engineered donor cell populations, e.g., engineered immune cell populations, such as CAR T cell populations. For example, the instant disclosure relates to, *inter alia*, methods, compositions, and kits for detecting the presence or absence of biomarkers and/or secretion profiles of donor cell populations, selecting donor cells with certain biomarkers and/or secretion profiles, and engineering CAR T cells from the selected donor cells.

SUMMARY

[0005] The instant disclosure relates to methods and reagents for analyzing the attributes of donor cell populations prior to modification to manufacture engineered cells, e.g., engineered immune cell populations, such as CAR T cell populations. Engineered immune cell populations derived from such donor cell populations may also be analyzed for one or more of the same attributes at one or more timepoints along the engineering process, e.g., the CAR-T cell manufacturing process.

[0006] In one aspect, the present disclosure describes methods of manufacturing engineered immune cells. In one example, the method comprises detecting an HLA-DR expression level of 65% or less in an immune cell population. In another example, the method further comprises modifying the immune cell population to express an exogenous nucleic acid sequence, thereby providing an engineered immune cell population. In a further example, the modifying step further comprises reducing or eliminating expression of an endogenous gene (such as for example TCRα and/or CD52 as further described herein).

[0007] In other examples, (i) the exogenous nucleic acid sequence comprises a chimeric antigen receptor (CAR) nucleic acid sequence or (ii) the exogenous nucleic acid sequence further comprises one or more nucleic acid sequences selected from the group consisting of a chimeric antigen receptor (CAR), a transmembrane domain nucleic acid sequence, a costimulatory domain nucleic acid sequence and a signaling domain nucleic acid sequence. In another example, the exogenous nucleic acid sequence is expressed as a single transcript. In a further example, the CAR nucleic acid sequence expresses a CAR that binds to BCMA, EGFRvIII, WT-1, CD20, CD23, CD30, CD38, CD33, CD133, MHC-WT1, TSPAN10, MHC-PRAME, Liv1, ADAM10, CHRNA2, LeY, NKGD2D, CS1, CD44v6, ROR1, Claudin-18.2, Muc17, FAP alpha, Ly6G6D, c6orf23, G6D, MEGT1, NG25, CD19, FLT3, CD70, DLL3, CD52 or CD34.

[0008] In one example, the engineered immune cell population comprises, exhibits, shows, or has improved in vitro functionality as compared to a non-engineered immune cell population. In another example, the engineered immune cell population comprises, exhibits, shows, or has improved in vitro functionality as compared to an additional engineered immune cell population that originated from (or was originated from or originated from) an additional immune cell population expressing HLA-DR at a level greater than about 65%. In other examples, the improved in vitro functionality comprises one or more of improved in vitro cytotoxicity, improved cell fitness, and reduced cytokine secretion. In one additional example, cytotoxicity is demonstrated by an in vitro killing assay. In other examples, the in vitro killing assay comprises the killing of cells that express a target of the CAR. The in vitro killing assay described herein may be a long-term killing assay or a short-term killing assay.

[0009] In a further example, the immune cell population is obtained from or derived from a donor prior to the detecting step. In one example, the donor is a healthy donor or a patient in need of treatment (such as for example a human patient). In another example, the patient is a patient in need of treatment with an autologous cell therapy. In one other example, the autologous cell therapy comprises the engineered immune cell population.

[0010] In other examples, the detecting step comprises detecting a protein level of a molecule, e.g., HLA-DR or TIGIT, using flow cytometry (FACS), an Enzyme-Linked Immunosorbent Assay (ELISA), an immunoblotting assay, an immunofluorescence assay, or an immunochemistry (IHC) assay.

[0011] In one example, the donor from which the immune cell population is obtained or derived from prior to the detecting step is a healthy human donor. In another example, the healthy human donor is aged between about 18 and about 30 years old.

[0012] In other examples, the method further comprises detecting a level of expression of one or more biomarkers selected from the group consisting of TIGIT, CD16, CD56, CCR7, CD27, and CD45RA. In one example, the method further comprises detecting a level of expression of TIGIT. In another example, the TIGIT expression level that is detected is 30% or less in the immune cell population.

[0013] In an additional example, the method further comprises depleting HLA-DR-positive immune cells from the immune cell population to provide an HLA-DR-

depleted immune cell population and/or depleting TIGIT-positive immune cells from the immune cell population to provide a TIGIT-depleted immune cell population. In one example, the depleting step is performed prior to the modifying step.

