882, chr5:157106803-157106823, chr5:157106850-157106870, chr5:157106668-157106688, chr5:157104681-157104701, chr5:157104681-157104701, chr5:157095432-157095452, chr5:157095361-157095381, chr5:157095360-157095380, chr5:157108945-157108965, chr5:157106824-157106844, chr5:157087117-157087137, chr5:157106864-157106884, chr5:157106888-157106908, chr5:157087253-157087273, chr5:157106935-157106955, chr5:157106641-157106661, chr5:157104663-157104683, and chr5:157106936-157106956; or

the genomic coordinates selected from those targeted by TIM3-2, TIM3-4, TIM3-15, TIM3-23, TIM3-56, TIM3-59, TIM3-63, TIM3-75, and TIM3-87: chr5:157106862-157106882, chr5:157106850-157106870, chr5:157108945-157108965, chr5:157106824-157106844, chr5:157106888-157106908, chr5:157087253-157087273, chr5:157106935-157106955, chr5:157104663-157104683, and chr5:157106936-157106956, respectively; or

the genomic coordinates selected from those targeted by TIM3-1 through TIM3-4: chr5:157106867-157106887, chr5:157106862-157106882, chr5:157106803-157106823, and

chr5:157106850-157106870; or

the genomic coordinates selected from those targeted by TIM3-2, TIM-4, and TIM3-15:

chr5:157106862-157106882, chr5:157106850-157106870, and chr5:157108945-157108965;

or

the genomic coordinates selected from those targeted by TIM3-2, TIM-4, TIM3-15, TIM3-

63, and TIM3-87: chr5:157106862-157106882, chr5:157106850-157106870,

chr5:157108945-157108965, chr5:157106935-157106955, and chr5:157106936-

157106956y; or

the genomic coordinates selected from those targeted by TIM3-2 and TIM3-15:

chr5:157106862-157106882 and chr5:157108945-157108965; or

the genomic coordinates selected from those targeted by TIM3-63 and TIM3-87:

chr5:157106935-157106955 and chr5:157106936-157106956; or

the genomic coordinates selected from those targeted by TIM3-15: chr5:157108945-

157108965.

18. The engineered cell of any one of claims 1-17, comprising a genetic modification in a human PD-1 sequence, within the genomic coordinates of chr2: 241849881-241858908.

19. The engineered cell of claim 18, wherein the genetic modification comprises a modification of at least one nucleotide within the genomic coordinates selected from:

PD1 NO	Genomic Coordinates (hg38)
PD1-29	chr2:241852703-241852723
PD1-43	chr2:241858807-241858827
PD1-5	chr2:241858789-241858809
PD1-6	chr2:241858788-241858808
PD1-8	chr2:241858755-241858775
PD1-11	chr2:241852919-241852939
PD1-12	chr2:241852915-241852935
PD1-22	chr2:241852755-241852775
PD1-23	chr2:241852751-241852771
PD1-24	chr2:241852750-241852770
PD1-36	chr2:241852264-241852284
PD1-57	chr2:241852201-241852221
PD1-58	chr2:241852749-241852769
PD1-17	chr2:241852821-241852841

PD1-38	chr2:241852265-241852285
PD1-56	chr2:241851221-241851241
PD1-41	chr2:241852188-241852208

; or

the genomic coordinates selected from chr2:241852919-241852939, chr2:241852915-241852935, chr2:241852750-241852770, chr2:241852264-241852284, chr2:241852265-241852285, chr2:241858807-241858827, chr2:241852201-241852221, chr2:241858789-241858809, chr2:241858788-241858808, chr2:241858755-241858775, chr2:241852755-241852775, chr2:241852751-241852771, and chr2:241852703-241852723, respectively; or

the genomic coordinates selected from chr2:241858788-241858808, chr2:241858755-241858775, chr2:241852919-241852939, chr2:241852915-241852935, chr2:241852751-241852771, chr2:241858807-241858827, and chr2:241852703-241852723, respectively; or

the genomic coordinates selected from chr2: 241858789-241858809, chr2:241852919-241852939, chr2:241852915-241852935, chr2:241852755-241852775, chr2:241852751-241852771, and chr2:241858807-241858827, respectively; or

the genomic coordinates selected from chr2:241858788-241858808, chr2:241858755-241858775, chr2:241852751-241852771, and chr2:241852703-241852723, respectively; or

the genomic coordinates selected from chr2:241858788-241858808 and chr2:241852703-241852723, respectively; or

the genomic coordinates selected from chr2:241858788-241858808, chr2:241852751-241852771, chr2:241852703-241852723, chr2:241852188-241852208, and chr2:241852201-241852221, respectively; or

the genomic coordinates selected from chr2:241858788-241858808, chr2:241852703-241852723, and chr2:241852201-241852221, respectively; or

the genomic coordinates of chr2:241858807-241858827.

20. The engineered cell of any one of claims 1-19, wherein the genetic modification comprises an indel.

21. The engineered cell of any one of claims 1-20, wherein the genetic modification comprises an insertion of a heterologous coding sequence.

22. The engineered cell of any one of claims 1-21, wherein the genetic modification comprises a substitution, optionally wherein the substitution comprises a C to T substitution or an A to G substitution.

23. The engineered cell of any one of claims 1-22, wherein the genetic modification results in a change in the nucleic acid sequence that prevents translation of a full-length protein having an amino acid sequence of the full-length protein prior to genetic modification, optionally wherein the genetic modification results in a change in the nucleic acid sequence that results in a premature stop codon in a coding sequence of the full-length protein, or results in a change in splicing of a pre-mRNA from the genomic locus.
24. The engineered cell of any one of claims 1-23, wherein the cell comprises an exogenous nucleic acid encoding a targeting receptor that is expressed on the surface of the engineered cell, optionally wherein the targeting receptor is a CAR or a TCR.
25. The engineered cell of any one of claims 1-24, wherein the engineered cell is a T cell.
26. A pharmaceutical composition comprising the engineered cell of any one of claims 1-25.
27. A population of cells comprising the engineered cell of any one of claims 1-25.
28. A method of administering the engineered cell, population of cells, or pharmaceutical composition of any one of claims 1-27 to a subject in need thereof.
29. A method of administering the engineered cell, population of cells, or pharmaceutical composition of any one of claims 1-27 to a subject as an adoptive cell transfer (ACT) therapy.
30. An engineered cell, population of cells, or pharmaceutical composition of any one of claims 1-27, for use as an ACT therapy.
31. A LAG3 guide RNA that specifically hybridizes to a LAG3 sequence comprising a nucleotide sequence selected from:
- a. a guide sequence comprising a nucleotide sequence selected from SEQ ID NOs: 1-17, 24, 26, 41, 59, and 83;
 - b. a guide sequence comprising a nucleotide sequence of at least 17, 18, 19, or 20 contiguous nucleotides of a nucleotide sequence selected from the sequence of SEQ ID NOs: 1-17, 24, 26, 41, 59, and 83;
 - c. a guide sequence comprising a nucleotide sequence at least 95% identical or at least 90% identical to a nucleotide sequence selected from SEQ ID Nos: 1-17, 24, 26, 41, 59, and 83;
 - d. a guide sequence comprising a nucleotide sequence selected from SEQ ID NOs: 1-15;
 - e. a guide sequence comprising a nucleotide sequence selected from SEQ ID NOs: 1-11;

f. a guide sequence comprising a nucleotide sequence selected from SEQ ID NOs: 1-4; and

g. a guide sequence comprising a nucleotide sequence selected from SEQ ID NOs: 1, 4, 5, and 9.

