use in cell therapy. In particular, methods are needed for the preparation and engineering of cells, e.g., a plurality of isolated cell types or sub-types, with improved efficiency, safety, variability, and conservation of resources. Provided are methods, cells, compositions, kits, and systems that meet such needs.

Summary

[0004a] In one aspect, there is provided a method for enriching CD4+ or CD8+ T cells, the method comprising: (a) performing a first selection in a closed system, said first selection comprising enriching for one of (i) CD4+ cells and (ii) CD8+ cells from a sample containing primary human T cells, the enrichment thereby generating a first selected population and a non-selected population; and (b) performing a second selection in the closed system, said second selection comprising enriching for the other of (i) CD4+ cells and (ii) CD8+ cells from the non-selected population, the enrichment thereby generating a second selected population.

[0004b] In another aspect, there is provided a kit produced by the method as described herein, comprising cells of the first selected population and cells of the second selected population at the desired CD4:CD8 ratio.

[0004c] In yet another aspect, there is provided a method for producing genetically engineered T cells, the method comprising: (a) performing a first selection in a closed system, said first selection comprising enriching for one of (i) CD4+ cells and (ii) CD8+ cells from a sample containing primary human T cells, the enrichment thereby generating a first selected population and a non-selected population; and (b) performing a second selection in the closed system, said second selection comprising enriching for the other of (i) CD4+ cells and (ii) CD8+ cells from the non-selected population, the enrichment thereby generating a second selected population; (c) incubating a culture-initiating composition, which comprises cells of the first selected population and cells of the second selected population, in a culture vessel under stimulating conditions, thereby generating stimulated cells; and (d) introducing a genetically engineered antigen receptor into stimulated cells generated in (c), wherein the method thereby generates an output composition comprising CD4+ T cells and CD8+ T cells expressing the genetically engineered antigen receptor.

[0004d] In yet another aspect, there is provided a method for enriching CD4+ and CD8+ T cells, the method comprising contacting cells of a sample containing primary human T cells with a first immunoaffinity reagent that specifically binds to CD4 and a second immunoaffinity reagent that specifically binds to CD8 in an incubation composition, under conditions whereby the immunoaffinity reagents specifically bind to CD4 and CD8 molecules, respectively, on the surface of cells in the sample; and recovering cells bound to the first and/or the second immunoaffinity reagent, thereby generating an enriched composition comprising CD4+ cells and CD8+ cells at a desired ratio, wherein: the first and/or second immunoaffinity reagent are present in the incubation composition at a sub-optimal yield concentration, whereby the enriched composition contains less than 70% of the total CD4+ cells in the incubation composition and/or less than 70% of the CD8+ cells in the incubation composition.

[0004e] In yet another aspect, there is provided a method for producing genetically engineered T cells, the method comprising: (a) enriching a sample for primary human T cells , the enriching comprising: contacting cells of said sample with a first immunoaffinity reagent that specifically binds to CD4 and a second immunoaffinity reagent that specifically binds to CD8 in an incubation composition, under conditions whereby the immunoaffinity reagents specifically bind to CD4 and CD8 molecules, respectively, on the surface of cells in the sample; and recovering cells bound to the first and/or the second immunoaffinity reagent, thereby generating an enriched composition comprising CD4+ cells and CD8+ cells at a culture-initiating ratio, wherein: the first and/or second immunoaffinity reagent are present in the incubation composition at a sub-optimal yield concentration, whereby the enriched composition contains less than 70% of the total CD4+ cells in the incubation composition and/or less than 70% of the CD8+ cells in the incubation composition; and (b) incubating cells of the enriched composition at or substantially at the culture-initiation ratio in a culture-initiation composition in a culture vessel under stimulating conditions; and (c) introducing a genetically engineered antigen receptor into cells stimulated in (b), thereby generating an output composition comprising CD4+ T cells and CD8+ T cells expressing the genetically engineered antigen receptor.

[0004f] In yet another aspect, there is provided a composition or kit comprising cells produced by the method as described herein.

[0004g] In yet another aspect, there is provided the composition or kit as described herein for use in treating a disease or condition in a subject.

[0004h] In yet another aspect, there is provided use of a composition as described herein for the manufacture of a medicament for treating a disease or condition in a subject.