[0014] In one other example, the present disclosure describes an engineered immune cell population comprising certain levels of biomarker-positive cells. In one example, the engineered immune cell population comprises 65% or less HLA-DR+ cells and/or 30% or less TIGIT+ cells. In some examples, the engineered immune cell population with biomarker-positive cells comprises an exogenous nucleic acid sequence. In other examples, (i) the exogenous nucleic acid sequence comprises a chimeric antigen receptor (CAR) nucleic acid sequence or (ii) the exogenous nucleic acid sequence further comprises one or more nucleic acid sequences selected from the group consisting of a chimeric antigen receptor (CAR), a transmembrane domain nucleic acid sequence, a costimulatory domain nucleic acid sequence and a signaling domain nucleic acid sequence. In another example, the exogenous nucleic acid sequence is expressed as a single transcript. In a further example, the CAR nucleic acid sequence expresses a CAR that binds to BCMA, EGFRvIII, WT-1, CD20, CD23, CD30, CD38, CD33, CD133, MHC-WT1, TSPAN10, MHC-PRAME, Liv1, ADAM10, CHRNA2, LeY, NKGD2D, CS1, CD44v6, ROR1, Claudin-18.2, Muc17, FAP alpha, Ly6G6D, c6orf23, G6D, MEGT1, NG25, CD19, FLT3, CD70, DLL3, CD52 or CD34.

[0015] In one additional example, the present disclosure describes methods of manufacturing immune cells with improved in vitro functionality. In some examples, the method comprises a step of detecting a level of HLA-DR expression in an immune cell population to provide a detected level of HLA-DR expression. In some examples, the detecting comprises detecting a protein level of HLA-DR using flow cytometry (FACS), an Enzyme-Linked Immunosorbent Assay (ELISA), an immunoblotting assay, an immunofluorescence assay, or an immunochemistry (IHC) assay. The method can further comprise a step of modifying the immune cell population to express an exogenous nucleic acid sequence, thereby providing an engineered immune cell population. In some examples, the engineered immune cell population comprises, exhibits, shows, or has improved in vitro functionality as compared to (i) an additional engineered immune cell population that originated from an additional immune cell population having a higher level of HLA-DR expression than the detected level, (ii) an additional engineered immune cell population originated from an additional immune cell population having a higher level of

HLA-DR expression than the detected level, or (iii) an additional engineered immune cell population that was originated from an additional immune cell population having a higher level of HLA-DR expression than the detected level. The method may further comprise detecting different levels of expression for different biomarkers and combinations thereof. In other examples, the detected level indicates HLA-DR is expressed in less than 65% of immune cells of the immune cell population. In another example, the lower level is more than 65% of immune cells of the additional immune cell population. In one example, the modifying step further comprises reducing or eliminating expression or activity of an endogenous gene.

[0016] In some examples, the exogenous nucleic acid sequence comprises a chimeric antigen receptor (CAR) nucleic acid sequence. The exogenous nucleic acid sequence may further comprise one or more nucleic acid sequences selected from the group consisting of a transmembrane domain nucleic acid sequence, a costimulatory domain nucleic acid sequence and a signaling domain nucleic acid sequence. In other examples, the exogenous nucleic acid sequence is expressed as a single transcript. In some examples, the CAR nucleic acid sequence expresses a CAR that binds to BCMA, EGFRvIII, WT-1, CD20, CD23, CD30, CD38, CD33, CD133, MHC-WT1, TSPAN10, MHC-PRAME, Liv1, ADAM10, CHRNA2, LeY, NKGD2D, CS1, CD44v6, ROR1, Claudin-18.2, Muc17, FAP alpha, Ly6G6D, c6orf23, G6D, MEGT1, NG25, CD19, FLT3, CD70, DLL3, CD52 or CD34.

[0017] In one example, the improved in vitro functionality of the engineered immune cell population comprises, exhibits, shows, or has improved in vitro cytotoxicity. The improved in vitro functionality may comprise improved in vitro cytotoxicity. In some examples, the cytotoxicity is demonstrated by an in vitro killing assay. In one example, the cytotoxicity is demonstrated by in vitro killing assay that comprises killing of cells that express a target of the CAR. In other examples, the in vitro killing assay is a long-term killing assay (LTKA) or a short-term killing assay (STKA).

