[0005] In one example, the present disclosure provides an *in vitro* method for the disruption of at least two genes in an immune cell, wherein the at least two genes are selected from the group consisting of NKG2A, SIGLEC-7, LAG3, TIM3, CISH, FOXO1, TGFBR2, TIGIT, CD96, ADORA2, NR3C1, PD1, PDL-1, PDL-2, CD47, SIRPA, SHIP1, ADAM17, RPS6, 4EBP1, CD25, CD40, IL21R, ICAM1, CD95, CD80, CD86, IL10R, CD5, CD7, and a combination thereof. In one aspect, the present disclosure refers to an *in vitro* method for the disruption of at least three genes in a natural killer cell, wherein the at least three genes are selected from the group consisting of (1) NKG2A, CISH, and TGFBRII, (2) TIGIT, FOXO1, and TGFBRII, (3) TGFBRII, CD96, and TIGIT, (4) TGFBRII, CISH, and TIGIT, (5) TIM3, CISH, and TGFBRII, (6) CD96, FOXO1, and TGFBRII, (7) TGFBRII, TIM3, and TIGIT, (8) SIGLEC7, CISH, and TGFBRII, (9) CD47, CISH, and TGFBRII, (10) SIRPA, CISH, and TGFBRII, (11) TGFBRII, CD47, and TIGIT, (12) TGFBRII, CD47, and SIRPA, (13) A2AR, CISH, and TGFBRII, (14) TGFBRII, CISH, and ADAM17, (15) TGFBRII, TIM3, and TIGIT, (16) TGFBRII, A2AR, and TIGIT, (17) SHP1, CISH, and TGFBRII, (18) TGFBRII, CISH, and SHP1, (19) TGFBRII, SHP1, and TIGIT, and (20) TGFBRII, SHP1, and TIM3. In particular examples, three, four, five, or six or more genes are disrupted. In specific examples, the disruption of two or more genes is simultaneous, such as in the same method step. The method may comprise introducing a guide RNA (gRNA) for each gene to the immune cell.

[0006] The method can comprise the knockdown of particular combinations of genes, such as the following, for example: (a) NKG2A and CISH, (b) NKG2A and TGFBRII, (c) CISH and TGFBRII, (d) TIGIT and FOXO1, (e) TIGIT and TGFBRII, (f) CD96 and FOXO1, (g) CD96 and TGFBRII, (h) FOXO1 and TGFBRII, (i) CD96 and TIGIT, (j) CISH and TIGIT, (k) TIM3 and CISH, (l) TIM3 and TGFBRII, (m) FOXO1 and TGFBRII, (n) TIM3 and TIGIT, (o) SIGLEC7 and CISH, (p) SIGLEC7 and TGFBRII, (q) CD47 and CISH, (r) CD47 and TGFBRII, (s) SIRPA and CISH, (t) SIRPA and TGFBRII, (u) CD47 and TIGIT, (v) CD47 and SIRPA, (w) A2AR and CISH, (x) A2AR and TGFBRII, (y) ADAM17 and CISH, (z) TGFBRII and ADAM17, (a) A2AR and TIGIT, (b) SHP1 and CISH, (c) CISH and TGFBRII, (d) SHP1 and TGFBRII, (e) SHP1 and TIGIT, or (f) SHP1 and TIM3. The method can comprise the knockdown of (1) NKG2A, CISH, and TGFBRII, (2) TIGIT, FOXO1, and TGFBRII, (3) TGFBRII, CD96, and TIGIT, (4) TGFBR2, CISH, and TIGIT, (5) TIM3, CISH, and TGFBRII, (6) CD96, FOXO1, and TGFBRII, (7) TGFBRII, TIM3, and TIGIT, (8) SIGLEC7, CISH, and TGFBRII, (9) CD47, CISH, and TGFBRII, (10) SIRPA, CISH, and TGFBRII, (11) TGFBRII, CD47, and TIGIT, (12) TGFBRII, CD47, and SIRPA, (13) A2AR, CISH, and TGFBRII, (14) TGFBRII, CISH, and ADAM17, (15) TGFBRII, TIM3, and TIGIT, (16) TGFBRII, A2AR, and TIGIT, (17) SHP1, CISH, and TGFBRII, (18) TGFBRII, CISH, and SHP1, (19) TGFBRII, SHP1, and TIGIT, or (20) TGFBRII, SHP1, and TIM3. Any of the above subgroups may be combined with a second subgroup as disclosed above. For example, any one of subgroups a-j1 may be combined with any one or more of the other subgroups a-j1, any one or more of subgroups a-j1 may be combined with any one or more of the other subgroups 1-23, or any one or more of subgroups 1-23 may be combined with any one or more of the other subgroups 1-23.

compared to in the absence of the modification(s). In some examples, the immune cell has increased production of perforin and/or granzyme B, compared to in the absence of the modification(s).