[0004i] In yet another aspect, there is provided a closed apparatus system for purification of target cells, comprising: a) a first affinity chromatography matrix comprising a first binding agent immobilized thereon, which binding agent specifically binds to a first cell surface marker present on a first cell type, said first affinity matrix not comprising a second binding agent that specifically binds to a second cell surface marker, wherein: the first affinity chromatography matrix is operably connected to a storage reservoir comprising a cell sample; and is operably connected to an output vessel; b) a second affinity chromatography matrix comprising a second binding agent immobilized thereon, which second binding agent specifically binds to a second cell surface marker present on a second cell type, wherein: the first affinity chromatography matrix is operably connected to the second affinity chromatography matrix; and the second affinity chromatography matrix is operably connected to the output vessel, and is operably linked to a waste vessel; c) the output vessel; and d) the waste vessel, wherein the system is configured to permit, within the closed system, collection of, in the output vessel in a single composition, (i) cells having bound and been recovered from the first affinity chromatography matrix and (ii) cells having passed through and not bound to the first affinity chromatography matrix and having bound and been recovered from the second chromatography matrix.

[0005] Provided are methods for the preparation and engineering of cells and populations of cells and cells and compositions produced by the methods. In some examples, the cells can be used for immunotherapy, such as in connection with adoptive immunotherapy methods. In some example, the provided methods include isolation, selection or enrichment of CD4+ and CD8+ cells, or sub-populations thereof, from the same starting sample, such as a single sample, for example a single apheresis sample, leukapheresis sample or a sample containing peripheral blood mononuclear

cells (PBMCs). In some examples, the methods include selection or enrichment of at least two populations of cells, such as a CD4+ cell population and a CD8+ population, in a single processing stream in which no negative fraction sample is discarded from a first selection or enrichment of one of the CD4+ or CD8+ in the process, prior to performing the second selection for the other of the CD4+ or CD8+ cells. In some examples, the first and second selection can occur simultaneously or sequentially.

[0006] In some examples, the selection, enrichment and/or isolation of both populations of cells, such as CD4+ and CD8+ cells, is performed simultaneously, such as in the same vessel or using the same apparatus, or sequentially as part of a system or apparatus in which vessels, e.g. columns, chambers, used in performing the first and second selection, are operably connected. In some examples, the simultaneous and/or sequential enrichments or selections can occur as a single process stream without handling of any of the positive or negative fractions prepared as part of the first and/or second selection or enrichment. In some examples, the isolation, culture, and/or engineering of the different populations is carried out from the same starting composition or material, such as from the same sample.

[0007] In some examples, the methods include performing a first selection by enriching from a sample containing primary human T cells one of CD4+ or CD8+ cells to generate a first

selected population and a non-selected population, and from the non-selected population performing a second selection by enriching for the other of CD4+ cell or CD8+ cell, wherein the method produces a composition of cells containing cells enriched for CD4+ cells and cells enriched for CD8+.

[0008] In some examples, the second selection is carried out by enriching for the other of the T cell subtypes from the non-selected population generated by the first selection. Thus, in some examples, the provided methods differ from other selection methods in that the negative fraction from a first selection is not discarded but rather is used as the basis for a further selection to enrich for another cell type. In general, where the T cell subset enriched for in the first selection is a CD4+ subset (or where the first selection enriches for CD4+ cells), it will follow that the first selection is designed such that it does not enrich for cells of the other subtype to be enriched for in the second selection. For example, in some examples, the first selection enriches for CD4+ cells and does not enrich for CD8+ cells, and the second selection enriches for CD8+ cells from the negative fraction recovered from the first selection. Likewise, in general, where the T cell subset enriched for in the first selection is a CD8+ subset (or where the first selection enriches for CD8+ cells), it will follow that the first selection is designed such that it does not enrich for cells of the other subtype to be enriched for in the second selection. For example, in some examples, the first selection enriches for CD8+ cells and does not enrich for CD4+ cells, and the second selection enriches for CD4+ cells from the negative fraction recovered from the first selection.

[0009] In some examples, the methods further involve third, fourth, and so-forth further selections, which may enrich for cells from the selected and/or the non-selected populations from any previous selection step. For example, in some examples, cells from either the selected population or the non-selected population from a given step (e.g., the second selection step, are further enriched. For example, in some examples, selected CD8+ cells are further enriched for a subtype of CD8+ cells, such as resting cells or central memory cells.