32. A LAG3 guide RNA comprising a guide sequence that directs an RNA-guided DNA binding agent to a chromosomal location within the genomic coordinates selected from those targeted by SEQ ID NO: 1-17, 24, 26, 41, 59, and 83; or selected from the genomic coordinates targeted by SEQ ID NOs: 1-15; or selected from the genomic coordinates targeted by SEQ ID NOs: 1-11; or selected from the genomic coordinates targeted by SEQ ID NOs: 1-4; or selected from genomic coordinates targeted by SEQ ID NOs: 1, 4, 5, or 9.

33. The guide RNA of claim 31 or 32, wherein the guide RNA is a single guide RNA (sgRNA).

34. The guide RNA of claim 33, further comprising the nucleotide sequence of SEQ ID NO: 201 3' to the guide sequence, wherein the guide RNA comprises a 5' end modification or a 3' end modification.

35. The guide RNA of claim 33, further comprising 5' end modification or a 3' end modification and a conserved portion of an gRNA comprising one or more of:

A. a shortened hairpin 1 region or a substituted and optionally shortened hairpin 1 region relative to SEQ ID NO: 201, wherein

1. at least one of the following pairs of nucleotides are substituted in the substituted and optionally shortened hairpin 1 with Watson-Crick pairing nucleotides: H1-1 and H1-12, H1-2 and H1-11, H1-3 and H1-10, or H1-4 and H1-9, and the hairpin 1 region optionally lacks

a. any one or two of H1-5 through H1-8,

b. one, two, or three of the following pairs of nucleotides: H1-1 and H1-12, H1-2 and H1-11, H1-3 and H1-10, and H1-4 and H1-9, or

c. 1-8 nucleotides of hairpin 1 region; or

2. the shortened hairpin 1 region lacks 4-8 nucleotides, preferably 4-6 nucleotides; and

a. one or more of positions H1-1, H1-2, or H1-3 is deleted or substituted relative to SEQ ID NO: 201; or

b. one or more of positions H1-6 through H1-10 is substituted relative to SEQ ID NO: 201; or

3. the shortened hairpin 1 region lacks 5-10 nucleotides, preferably 5-6 nucleotides, and one or more of positions N18, H1-12, or n is substituted relative to SEQ ID NO: 201; or
- B. a shortened upper stem region, wherein the shortened upper stem region lacks 1-6 nucleotides and wherein the 6, 7, 8, 9, 10, or 11 nucleotides of the shortened upper stem region include less than or equal to 4 substitutions relative to SEQ ID NO: 201; or
- C. a substitution relative to SEQ ID NO: 201 at any one or more of LS6, LS7, US3, US10, B3, N7, N15, N17, H2-2 and H2-14, wherein the substituent nucleotide is neither a pyrimidine that is followed by an adenine, nor an adenine that is preceded by a pyrimidine; or
- D. an upper stem region, wherein the upper stem modification comprises a modification to any one or more of US1-US12 in the upper stem region relative to SEQ ID NO: 201.
36. The guide RNA of claim 33 or 34, wherein the guide RNA is modified according to the pattern
 mN*mN*mN*NNNNNNNNNNNNNNNNNGUUUAGAmGmCmUmAmGmAmAmAmU
 mAmGmCAAGUUA AAAUAAGGCUAGUCCGUUAUCAmAmCmUmUmGmAmAmAm
 AmAmGmUmGmGmCmAmCmCmGmAmGmUmCmGmGmUmGmCmU*mU*mU*mU
 (SEQ ID NO: 300), where “N” may be any natural or non-natural nucleotide, m is a 2'-O-methyl modified nucleotide, and * is a phosphorothioate linkage between nucleotide residues; and wherein the N's are collectively the nucleotide sequence of a guide sequence of any preceding claim, optionally wherein each N is independently any natural or non-natural nucleotide and the guide sequence targets Cas9 to the LAG3 gene.
37. The guide RNA of any one of claims 33-36, wherein the guide RNA comprises a modification.
38. The guide RNA of claim 37, wherein the modification comprises (1) a 2'-O-methyl (2'-O-Me) modified nucleotide, (ii) a 2'-F modified nucleotide, (iii) a phosphorothioate (PS) bond between nucleotides, (iv) a modification at one or more of the first five nucleotides at the 5' end of the guide RNA, (v) a modification at one or more of the last five nucleotides at the 3' end of the guide RNA, (vi) a PS bond between each of the first four nucleotides of the guide RNA, (vii) a PS bond between each of the last four nucleotides of the guide RNA, (viii) a 2'-O-Me modified nucleotide at each of the first three nucleotides at the 5' end of the guide

RNA, (ix) a 2'-O-Me modified nucleotide at each of the last three nucleotides at the 3' end of the guide RNA, or combinations of one or more of (i)-(ix).

39. A composition comprising a guide RNA of any one of claims 31-38 and an RNA guided DNA binding agent wherein the RNA guided DNA binding agent is a polypeptide RNA guided DNA binding agent or a nucleic acid encoding an RNA guided DNA binding agent polypeptide, optionally the RNA guided DNA-binding agent is a Cas9 nuclease.

40. The guide RNA of any one of claims 31-38 or the composition of claim 39, wherein the composition further comprises a pharmaceutically acceptable excipient.

41. The guide RNA or composition of any one of claims 31-40, wherein the guide RNA is associated with a lipid nanoparticle (LNP).

42. A method of making a genetic modification in a LAG3 sequence within a cell, comprising contacting the cell with the guide RNA or composition of any one of claims 31-41.

43. The method of claim 42, further comprising making a genetic modification in a TCR sequence to inhibit expression of a TCR gene.

44. A method of preparing a population of cells for immunotherapy comprising:

a. making a genetic modification in a LAG3 sequence in the cells in the population with a LAG3 guide RNA or composition of any one of claims 31-41;

b. making a genetic modification in a TCR sequence in the cells of the population to reduce expression of the TCR protein on the surface of the cells in the population;

c. expanding the population of cells in culture.

45. A population of cells made by the method of any one of claims 42-44.

46. The population of cells of claim 45, wherein the population of cells is altered *ex vivo*.

47. A method of administering the population of cells of claim 45 or 46 to a subject in need thereof.

48. A method of administering the population of cells of claim 45 or 46 to a subject as an adoptive cell transfer (ACT) therapy.

49. A population of cells of claim 45 or 46, for use as an ACT therapy.

50. A population of cells comprising a genetic modification of a LAG3 gene, wherein at least 40%, 45%, 50%, 55%, 60%, 65%, preferably at least 70%, 75%, 80%, 85%, 90%, or 95% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence.

51. The population of cells of claim 50, wherein expression of LAG3 is decreased by at least 40%, 45%, 50%, 55%, 60%, 65%, preferably at least 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 LAG3 gene has not been modified.

52. The population of cells of claim 50 or 51, wherein at least 70%, at least 80%, at least 90%, or at least 95% of cells in the population comprise a modification selected from an insertion, a deletion, and a substitution in the endogenous LAG3 sequence.