[0018] In other examples, the immune cell population is obtained from or derived from a donor prior to the detecting step. The donor may be a healthy donor or a patient in need of treatment. In one example, the patient is a patient in need of treatment with an autologous cell therapy. The autologous cell therapy may comprise the engineered immune cell population.

[0019] In another example, the present disclosure describes methods for selecting a donor immune cell population for engineering. In one example, the method comprises a step of detecting a first level of HLA-DR expression in a first immune cell population to provide a first detected level of HLA-DR. In another example, the method comprises a step of detecting a second level of HLA-DR expression in a second immune cell population to provide a second detected level of HLA-DR. In one other example, the second detected level is greater than the first detected level. The method can comprise selecting the first immune cell population for engineering. In some examples, the method may further comprise discarding the second cell population and/or preserving the first cell population. In another example, the detecting steps comprise detecting a protein level of HLA-DR using flow cytometry (FACS), an Enzyme-Linked Immunosorbent Assay (ELISA), an immunoblotting assay, an immunofluorescence assay, or an immunochemistry (IHC) assay. In other examples, the first detected level indicates that HLA-DR is expressed in less than 65% of immune cells of the immune cell population. In another example, the second detected level indicates that HLA-DR is expressed in more than 65% of immune cells of the immune cell population.

[0020] In one example, the method can further comprise a step of modifying the first immune cell population to express an exogenous nucleic acid sequence, thereby providing an engineered immune cell population. In some examples, the engineered immune cell population comprises, exhibits, shows, or has improved in vitro functionality as compared to an additional engineered immune cell population that originated from (or was originated from) the second immune cell population. The method may further comprise detecting different levels of expression for different biomarkers and combinations thereof. In one example, the modifying step further comprises reducing or eliminating expression or activity of an endogenous gene.

[0021] In some examples, the exogenous nucleic acid sequence comprises a chimeric antigen receptor (CAR) nucleic acid sequence. The exogenous nucleic acid sequence may further comprise one or more nucleic acid sequences selected from the group consisting of a transmembrane domain nucleic acid sequence, a costimulatory domain nucleic acid sequence and a signaling domain nucleic acid sequence. In other examples, the exogenous nucleic acid sequence is expressed as a single transcript. In some examples, the CAR nucleic acid sequence expresses a CAR that binds to BCMA, EGFRvIII, WT-1, CD20, CD23, CD30, CD38, CD33, CD133, MHC-WT1, TSPAN10,

MHC-PRAME, Liv1, ADAM10, CHRNA2, LeY, NKGD2D, CS1, CD44v6, ROR1, Claudin-18.2, Muc17, FAP alpha, Ly6G6D, c6orf23, G6D, MEGT1, NG25, CD19, FLT3, CD70, DLL3, CD52 or CD34.

[0022] In one example, the improved in vitro functionality of the engineered immune cell population comprises improved in vitro cytotoxicity. The improved in vitro functionality may comprise improved in vitro cytotoxicity. In some examples, the cytotoxicity is demonstrated by an in vitro killing assay. In one example, the cytotoxicity is demonstrated by in vitro killing assay that comprises killing of cells that express a target of the CAR. In other examples, the in vitro killing assay is a long-term killing assay or a short-term killing assay.

[0023] In other examples, the immune cell population is obtained from or derived from a donor prior to the detecting step. The donor may be a healthy donor or a patient in need of treatment. In one example, the patient is a patient in need of treatment with an autologous cell therapy. The autologous cell therapy may comprise the engineered immune cell population.

[0024] In a further example, the present disclosure describes methods for manufacturing immune cells with improved in vitro functionality. In one example, the method comprises a step of modifying an immune cell population to express an exogenous nucleic acid sequence, thereby providing an engineered immune cell population. The method may further comprise depleting HLA-DR-positive engineered immune cells from the engineered immune cell population to provide an HLA-DR-depleted engineered immune cell population. In another example, the HLA-DR-depleted engineered immune cell population comprises, exhibits, shows, or has improved in vitro functionality as compared to an engineered immune cell population that has not been depleted of HLA-DR-positive engineered immune cells. In one additional example, the method further comprises depleting additional immune cells from the engineered immune cell population. In other examples, the additional immune cells express one or more of TIGIT, CD16, and CD56. In a further example, the HLA-DR-depleted and TIGIT-, CD16-, or CD56-depleted engineered immune cell population comprises, exhibits, shows, or has improved in vitro functionality as compared to an engineered immune cell population that has not been depleted of HLA-DR-positive and TIGIT-, CD16- or CD56-positive immune cells. In another example, the modifying step further comprises reducing or eliminating

expression or activity of an endogenous gene. In one other example, the depleting comprises a flow cytometry (FACS) method. In another example, the method further comprises detecting a level of HLA-DR expression in the HLA-DR-depleted engineered immune cell population and/or detecting a level of TIGIT, CD16, and/or CD56 in the TIGIT-, CD16-, and/or CD56-depleted engineered immune cell population.