[0010] In some examples, provided is a method for producing genetically engineered T cells, comprising (a) providing a culture-initiation composition, said composition produced by performing a first selection in a closed system, said first selection comprising enriching for one of

$CD4^+$ cells and CD8+ cells from a sample containing primary human T cells, thereby generating a first selected population and a non-selected population, and performing a second selection in the closed system, said second selection comprising enriching for the other of CD4+ cells and CD8+ cells from the non-selected population, thereby generating a second selected population; (b) incubating a culture-initiating composition, which comprises cells of the first selected population and cells of the second selected population, in a culture vessel under stimulating conditions, thereby generating stimulated cells; and (d) introducing a genetically engineered antigen receptor into stimulated cells generated in (b), wherein the method thereby generates an output composition comprising $CD4^+$ T cells and $CD8^+$ T cells expressing the genetically engineered antigen receptor.

[0011] Also provided, in some examples, are methods that include a simultaneous enrichment or selection of a first and second population of cells, such as a CD4+ and CD8+ cell population. In some examples, the method includes contacting cells of a sample containing primary human T cells with a first immunoaffinity reagent that specifically binds to CD4 and a second immunoaffinity reagent that specifically binds to CD8 in an incubation composition, under conditions whereby the immunoaffinity reagents specifically bind to CD4 and CD8 molecules, respectively, on the surface of cells in the sample, and recovering cells bound to the first and/or the second immunoaffinity reagent, thereby generating an enriched composition comprising CD4+ cells and CD8+ cells. In some example, the methods are performed to include in the incubation composition a concentration of the first and/or second immunoaffinity reagent that is at a sub-optimal yield concentration so that the enriched composition contains less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20% or less of the total CD4+ cells in the incubation composition or less than 70% less than 60%, less than 50%, less than 40%, less than 30%, less than 20% or less of the CD8+ cells in the incubation composition.

[0012] In some examples, provided is a method for enriching CD4+ and CD8+ T cells, comprising providing an enriched composition of CD4+ and CD9+ T cells, said enriched composition produced by contacting cells of a sample containing primary human T cells with a first immunoaffinity reagent that specifically binds to CD4 and a second immunoaffinity reagent that specifically binds to CD8 in an incubation composition, under conditions whereby the

immunoaffinity reagents specifically bind to CD4 and CD8 molecules, respectively, on the surface of cells in the sample; and recovering cells bound to the first and/or the second immunoaffinity reagent, thereby generating an enriched composition comprising CD4+ cells and CD8+ cells at a culture-initiating ratio, wherein: the first and/or second immunoaffinity reagent are present in the incubation composition at a sub-optimal yield concentration, whereby the enriched composition contains less than 70% of the total CD4+ cells in the incubation composition and/or less than 70% of the CD8+ cells in the incubation composition, thereby producing a composition enriched for CD4+ and CD8+ T cells.

[0013] In some examples of any of such provided examples, the methods are performed by immunoaffinity-based selection, such as by contacting cells with an antibody that specifically binds a cell surface marker, such as CD4, CD8 or other cell surface marker, such as expressed on naïve, resting or central memory T cells. In some examples, the solid support is a sphere, such as a bead, such as a microbead or nanobead. In some examples, the bead can be a magnetic bead. In some examples, the solid support can be a column or other vessel to effect column chromatography.

[0014] In some examples, the antibody contains one or more binding partners capable of forming a reversible bond with a binding reagent immobilized on the solid surface, such as a sphere or chromatography matrix, wherein the antibody is reversibly mobilized to the solid surface. In some examples, cells expressing a cell surface marker bound by the antibody on said solid surface are capable of being recovered from the matrix by disruption of the reversible binding between the binding reagent and binding partner. In some examples, the binding reagent is streptavidin or is a streptavidin analog or mutant, such as a streptavidin, analog or mutant set forth in any of SEQ ID NOS:11-16 or a sequence of amino acids that exhibits at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequence set forth in any of SEQ ID NOS: 11-16 and retains binding to a binding partner, such as biotin or peptide. In some examples, the binding partner is biotin, a biotin analog or a peptide capable of binding to the binding reagent. In some examples, the binding partner is or contains a peptide capable of binding the binding reagent, such as a streptavidin binding peptide, such as a peptide containing the

sequence set forth in any of SEQ ID NOS: 1-10. In some examples, the methods include after contacting cells in the sample to the solid support containing the antibody immobilized thereon as part of the first and/or second selection, applying a competition reagent to disrupt the bond between the binding partner and binding reagent, thereby recovering the selected cells from the solid surface. In some examples, the competition reagent is biotin or biotin analog.