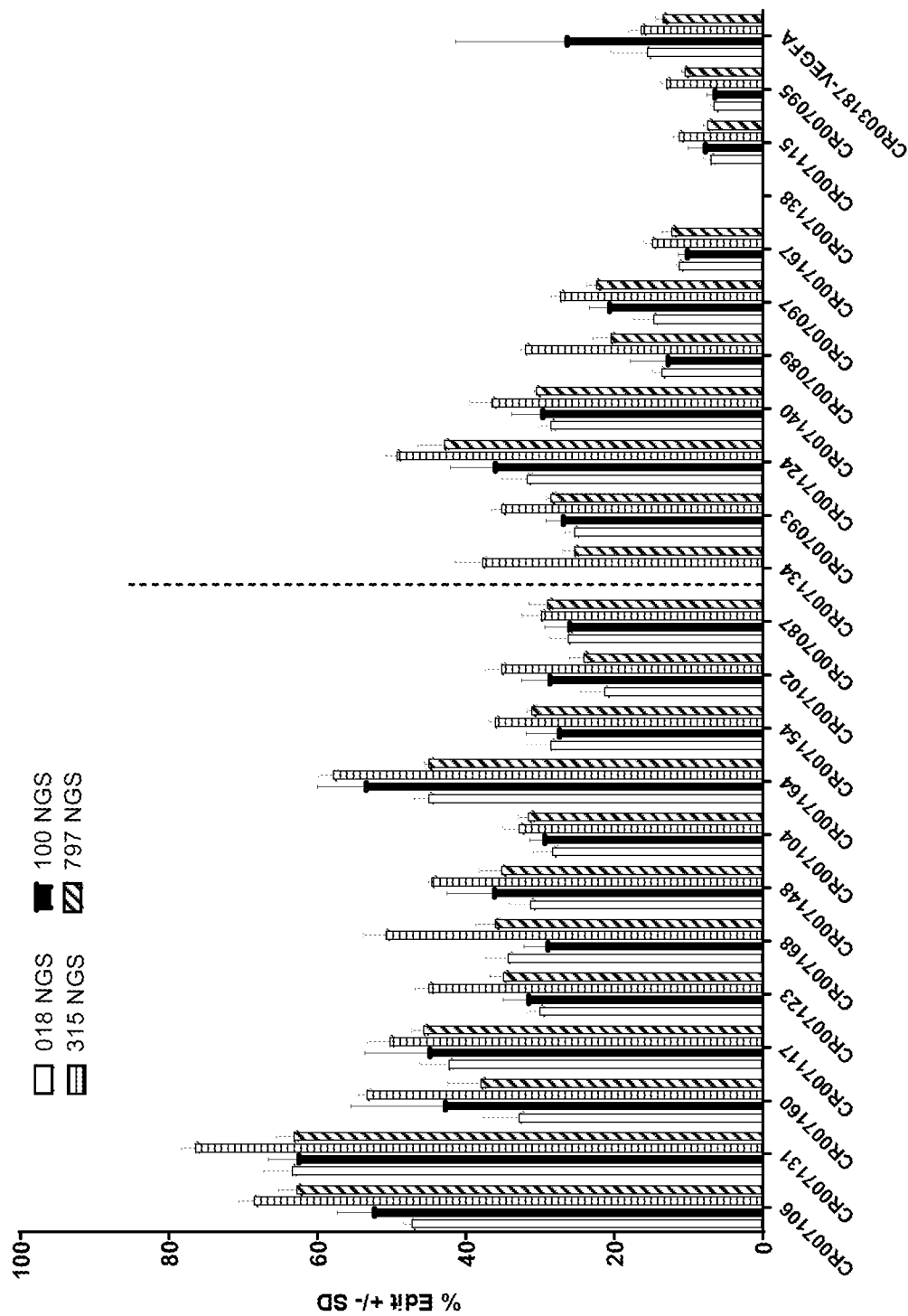


Fig. 1

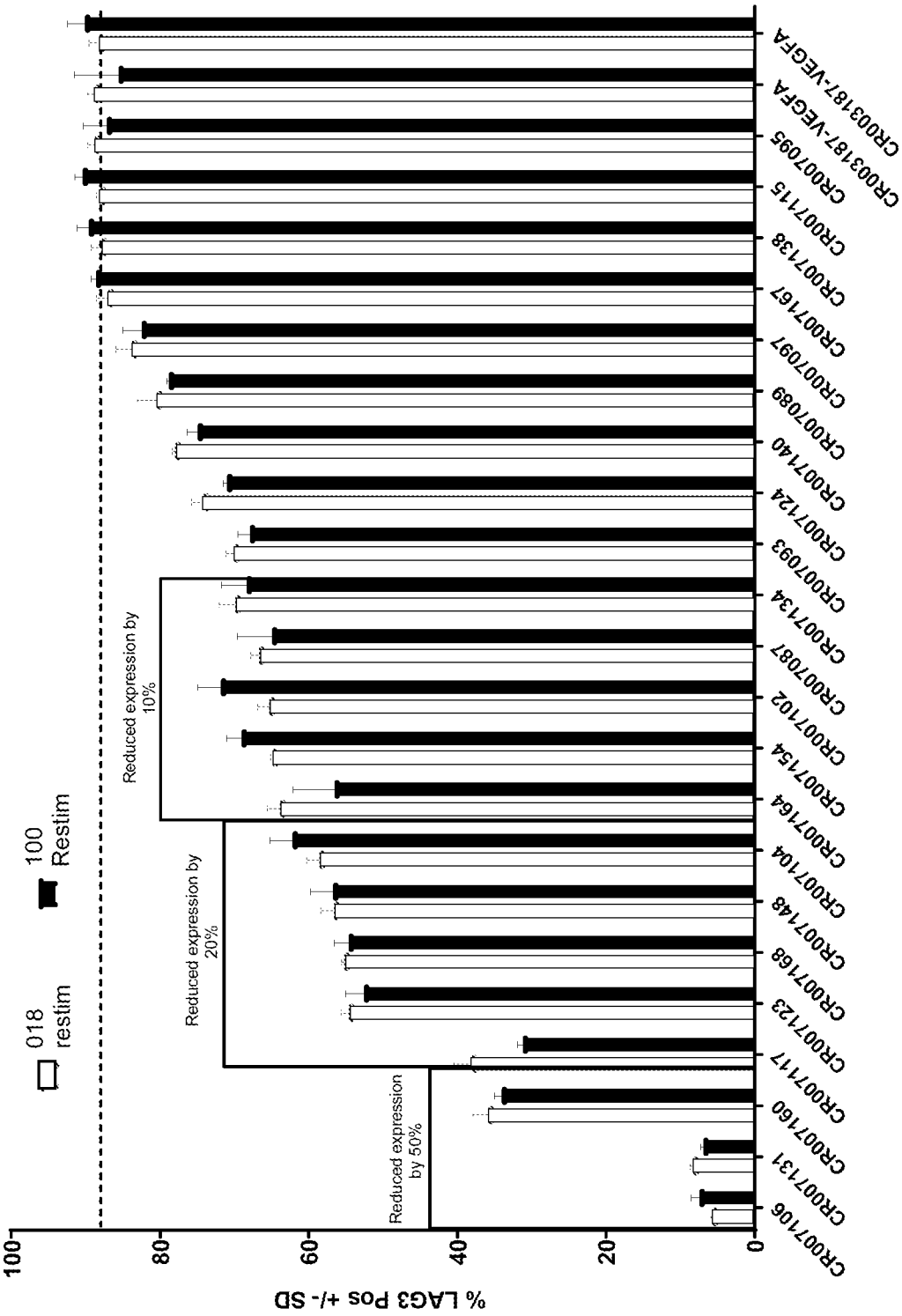


Fig. 2A

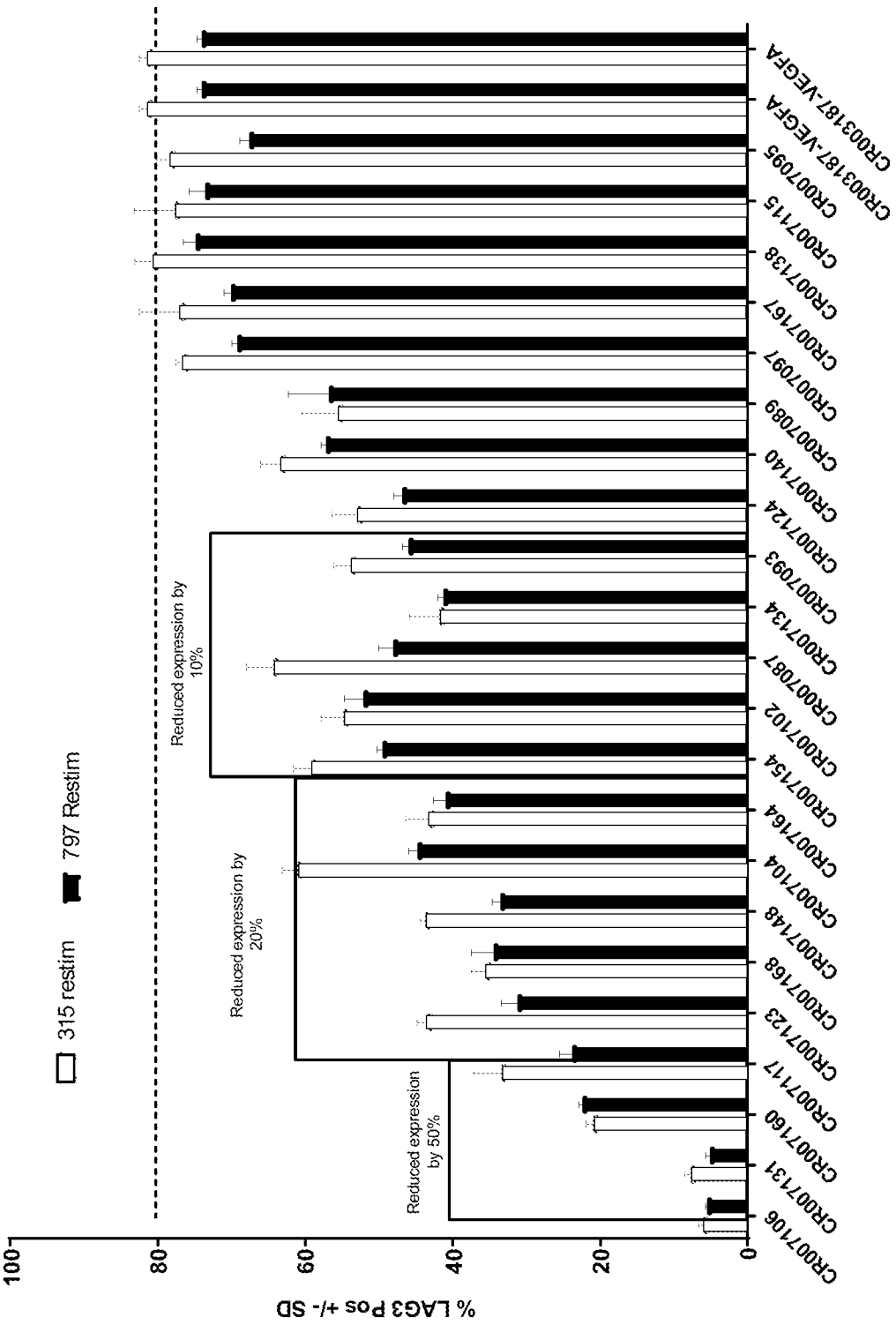


Fig. 2B

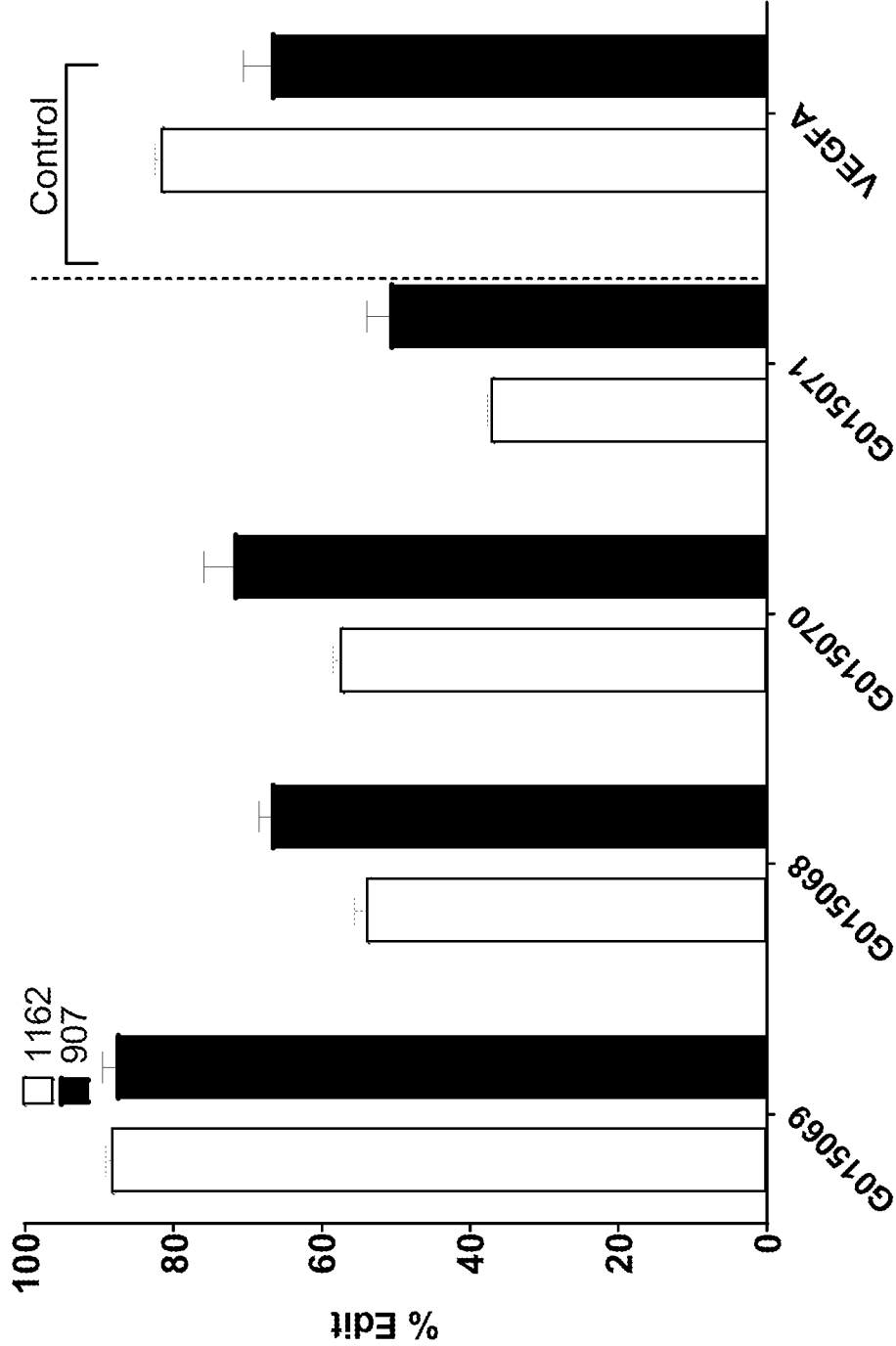