[0025] In some examples, the exogenous nucleic acid sequence comprises a chimeric antigen receptor (CAR) nucleic acid sequence. The exogenous nucleic acid sequence may further comprise one or more nucleic acid sequences selected from the group consisting of a transmembrane domain nucleic acid sequence, a costimulatory domain nucleic acid sequence and a signaling domain nucleic acid sequence. In other examples, the exogenous nucleic acid sequence is expressed as a single transcript. In some examples, the CAR nucleic acid sequence expresses a CAR that binds to BCMA, EGFRvIII, WT-1, CD20, CD23, CD30, CD38, CD33, CD133, MHC-WT1, TSPAN10, MHC-PRAME, Liv1, ADAM10, CHRNA2, LeY, NKGD2D, CS1, CD44v6, ROR1, Claudin-18.2, Muc17, FAP alpha, Ly6G6D, c6orf23, G6D, MEGT1, NG25, CD19, FLT3, CD70, DLL3, CD52 or CD34.

[0026] In other examples, the immune cell population is obtained from or derived from a donor prior to the detecting step. The donor may be a healthy donor or a patient in need of treatment. In one example, the patient is a patient in need of treatment with an autologous cell therapy. The autologous cell therapy may comprise the engineered immune cell population.

[0027] In one other example, the present disclosure describes a chimeric antigen receptor T (CAR-T) cell populations. In one example, the CAR-T cell population is a cell population in which HLA-DR is expressed at a first level and the CAR-T cell population has improved in vitro functionality as compared to a CAR-T cell population in which HLA-DR is expressed at a second level. In other examples, the first level is lower than the second level. In one other example, the first level is more than 75% of the CAR-T cell population and/or wherein the second level is less than 75% of the CAR-T cell population.

[0028] In another example, the CAR-T cell population has an exogenous nucleic acid sequence comprising a chimeric antigen receptor (CAR) nucleic acid sequence. The exogenous nucleic acid sequence may further comprise one or more nucleic acid sequences

selected from the group consisting of a transmembrane domain nucleic acid sequence, a costimulatory domain nucleic acid sequence and a signaling domain nucleic acid sequence. In other examples, the exogenous nucleic acid sequence is expressed as a single transcript. In some examples, the CAR nucleic acid sequence expresses a CAR that binds to BCMA, EGFRvIII, WT-1, CD20, CD23, CD30, CD38, CD33, CD133, MHC-WT1, TSPAN10, MHC-PRAME, Liv1, ADAM10, CHRNA2, LeY, NKGD2D, CS1, CD44v6, ROR1, Claudin-18.2, Muc17, FAP alpha, Ly6G6D, c6orf23, G6D, MEGT1, NG25, CD19, FLT3, CD70, DLL3, CD52 or CD34.

[0029] In one example, the improved in vitro functionality of the CAR-T cell population comprises improved in vitro cytotoxicity. The improved in vitro functionality may comprise improved in vitro cytotoxicity. In some examples, the cytotoxicity is demonstrated by an in vitro killing assay. In one example, the cytotoxicity is demonstrated by in vitro killing assay that comprises killing of cells that express a target of the CAR. In other examples, the in vitro killing assay is a long-term killing assay or a short-term killing assay.

[0030] In an additional example, the preset disclosure describes a kit or an article of manufacture for in vitro functionality analysis of cell populations, e.g., donor cell populations and/or engineered cell populations. In one example, the kit comprises an anti-HLA-DR binding agent. The kit may further comprise instructions to use the binding agent to detect a level of HLA-DR expression in the cell population. In other examples, the kit further comprises one or more additional binding agents to detect one or more of TIGIT, CD16, and CD56. The kit may further comprise instructions to use the binding agent(s) to detect a level of expression of one or more of TIGIT, CD16, CD56, CCR7, CD27, CD45RA, and any combination thereof in the cell population. In one example, the kit further comprises reagents for measuring in vitro cytotoxicity of a CAR T cell engineered from the cell population. The binding agent(s) may be an antigen binding molecule, which may be an antibody or fragment thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

WHAT IS CLAIMED IS

1. A method of manufacturing engineered immune cells comprising:

a) detecting an HLA-DR expression level of 65% or less in an immune cell population; and

b) modifying the immune cell population to express an exogenous nucleic acid sequence, thereby providing an engineered immune cell population.