[0015] In some examples, the methods generate a selected or enriched composition containing selected or enriched CD4+ cells to CD8+ cells, or sub-populations thereof, present at a culture-initiating ratio. In some examples, the culture-initiating ratio of CD4+ to CD8+ cells is between at or about 10:1 and at or about 1:10, between at or about 5:1 and at or about 1:5 or is between at or about 2:1 and at or about 1:2, such as is at or about 1:1. It is within the level of a skilled artisan to choose or select a sufficient amount or a sufficient relative amount of immunoaffinity-based reagent, such as antibody-coated beads (e.g. magnetic beads) or an affinity chromatography matrix or matrices, to achieve or produce a culture-initiating ratio in the generated composition, such as culture-initiation composition, containing cells enriched or selected for CD4, CD8 or sub-populations thereof. Exemplary of such methods are described in subsections below.

[0016] In some examples, the cells include cells of the immune system, such as lymphocytes, e.g., T cells (e.g., CD4+ and CD8+ T cells and isolated subpopulations thereof), and NK cells. In some examples, the cells are present in combinations of multiple cell populations or cell types, which in some aspects are included in the compositions at particular ratios or numbers of the cells or cell types. Also provided are methods for optimizing the methods, such as by selecting or determining appropriate ratios and numbers, such as culture-initiating ratios and desired output ratios and doses, of the cell types and population, for use in connection with the methods.

[0017] Also provided are cells, cell populations, and compositions thereof, for use in and produced by the methods. Also provided are systems, devices, apparatuses, reagents, compounds, and kits for carrying out the methods. Also provided are therapeutic methods and uses for cells and compositions produced by the methods, such as methods for adoptive cell therapy.

[0018] In some examples, provided are methods for producing cells for adoptive cell therapy, methods for producing cells for genetic engineering, and methods for producing genetically

engineered cells. In some examples, the cells are T cells, such as $CD4^+$ and $CD8^+$ T cells and/or sub-types thereof. In some aspects, the cell therapy is T cell therapy.

[0019] In some examples, the methods are carried out by (a) isolating cell populations from a sample, and (b) incubating a culture-initiating composition in a culture vessel containing cells of the isolated populations. In some examples, the methods further include genetically engineering the incubated cells or cells in the culture vessel, such as by (c) introducing a genetically engineered antigen receptor into cells in the culture vessel.

[0020] In some examples, the methods are carried out by (a) incubating a culture-initiating composition in a culture vessel containing a plurality of cell populations at a particular culture-initiation ratio or particular number of cells; and (b) genetically engineering the cells, such as by introducing a genetically engineered antigen receptor into cells in the culture vessel.

[0021] In some examples, the genetically engineered antigen receptor is introduced into cells in the culture vessel, such as to different cell types or sub-populations within the culture vessel, e.g., to $CD4^+$ and $CD8^+$ cells in the culture vessel.

[0022] In some examples, the methods produce an output composition for genetic engineering or adoptive cell therapy or comprising cells expressing the genetically engineered antigen receptor.

[0023] In some examples, the isolation includes or is carried out by isolating a $CD4^+$ primary human T cell population and/or a $CD8^+$ primary human T cell population from the sample. In some aspects, it includes depleting or enriching for a sub-population of $CD4^+$ cells, and/or depleting or enriching for a sub-population of $CD8^+$ cells. Thus, in some examples, the culture-initiating composition includes isolated $CD4^+$ and $CD8^+$ primary human T cells.

[0024] In some examples, the enriching or depleting is carried out by immunoaffinity-based selection, such as binding to antibodies or other binding molecules recognizing surface markers on the cells. In some examples, the antibody or other molecule is coupled to a magnetically responsive or magnetic particle, e.g., bead. In some examples, the selection includes positive and/or negative selection steps.