Fig. 3A

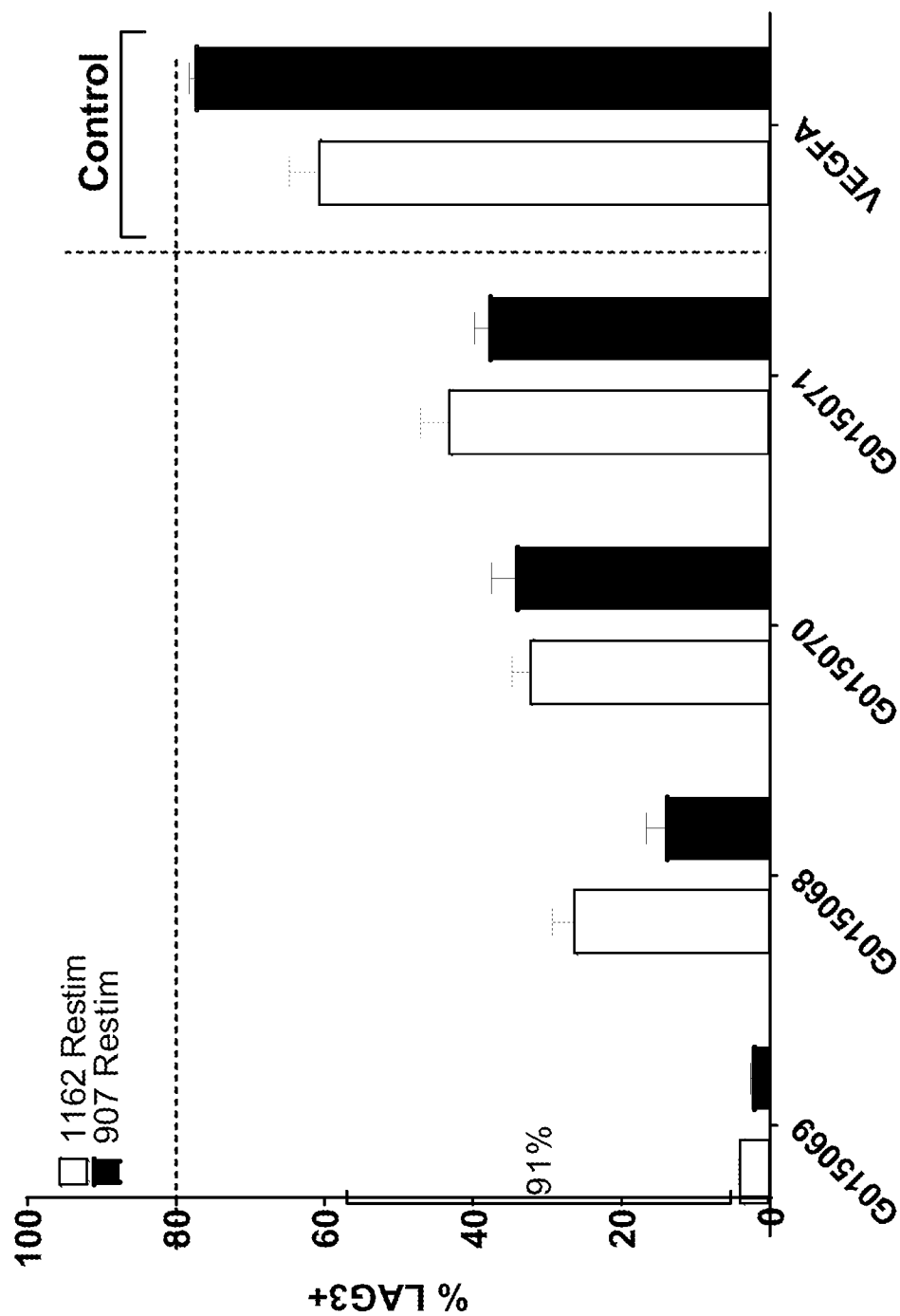


Fig. 3B

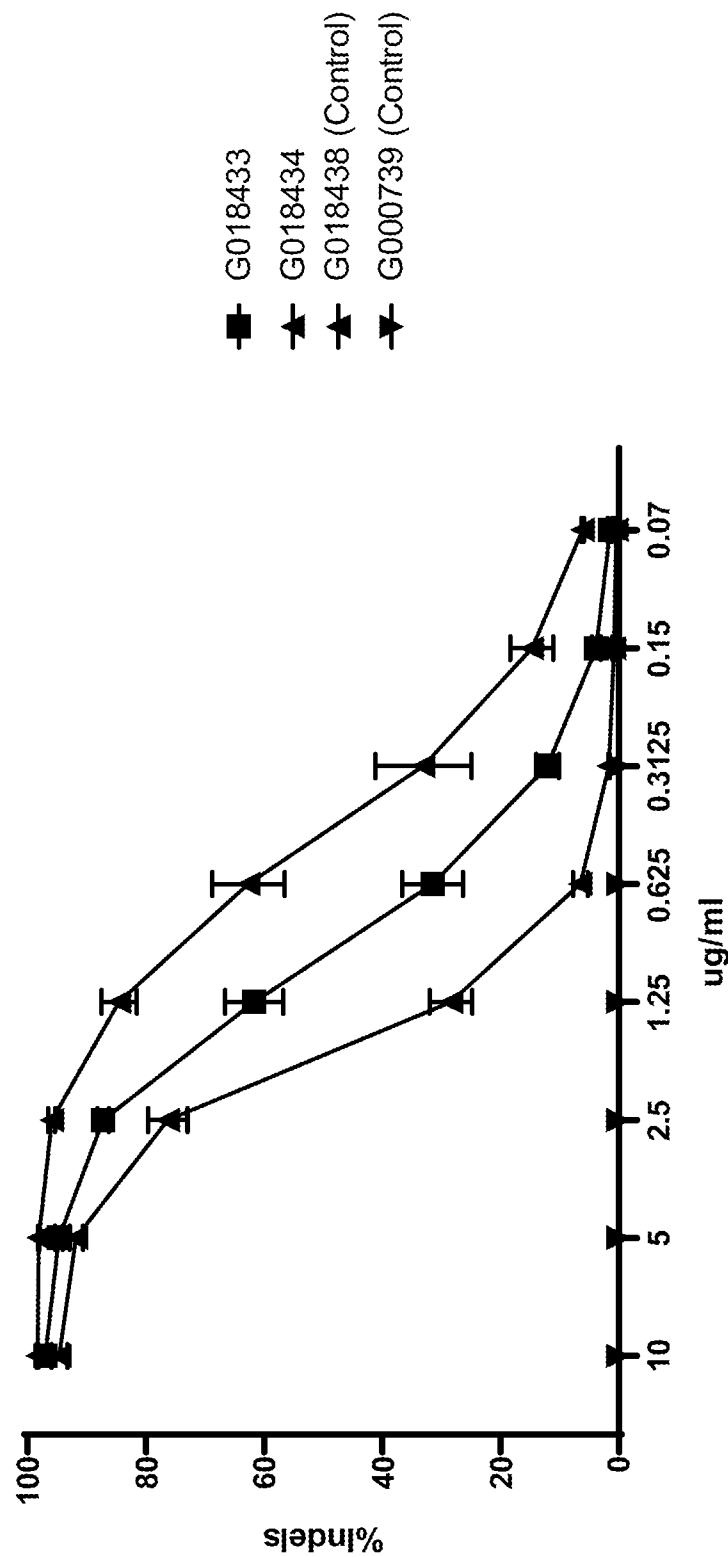


Fig. 4

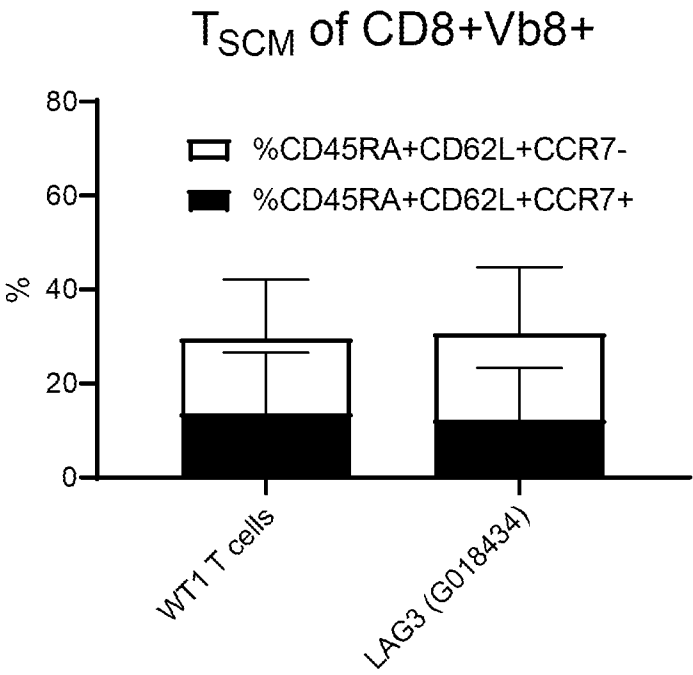


Fig. 5A