[0012] In additional examples, the method further comprises introducing a CAR and/or TCR to an immune cell, such as introducing a nucleic acid encoding the CAR and/or TCR into the immune cell. In some examples, the nucleic acid is in an expression vector, such as a retroviral vector. In certain examples, the vector is an adenovirus-associated vector, such as AAV6. In some examples, the vector further comprises an inhibitory gene sequence, such as an inhibitory gene sequence selected from the group consisting of NKG2A, SIGLEC-7, LAG3, TIM3, CISH, FOXO1, TGFBR2, TIGIT, CD96, ADORA2, NR3C1, PD1, PDL-1, PDL-2, CD47, SIRPA, SHIP1, ADAM17, RPS6, 4EBP1, CD25, CD40, IL21R, ICAM1, CD95, CD80, CD86, IL10R, CD5, CD7, and a combination thereof. In particular examples, the vector further comprises a guide RNA for the inhibitory gene. The CAR may be flanked by homology arms for the inhibitory gene. In some examples, introducing the vector comprising the CAR sequence results in insertion of the CAR at an inhibitory gene locus, such as an exon of an inhibitory gene, in the immune cell, such that the CAR is under the control of the endogenous promoter of the inhibitory gene. In particular examples, introducing the vector further disrupts expression of the inhibitory gene.

[0013] In another example, there is provided an immune cell, such as an immune cell of the disclosed examples, with disrupted expression of at least two genes in the immune cell, produced at least by the step comprising introducing a CRISPR guide RNA (gRNA) for each gene to said immune cell, wherein at least two genes are selected from the group consisting of NKG2A, SIGLEC-7, LAG3, TIM3, CISH, FOXO1, TGFBR2, TIGIT, CD96, ADORA2, NR3C1, PD1, PDL-1, PDL-2, CD47, SIRPA, SHIP1, ADAM17, RPS6, 4EBP1, CD25, CD40, IL21R, ICAM1, CD95, CD80, CD86, IL10R, CD5, CD7, and a combination thereof. In some examples, three, four, five, or six or more genes are disrupted.

[0013a] In another aspect, the present disclosure refers to a natural killer cell with disrupted expression of at least three genes in the natural killer cell, wherein the at least three genes are selected from the group consisting of (1) NKG2A, CISH, and TGFBRII, (2) TIGIT, FOXO1, and TGFBRII, (3) TGFBRII, CD96, and TIGIT, (4) TGFBRII, CISH, and TIGIT, (5) TIM3, CISH, and TGFBRII, (6) CD96, FOXO1, and TGFBRII, (7) TGFBRII, TIM3, and TIGIT, (8) SIGLEC7, CISH, and TGFBRII, (9) CD47, CISH, and TGFBRII, (10) SIRPA, CISH, and TGFBRII, (11) TGFBRII, CD47, and TIGIT, (12) TGFBRII, CD47, and SIRPA, (13) A2AR, CISH, and TGFBRII, (14) TGFBRII, CISH, and ADAM17, (15) TGFBRII, TIM3, and TIGIT, (16) TGFBRII, A2AR, and TIGIT, (17) SHP1, CISH, and TGFBRII, (18) TGFBRII, CISH, and SHP1, (19) TGFBRII, SHP1, and TIGIT, and (20) TGFBRII, SHP1, and TIM3.

[0014] In certain examples, the immune cell is a T cell, NK cell, B cell, or stem cell. In some examples, the immune cell is engineered to express a chimeric antigen receptor (CAR) and/or T cell receptor (TCR). The immune cell may be virus-specific, such as a virus-specific T cell. The T cell may be a regulatory T cell. The B cell may be a regulatory B cell. In some examples, the stem cell is a mesenchymal stem cell (MSC) or an induced pluripotent stem (iPS) cell. In particular examples, the T cell is a $CD8^+$ T cell, $CD4^+$ T cell, or gamma-delta T cell. The

of IL-7, IL-2, IL-15, IL-12, IL-18, and IL-21. In certain examples, the CAR further comprises a suicide gene, such as a membrane bound nonsecretable TNF-alpha mutant or inducible caspase 9.