[0025] In some examples, the isolation of the $CD8^+$ primary human T cell population comprises depleting or enriching for a sub-population of $CD8^+$ cells. In some examples, the

isolation of the $CD4^+$ primary human T cell population comprises depleting or enriching for a sub-population of $CD4^+$ cells. In some examples, the isolation of a T cell population comprises enriching for T_{CM} cells. In some examples, the isolation of the $CD8^+$ and/or the $CD4^+$ primary human T cell population comprises enriching for central memory T (T_{CM}) cells. In some examples, the enrichment for central memory T (T_{CM}) cells comprises negative selection for cells expressing a surface marker present on naïve T cells, such as CD45RA, or positive selection for cells expressing a surface marker present on central memory T cells and not present on naïve T cells, such as CD45RO; and/or positive selection for cells expressing surface marker present on central memory T (T_{CM}) cells and not present on another memory T cell sub-population, such as CD62L, CCR7, CD27, CD127, and/or CD44.

[0026] In some examples, the isolation includes (i) subjecting the sample to positive selection based on surface expression of CD4, resulting in a positive and first negative fraction, where the positive fraction is the isolated $CD4^+$ population; and (ii) subjecting the first negative fraction to negative selection based on surface expression of a non-T cell marker and a surface marker present on naïve T cells, thereby generating a second negative fraction; and (iii) subjecting the second negative fraction to positive selection based on surface expression of a marker present on the surface of central memory T (T_{CM}) cells and not present on the surface of another memory T cell sub-population.

[0027] In some examples, the marker present on naïve T cells includes CD45RA. In some examples, the surface marker present on central memory T (T_{CM}) cells and not present on another memory T cell sub-population includes CD62L, CCR7, CD27, CD127, and/or CD44.

[0028] In some examples, the isolation or selections are carried out in the same separation vessel. In some examples the isolation comprises (i) subjecting the sample to a first selection, thereby generating one of the $CD4^+$ and $CD8^+$ primary human T cell populations and a non-selected sample; and (ii) subjecting the non-selected sample to a second selection, thereby generating the other of the $CD4^+$ and $CD8^+$ primary human T cell populations. In some examples, the $CD4^+$ primary human T cell population is generated in the first selection and the $CD8^+$ primary human T cell

population is generated in the second selection. In some examples, the first and/or second selection comprises a plurality of positive or negative selection steps.

[0029] In some examples, the isolation of one or more of the populations, such as the primary human T cell population, the primary human CD4+ T cell population, or the primary human CD8+ T cell population, includes positive selection based on surface expression CD62L, CCR7, CD44, or CD27. In some examples, the isolation of one or more of the populations, such as the primary human T cell population, the primary human $CD4^+$ T cell population, or the primary human $CD8^+$ T cell population, includes negative selection based on surface expression of CD45RA or positive selection based on surface expression of CD45RO.

[0030] In some examples, the various isolations, such as isolation of the plurality of cell populations, e.g., the $CD4^+$ and the $CD8^+$ populations, are carried out in the same separation vessel. In some examples, the separation vessel is or includes a tube, tubing set, chamber, unit, well, culture vessel, bag, and/or column. In some aspects, the separation vessel maintains the cells in a contained or sterile environment during the separation.

[0031] In some examples, the incubating is carried out under stimulating conditions. In some examples, the culture-initiating composition comprises the $CD4^+$ and $CD8^+$ primary human T cell populations at a culture-initiating ratio. In some examples, the culture-initiating ratio is designed to yield a desired output ratio of $CD4^+$ to $CD8^+$ cells (or desired total number of T cells or number(s) of the sub-population(s)) following said incubation, or at some later time, such as following engineering, cryopreservation, or at the time just prior to administration, such as at thaw at bedside.

[0032] In some examples, the desired output ratio is between at or about 5:1 and at or about 1:5 (or greater than about 1:5 and less than about 5:1), such as between at or about 1:3 and at or about 3:1 (or greater than about 1:3 and less than about 3:1), such as between at or about 2:1 and at or about 1:5 (or greater than about 1:5 and less than about 2:1), or is the range of between at or about 2:1 and at or about 1:5. In some examples, the desired output ratio is at or about 3:1, 2.9:1, 2.8:1, 2.7:1, 2.6:1, 2.5:1, 2.4:1, 2.3:1, 2.2:1, 2.1:1, 2:1, 1.9:1, 1.8:1, 1.7:1, 1.6:1, 1.5:1, 1.4:1, 1.3:1,

1.2:1, 1.1:1, 1:1, 1:1.1, 1:1.2, 1:1.3, 1:1.4, 1:1.5, 1:1.6, 1:1.7, 1:1.8, 1:1.9: 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, or 1:5.