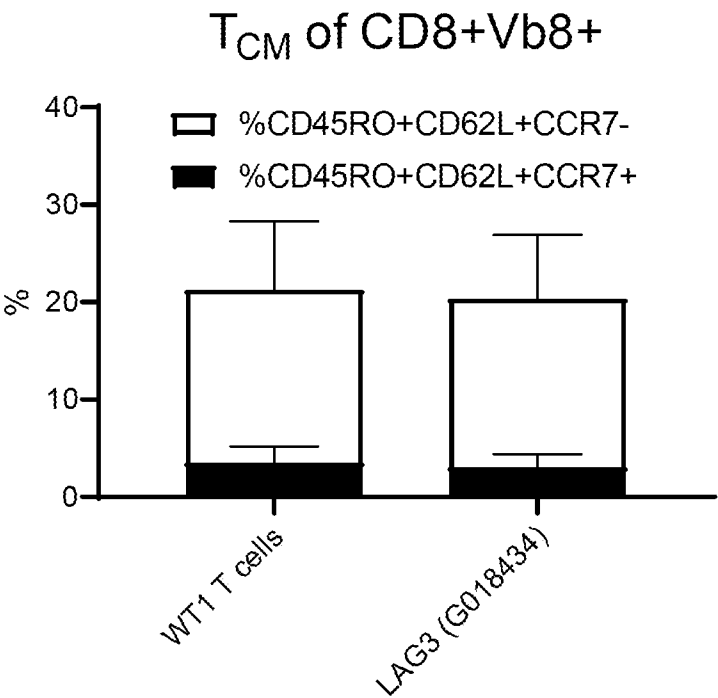


Fig. 5B

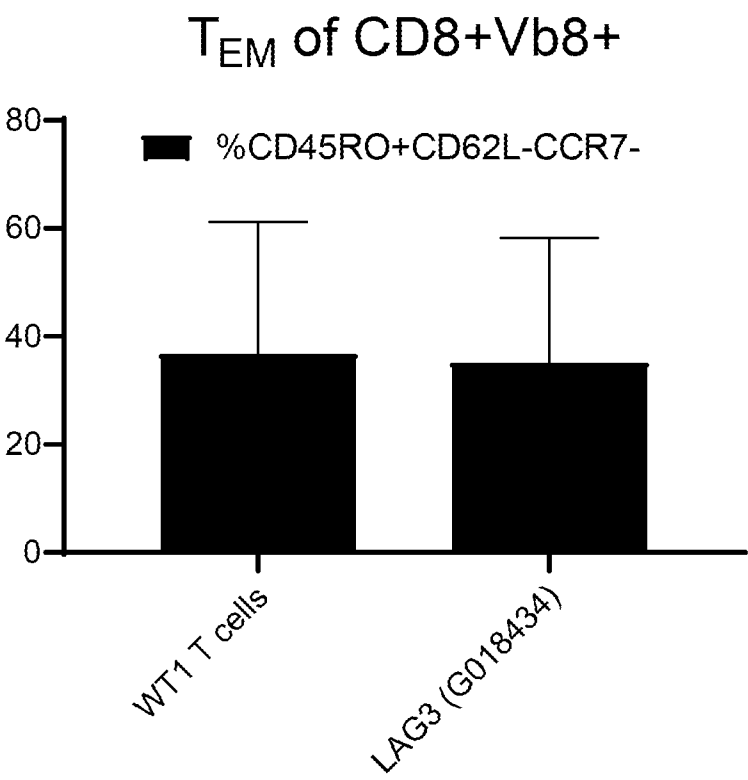


Fig. 5C

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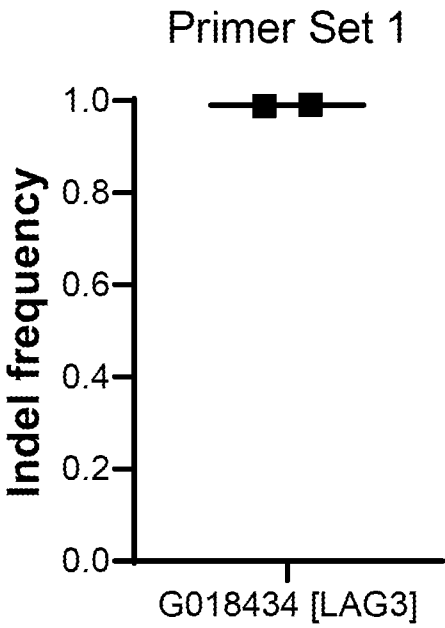