2. The method of claim 1, wherein the exogenous nucleic acid sequence comprises a chimeric antigen receptor (CAR) nucleic acid sequence.

3. The method of claim 2, wherein the exogenous nucleic acid sequence further comprises one or more nucleic acid sequences selected from the group consisting of a chimeric antigen receptor (CAR), a transmembrane domain nucleic acid sequence, a costimulatory domain nucleic acid sequence and a signaling domain nucleic acid sequence.

4. The method of claim 3, wherein the exogenous nucleic acid sequence is expressed as a single transcript.

5. The method of claim 1, wherein the engineered immune cell population has improved in vitro functionality as compared to a non-engineered immune cell population.

6. The method of claim 1, wherein the engineered immune cell population has improved in vitro functionality as compared to an additional engineered immune cell population that originated from an additional immune cell population expressing HLA-DR at a level greater than about 65%.

7. The method of claim 5 or 6, wherein the improved in vitro functionality comprises one or more of improved in vitro cytotoxicity, improved cell fitness, and reduced cytokine secretion.

8. The method of claim 7, wherein the cytotoxicity is demonstrated by an in vitro killing assay.

9. The method of claim 8, wherein the cytotoxicity is demonstrated by in vitro killing assay that comprises killing of cells that express a target of the CAR.

10. The method of claim 8 or 9, wherein the in vitro killing assay is a long-term killing assay or a short-term killing assay.

11. The method of claim 8 or 9, wherein the in vitro killing assay is a long-term killing assay or a short-term killing assay.

12. The method of claim 2 or 3, wherein the CAR nucleic acid sequence expresses a CAR that binds to BCMA, EGFRvIII, WT-1, CD20, CD23, CD30, CD38, CD33, CD133, MHC-WT1, TSPAN10, MHC-PRAME, Liv1, ADAM10, CHRNA2, LeY, NKGD2D, CS1, CD44v6, ROR1, Claudin-18.2, Muc17, FAP alpha, Ly6G6D, c6orf23, G6D, MEGT1, NG25, CD19, FLT3, CD70, DLL3, CD52 or CD34.

13. The method of claim 1, wherein the modifying further comprises reducing or eliminating expression or activity of an endogenous gene.

14. The method of claim 1, wherein the immune cell population had been obtained from or derived from a donor prior to the detecting step.

15. The method of claim 14, wherein the donor is a healthy donor or a patient in need of treatment.

16. The method of claim 15, wherein the patient is a patient in need of treatment with an autologous cell therapy.

17. The method of claim 16, wherein the autologous cell therapy comprises the engineered immune cell population.

18. The method of claim 1, wherein the detecting comprises detecting a protein level of HLA-DR using flow cytometry (FACS), an Enzyme-Linked Immunosorbent Assay (ELISA), an immunoblotting assay, an immunofluorescence assay, or an immunochemistry (IHC) assay.

19. The method of any one of claims 1 to 15 and 18, wherein the immune cell population had been obtained from a healthy human donor.

20. The method of claim 19, wherein the healthy human donor is aged between about 18 and about 30 years old.

21. The method of any one of claims 1-20, further comprising detecting a level of expression of one or more biomarkers selected from the group consisting of TIGIT, CD16, CD56, CCR7, CD27, and CD45RA.

22. The method of any one of claims 1-20, further comprising detecting a level of expression of TIGIT.

23. The method of claim 22, wherein the TIGIT expression level is 30% or less in the immune cell population.

24. The method of any one of claims 1-23, further comprising depleting HLA-DR-positive immune cells from the immune cell population to provide an HLA-DR-depleted immune cell population.

25. The method of any one of claims 1-24, further comprising depleting TIGIT-positive immune cells from the immune cell population to provide a TIGIT-depleted immune cell population.

26. The method of claim 24 or 25, wherein the depleting step is performed prior to the modifying step.