[0019] Further provided herein is an expression vector encoding at least one CAR and/or TCR, at least one inhibitory gene sequence, and at least one gRNA. In some examples, the inhibitory gene sequence is from an inhibitory gene selected from the group consisting of NKG2A, SIGLEC-7, LAG3, TIM3, CISH, FOXO1, TGFBR2, TIGIT, CD96, ADORA2, NR3C1, PD1, PDL-1, PDL-2, CD47, SIRPA, SHIP1, ADAM17, RPS6, 4EBP1, CD25, CD40, IL21R, ICAM1, CD95, CD80, CD86, IL10R, CD5, and CD7. In particular examples, the gRNA is specific to the inhibitory gene. In some examples, the vector is a viral vector, such as an AAV vector. The CAR may be flanked by homology arms for the inhibitory gene. Also provided herein is a host cell, such as a cell of the examples, engineered to express the vector of the examples. In examples, the cell is a T cell, NK cell, B cell, or stem cell.

[0020] Also provided herein is a pharmaceutical composition comprising a population of immune cells of the disclosed examples. In another aspect, the present disclosure refers to a pharmaceutical composition comprising a population of cells as disclosed herein. In another aspect, the present disclosure refers to a composition comprising a population of cells as disclosed herein for the treatment of an immune-related disorder, infectious disease, and/or cancer.

[0021] In a further example, there is provided a method of treating a disease or disorder in a subject comprising administering an effective amount of immune cells of the disclosed examples to the subject. In some examples, the disease or disorder is an infectious disease, cancer, such as a solid cancer or a hematologic malignancy, or an immune-related disorder. In another aspect, the present disclosure refers to use of natural killer cells as disclosed herein in the manufacture of a medicament for treating a disease or disorder in a subject. The immune-related disorder may be an autoimmune disorder, graft versus host disease, allograft rejection, or inflammatory condition, for example. In some examples, the immune-related disorder is an inflammatory condition and the immune cells have essentially no expression of glucocorticoid receptor. In certain examples, the immune cells are autologous or allogeneic with respect to a recipient individual.

[0022] In additional examples, the method further comprises administering at least a second therapeutic agent to the individual receiving the immune cells. In some examples, the at least a second therapeutic agent comprises chemotherapy, immunotherapy, surgery, radiotherapy, hormone therapy, or biotherapy. In certain examples, the immune cells and/or the at least a second therapeutic agent are administered intravenously, intraperitoneally,

intratracheally, intratumorally, intramuscularly, endoscopically, intralesionally, percutaneously, subcutaneously, regionally, or by direct injection or perfusion.

[0023] In another example, the present disclosure refers to a method for engineering an immune cell to express a CAR, said method comprising using a CRISPR gRNA to insert the CAR at an inhibitory gene locus of said immune cell. In some examples, the CAR is encoded by an expression vector, such as a retroviral vector, plasmid, lentiviral vector, adenoviral vector, adenovirus-associated viral vector, and so forth. In certain examples, the viral vector is an adenovirus-associated vector, such as AAV6.

[0024] In some examples, the vector further comprises an inhibitory gene sequence, such as an inhibitory gene sequence is selected from the group consisting of NKG2A, SIGLEC-7, LAG3, TIM3, CISH, FOXO1, TGFBR2, TIGIT, CD96, ADORA2, NR3C1, PD1, PDL-1, PDL-2, CD47, SIRPA, SHIP1, ADAM17, RPS6, 4EBP1, CD25, CD40, IL21R, ICAM1, CD95, CD80, CD86, IL10R, CD5, CD7 and a combination thereof. In some examples, the CRISPR gRNA is to the inhibitory gene. In certain examples, the CAR is flanked by homology arms for the inhibitory gene. In particular examples, the CAR is inserted at an inhibitory gene locus at any part of the gene, such as an exon of the inhibitory gene. The CAR may be under the control of the endogenous promoter of the inhibitory gene. In specific examples, the CAR disrupts the expression of the inhibitory gene.