Fig. 6A

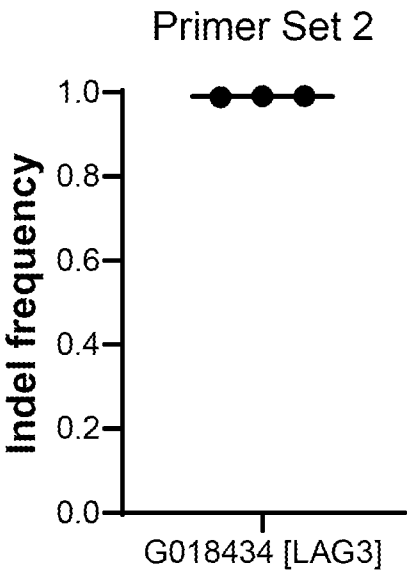


Fig. 6B

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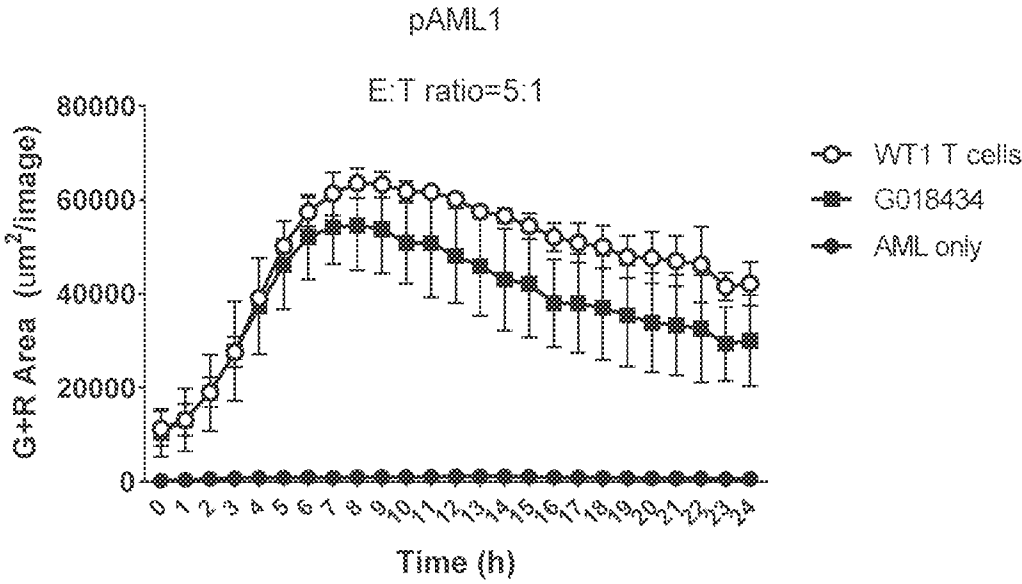