[0033] In some examples, the method results in a ratio of two different cell types or populations, such as a ratio of $CD4^+$ to $CD8^+$ cells, in the output composition that is between at or about 5:1 and at or about 1:5 (or greater than about 1:5 and less than about 5:1), such as between at or about 1:3 and at or about 3:1 (or greater than about 1:3 and less than about 3:1), such as between at or about 2:1 and at or about 1:5 (or greater than about 1:5 and less than about 2:1), or that is at or about 3:1, 2.9:1, 2.8:1, 2.7:1, 2.6:1, 2.5:1, 2.4:1, 2.3:1, 2.2:1, 2.1:1, 2:1, 1.9:1, 1.8:1, 1.7:1, 1.6:1, 1.5:1, 1.4:1, 1.3:1, 1.2:1, 1.1:1, 1:1, 1:1.1, 1:1.2, 1:1.3, 1:1.4, 1:1.5, 1:1.6, 1:1.7, 1:1.8, 1:1.9: 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, or 1:5.

[0034] In some examples, the desired output ratio is 1:1 or is about 1:1.

[0035] In some examples, the methods result in the desired output ratio or cell number(s) in the output composition, results in a ratio or number in the output composition that is within a certain tolerated difference or range of error of such a desired output ratio or number, and/or results in such a ratio or number a certain percentage of the time that the method is performed, such as at least at or about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more than 95% of the time.

[0036] In some examples, the tolerated difference is within about 1%, about 2%, about 3%, about 4% about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50% of the desired ratio. In some examples, the output ratio is within 20 % of the desired ratio and/or is within that ratio at least 80 % of the time the methods are performed.

[0037] In some examples, the tolerated difference and/or the desired output ratio or number(s) is or has been determined by administering different cell types, such as administering $CD4^+$ and $CD8^+$ cells, to one or more subjects at a plurality of test ratios or numbers, and assessing one or more parameters. In some examples, the determination of the desired output ratio or number or tolerated difference includes assessing one or more outcomes following administration to the subject. In some examples, the outcomes include those selected from among amelioration of a disease symptom and outcomes indicating safety and/or low or absence of toxicity.

[0038] In some examples, the culture-initiation ratio is selected based on a proliferation rate or survival capacity of various cell types isolated, such as the $CD4^+$ and/or $CD8^+$ primary human T cell population. In some examples, the culture-initiating ratio is selected based on a source of the sample, such as the subject from which the sample has been derived. For example, in some examples, the sample is derived from a subject and the culture-initiating ratio is selected based on a disease or condition affecting said subject and/or a treatment the subject is receiving, has received, or will receive, such as a co-treatment for administration with the adoptive cell therapy. In some examples, the culture-initiating ratio is selected based on a phenotype of one or more of the cells or sub-types of cells being isolated or cultured, such as a phenotype of the CD4+ and/or CD8+ cell populations, such as expression of a cell surface marker or synthesis or secretion of one or more factors, such as cytokines or chemokines.

[0039] In some examples, the methods comprise selecting the culture-initiation ratio prior to the incubation step. In some examples, the selection is carried out by measuring a proliferation rate or survival capacity of one or more of the isolated cell populations or populations of cells being incubated, such as the $CD4^+$ primary human T cells and/or the isolated $CD8^+$ primary human T cells. In some examples, the selection is carried out by assessing a phenotype of the isolated $CD4^+$ primary human T cells and/or the isolated $CD8^+$ primary human T cells. In some examples, the phenotype selected from among expression of a surface marker and secretion of a cytokine or other factor. In some examples, the selecting is carried out by assessing the source of the sample, such as where the culture-initiation ratio is selected based on a disease or condition affecting a subject from which the sample is derived.