[0025] In some examples, the CAR targets one or more tumor associated antigens selected from the group consisting of CD19, CD319 (CS1), ROR1, CD20, carcinoembryonic antigen, alphafetoprotein, CA-125, MUC-1, epithelial tumor antigen, melanoma-associated antigen, mutated p53, mutated ras, HER2/Neu, ERBB2, folate binding protein, HIV-1 envelope glycoprotein gp120, HIV-1 envelope glycoprotein gp41, GD2, CD5, CD123, CD23, CD30, CD56, c-Met, mesothelin, GD3, HERV-K, IL-11Ralpha, kappa chain, lambda chain, CSPG4, ERBB2, WT-1, TRAIL/DR4, VEGFR2, CD33, CD47, CLL-1, U5snRNP200, CD200, BAFF-R, BCMA, CD99, and a combination thereof. In particular examples, the CAR comprises at least one signaling domain selected from the group consisting of CD3ξ, CD28, OX40/CD134, 4-1BB/CD137, FcεRIγ, ICOS/CD278, ILRB/CD122, IL-2RG/CD132, DAP12, CD70, and CD40. In some examples, a vector that encodes the CAR also encodes a cytokine, such as IL-7, IL-2, IL-15, IL-12, IL-18, IL-21, or a combination thereof. In alternative cases, the cytokine is on a vector separate from the vector that encodes the CAR. In certain examples, an expression

construct that encodes the CAR further comprises a suicide gene, such as inducible caspase 9 or a membrane bound, nonsecretable TNF-alpha mutant.

[0026] Further provided herein is an immune cell with at least one CAR inserted at an inhibitory gene of the immune cell, such as an immune cell produced by the present methods. Also provided herein is a composition comprising a population of immune cells of examples of the disclosure, such as a population of T cells, B cells, NK cells, NK T cells, macrophages, stem cells, mixture thereof, and so forth.

[0027] Another example provides a composition comprising a population of cells of the examples, and in certain examples the population is utilized for treatment of a medical condition of any kind, including at least for the treatment of an immune-related disorder, infectious disease, and/or cancer.

[0028] A further example provides a method of treating a disease or disorder in a subject comprising administering an effective amount of immune cells of the examples to the subject. In some examples, the disease or disorder is an infectious disease; cancer, such as a solid cancer or a hematologic malignancy; and/or an immune-related disorder. The immune-related disorder may be an autoimmune disorder, graft versus host disease, allograft rejection, and/or inflammatory condition, in some cases. In some examples, the immune-related disorder is an inflammatory condition and the immune cells have essentially no expression of glucocorticoid receptor. In certain examples, the immune cells are autologous or allogeneic with respect to a recipient individual.

[0029] In additional examples, the method further comprises administering at least a second therapeutic agent to an individual. In some examples, the at least a second therapeutic agent comprises chemotherapy, immunotherapy, surgery, radiotherapy, hormone therapy, or biotherapy. In certain examples, the immune cells and/or the at least a second therapeutic agent are administered intravenously, intraperitoneally, intratracheally, intratumorally, intramuscularly, endoscopically, intralesionally, percutaneously, subcutaneously, regionally, or by direct injection or perfusion. The immune cells and the at least a second therapeutic agent may be administered at the same time or at different times, and when they are administered at different times or at the same time but not in the same formulation, they may or may not be administered by the same route.

WHAT IS CLAIMED IS:

1. An *in vitro* method for the disruption of at least three genes in a natural killer cell, wherein the at least three genes are selected from the group consisting of (1) NKG2A, CISH, and TGFBRII, (2) TIGIT, FOXO1, and TGFBRII, (3) TGFBRII, CD96, and TIGIT, (4) TGFBRII, CISH, and TIGIT, (5) TIM3, CISH, and TGFBRII, (6) CD96, FOXO1, and TGFBRII, (7) TGFBRII, TIM3, and TIGIT, (8) SIGLEC7, CISH, and TGFBRII, (9) CD47, CISH, and TGFBRII, (10) SIRPA, CISH, and TGFBRII, (11) TGFBRII, CD47, and TIGIT, (12) TGFBRII, CD47, and SIRPA, (13) A2AR, CISH, and TGFBRII, (14) TGFBRII, CISH, and ADAM17, (15) TGFBRII, TIM3, and TIGIT, (16) TGFBRII, A2AR, and TIGIT, (17) SHP1, CISH, and TGFBRII, (18) TGFBRII, CISH, and SHP1, (19) TGFBRII, SHP1, and TIGIT, and (20) TGFBRII, SHP1, and TIM3.
2. The method of claim 1, wherein the natural killer cell is engineered to express a chimeric antigen receptor (CAR) and/or T cell receptor (TCR).
3. The method of claim 1 or 2, wherein the natural killer cell is isolated from cord blood and the cord blood is pooled from 2 or more individual cord blood units.
4. The method of any one of claims 1-3, further comprising introducing a CAR or TCR to said natural killer cell.
5. The method of claim 4, wherein said introducing comprises introducing a nucleic acid encoding said CAR or TCR into said natural killer cells, wherein the nucleic acid is in an expression vector, wherein the vector further comprises a sequence that encodes a guide RNA for the at least three genes are selected from the group consisting of NKG2A, SIGLEC-7, LAG3, TIM3, CISH, FOXO1, TGFBRII, TIGIT, CD96, ADORA2, NR3C1, PD1, PDL-1, PDL-2, CD47, SIRPA, SHIP1, ADAM17, RPS6, 4EBP1, CD25, CD40, IL21R, ICAM1, CD95, CD80, CD86, IL10R, CD5, and CD7.