Fig. 7A

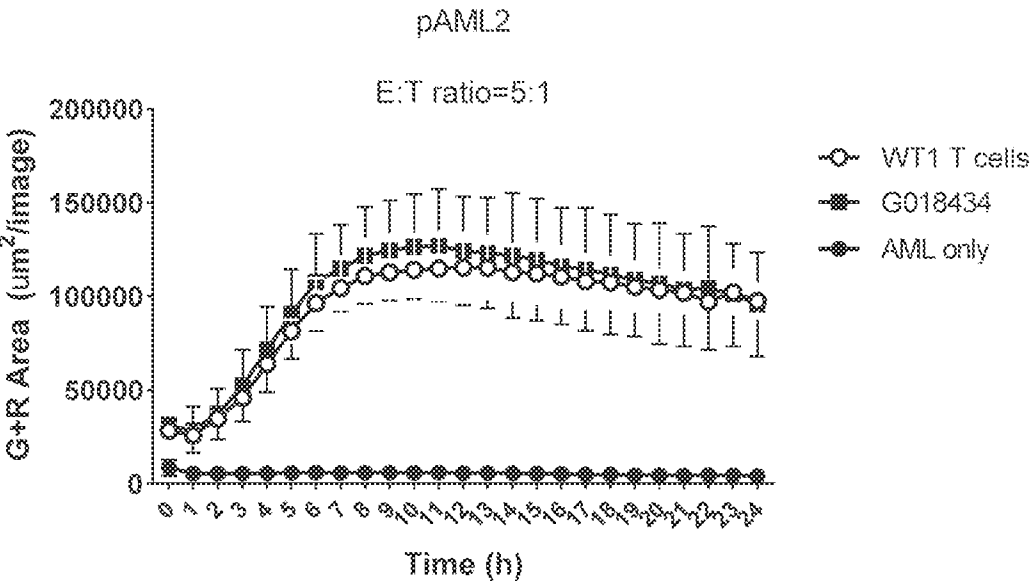


Fig. 7B

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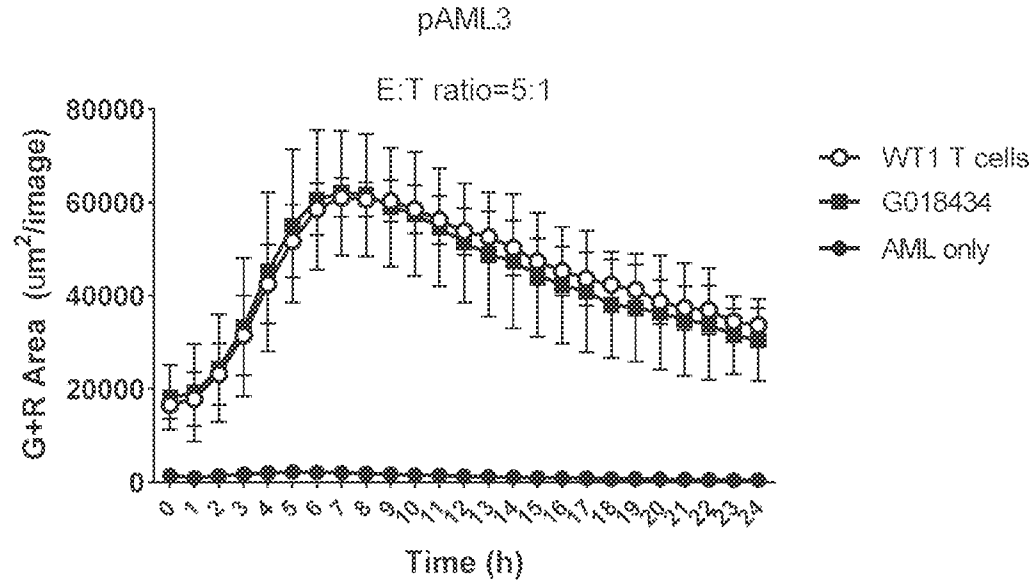


Fig. 7C

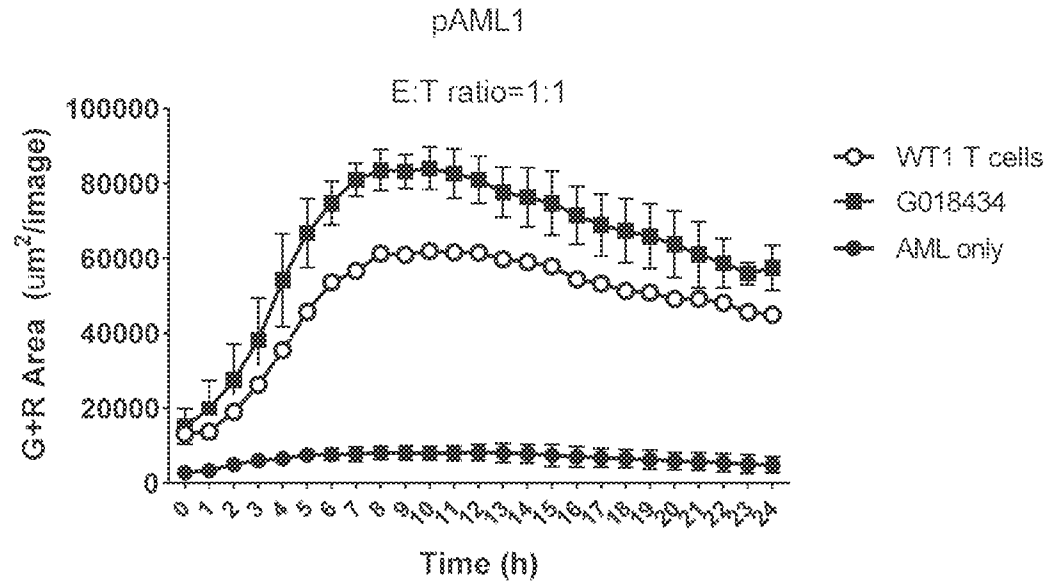


Fig. 7D

12/14

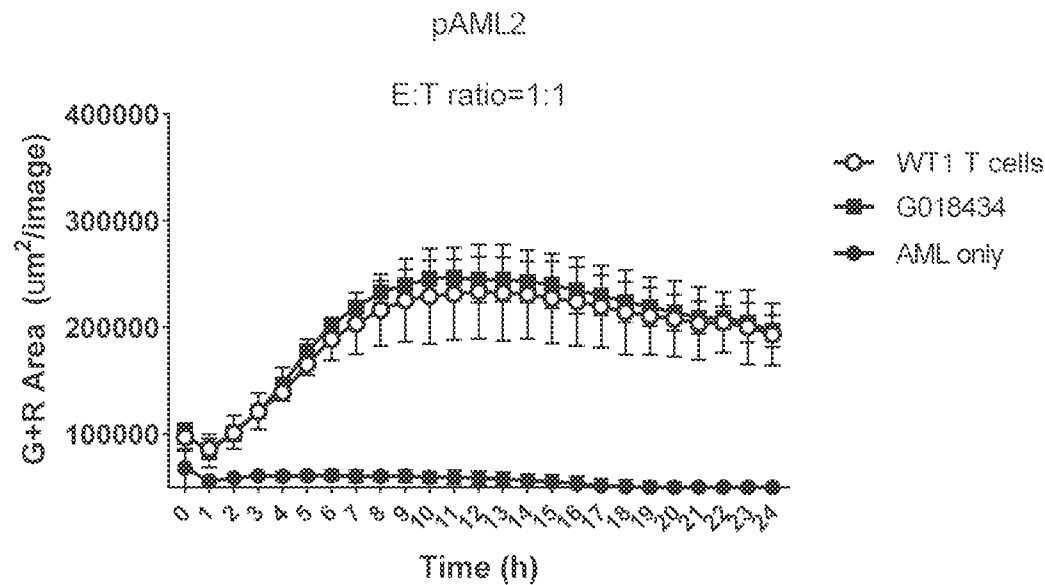


Fig. 7E

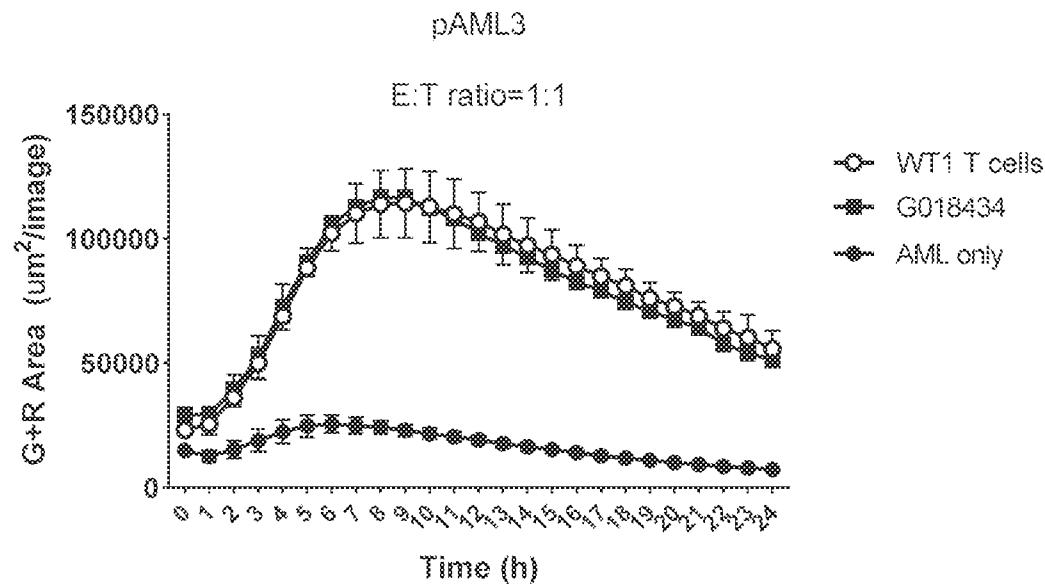


Fig. 7F

13/14

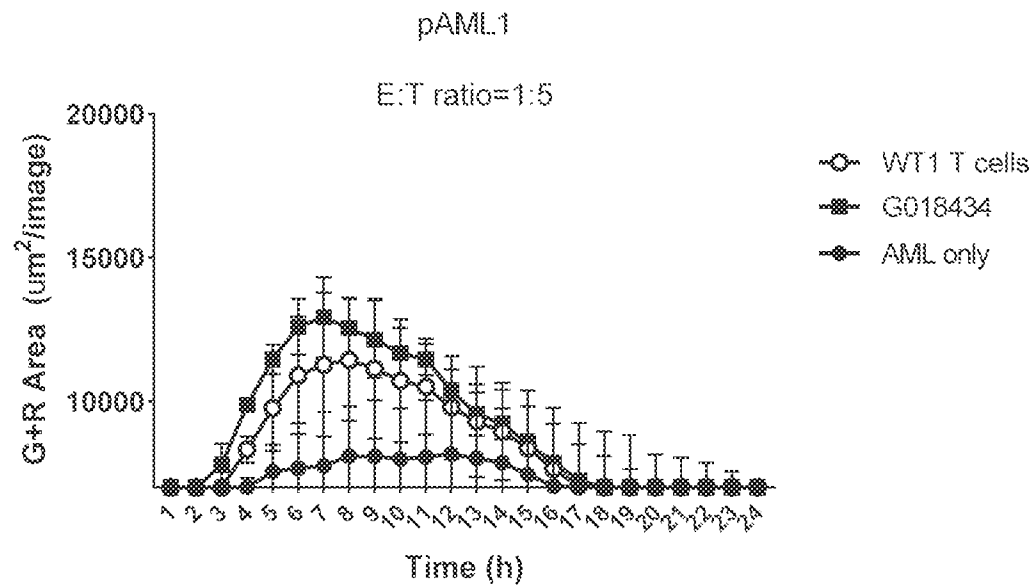


Fig. 7G

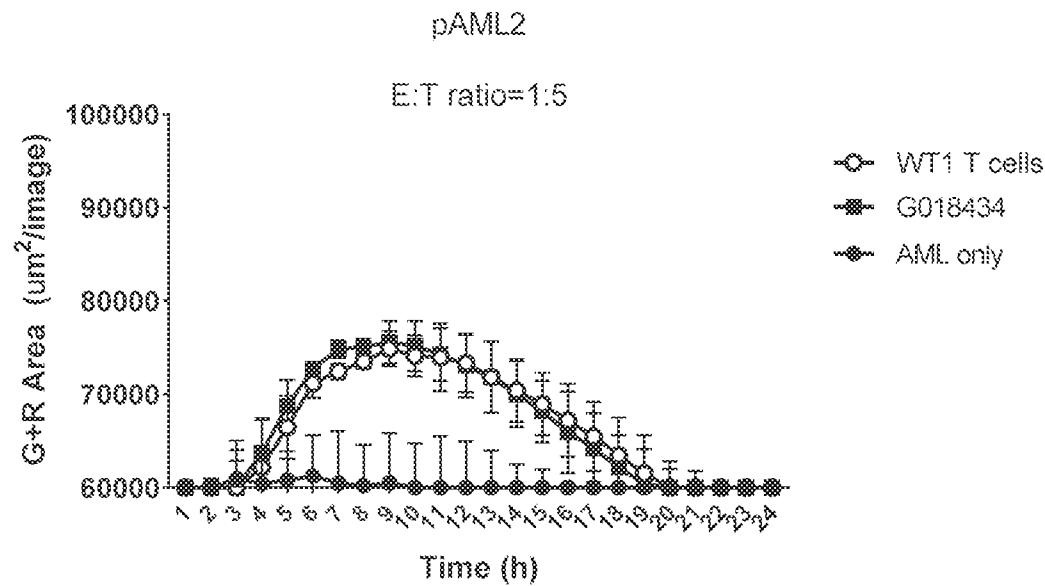


Fig. 7H

14/14

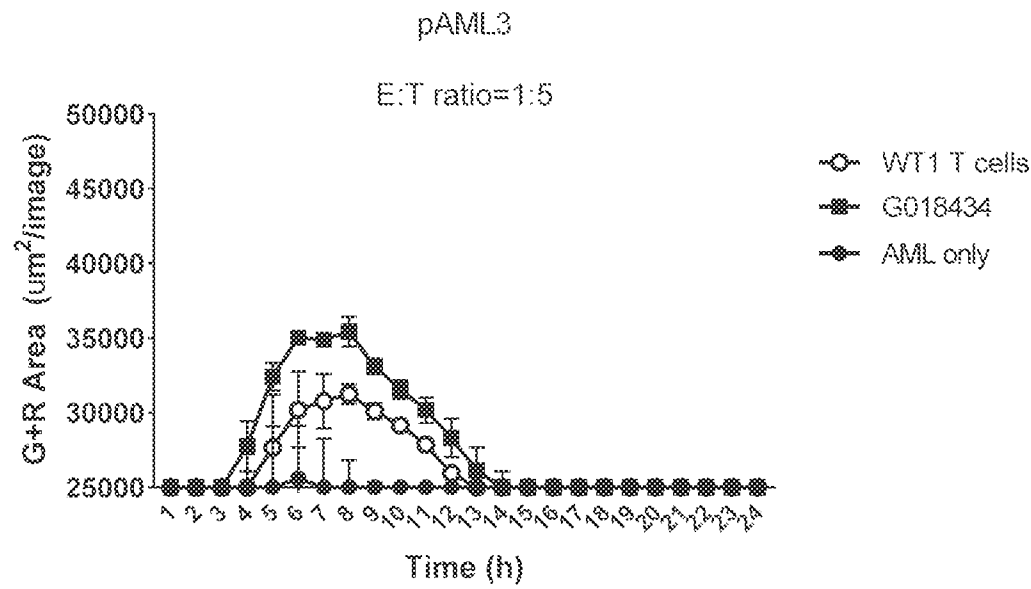


Fig. 7I