6. The method of claim 5, wherein the CAR is flanked by homology arms for said genes, wherein introducing the vector comprising the CAR sequence results in insertion of the CAR at the gene locus that encodes the inhibitory gene product in said immune cell.

7. The method of claim 6, wherein the CAR is inserted at an exon of said gene that encodes the inhibitory gene product.

8. The method of claim 6, wherein the CAR is under the control of the endogenous promoter of the gene that encodes the inhibitory gene product.

9. The method of claim 6, wherein introducing the vector further disrupts expression of said gene that encodes the inhibitory gene product.

10. A natural killer cell with disrupted expression of at least three genes in the natural killer cell, wherein the at least three genes are selected from the group consisting of (1) NKG2A, CISH, and TGFBRII, (2) TIGIT, FOXO1, and TGFBRII, (3) TGFBRII, CD96, and TIGIT, (4) TGFBRII, CISH, and TIGIT, (5) TIM3, CISH, and TGFBRII, (6) CD96, FOXO1, and TGFBRII, (7) TGFBRII, TIM3, and TIGIT, (8) SIGLEC7, CISH, and TGFBRII, (9) CD47, CISH, and TGFBRII, (10) SIRPA, CISH, and TGFBRII, (11) TGFBRII, CD47, and TIGIT, (12) TGFBRII, CD47, and SIRPA, (13) A2AR, CISH, and TGFBRII, (14) TGFBRII, CISH, and ADAM17, (15) TGFBRII, TIM3, and TIGIT, (16) TGFBRII, A2AR, and TIGIT, (17) SHP1, CISH, and TGFBRII, (18) TGFBRII, CISH, and SHP1, (19) TGFBRII, SHP1, and TIGIT, and (20) TGFBRII, SHP1, and TIM3.

11. The cell of claim 10, wherein the cell is produced according to the method of any one of claims 1-9.

12. The cell of claim 10, wherein the natural killer cell is engineered to express a chimeric antigen receptor (CAR) and/or T cell receptor (TCR).

13. The cell of claim 10, wherein the cell is engineered to express a CAR and/or TCR.

14. The cell of claim 13, wherein the CAR is inserted at an endogenous gene locus that encodes an inhibitory gene product of said cell.

15. The cell of claim 14, wherein the locus is selected from the group consisting of SIGLEC-7, LAG3, TIM3, CISH, FOXO1, TGFBRII, TIGIT, CD96, ADORA2, NR3C1, PD1, PDL-1, PDL-2, CD47, SIRPA, SHIP1, ADAM17, RPS6, 4EBP1, CD25, CD40, IL21R, ICAM1, CD95, CD80, CD86, IL10R, CD5, and CD7.

16. The cell of claim 14, wherein the CAR is under the control of the endogenous promoter of said gene that encodes an inhibitory gene product.

17. The cell of claim 14, wherein the CAR was inserted at the gene locus that encodes an inhibitory gene product by CRISPR-mediated gene editing.

18. The cell of any one of claims 10-17, wherein the cell is engineered to express a heterologous cytokine selected from the group consisting of IL-7, IL-2, IL-15, IL-12, IL-18, IL-21, and a combination thereof.

19. A pharmaceutical composition comprising a population of cells of any one of claims 10-18.

20. A composition comprising a population of cells of any one of claims 10-18 for the treatment of an immune-related disorder, infectious disease, or cancer.

21. Use of natural killer cells of any one of claims 10-18 in the manufacture of a medicament for treating a disease or disorder in a subject.

22. The use of claim 21, wherein the disease or disorder is an infectious disease, cancer, or immune-related disorder.