[0040] In some examples, the methods further include determining an intermediate ratio or number of cells, such as an intermediate ratio of $CD4^+$ to $CD8^+$ cells, present in the culture vessel at a time point subsequent to the initiation of said incubation. In some examples, the methods further include adjusting one or more parameters and/or or increasing or decreasing the time for carrying out the incubation and/or engineering steps, based on said intermediate ratio. In one examples, the adjusting comprises increasing or decreasing the number of or enriching for one or more cell populations in the culture vessel, such as increasing or enriching for $CD4^+$ or $CD8^+$ cells in the

culture vessel, adjusting temperature, adding a stimulant to the culture vessel, adjusting the concentration of one or more stimulants in the culture vessel, and/or adding and/or removing a sub-population of cells to or from the culture vessel. In some examples, the determining and/or adjusting are carried out while maintaining the composition being incubated within a sterile or contained environment. In some examples, the determination and/or adjusting are carried out in an automated fashion, such as controlled by a computer attached to a device in which the steps are performed.

[0041] In some examples, the isolating, incubating, and/or engineering steps are carried out in a sterile or contained environment and/or in an automated fashion, such as controlled by a computer attached to a device in which the steps are performed.

[0042] In some examples, the $CD8^+$ population in the culture-initiating composition comprises at least 50 % central memory T (T_{CM}) cells or comprises less than 20 % naïve T (T_N) cells.

[0043] In some examples, the sample is obtained from a subject. In some examples, the subject is a subject to whom said genetically engineered cells, e.g., T cells, or cells for adoptive cell therapy will be administered or subject in need of such administration. In other examples, the subject is a subject other than a subject to whom said genetically engineered cells, e.g., T cells, or cells for adoptive therapy will be administered or is a subject not in need of such therapy. Among the samples are blood and blood-derived samples, such as white blood cell samples, apheresis samples, leukapheresis samples, peripheral blood mononuclear cell (PBMC) samples, and whole blood.

[0044] In some examples, the stimulating conditions for the incubation or engineering include conditions whereby T cells of the culture-initiating composition proliferate or expand. For example, in some examples, the incubation is carried out in the presence of an agent capable of activating one or more intracellular signaling domains of one or more components of a TCR complex, such as a CD3 zeta chain, or capable of activating signaling through such a complex or component. In some examples, the incubation is carried out in the presence of an anti-CD3 antibody, and anti-CD28 antibody, anti-4-1BB antibody, for example, such antibodies coupled to or present on the surface of a solid support, such as a bead, and/or a cytokine, such as IL-2, IL-15, IL-7, and/or IL-21.

[0045] In some examples, the genetically engineered antigen receptor is or includes a T cell receptor (TCR), such as a high-affinity TCR, or functional non-TCR antigen receptor, such as a

chimeric antigen receptor (CAR). In some examples, the receptor specifically binds to an antigen expressed by cells of a disease or condition to be treated. In some examples, the CAR contains an extracellular antigen-recognition domain. In some examples, it further contains an intracellular signaling domain comprising an ITAM-containing sequence and an intracellular signaling domain of a T cell costimulatory molecule.

[0046] Also provided are cells and compositions, including pharmaceutical compositions, produced by any of the methods or embodiments, including genetically engineered cells and cells for adoptive cell therapy. Also provided are methods for administering such cells and compositions to subjects and uses of the cells and compositions in such methods. For example, provided are methods of treatment carried out by producing cells according to the cell production methods and administering cells of the output composition or a composition derived therefrom to a subject. Provided are methods of treatment including administering the provided cells or compositions to a subject. In some examples, the sample from which the cells are isolated is derived from the subject to which the cells are administered. In some examples, the sample is from a different subject. Thus, the methods include autologous and allogeneic methods. In some examples, the methods ameliorate, treat, or prevent one or more symptoms of a disease or condition in the subject. In some examples, the disease or condition is a cancer or associated symptom. In some examples, the cancers include leukemia, lymphoma, e.g., chronic lymphocytic leukemia (CLL), ALL, non-Hodgkin's lymphoma, acute myeloid leukemia, multiple myeloma, refractory follicular lymphoma, mantle cell lymphoma, indolent B cell lymphoma, B cell malignancies, cancers of the colon, lung, liver, breast, prostate, ovarian, skin (including melanoma), bone, and brain cancer, ovarian cancer, epithelial cancers, renal cell carcinoma, pancreatic adenocarcinoma, Hodgkin lymphoma, cervical carcinoma, colorectal cancer, glioblastoma, neuroblastoma, Ewing sarcoma, medulloblastoma, osteosarcoma, synovial sarcoma, and/or mesothelioma.

Brief Description of the Drawings

[0047] **Figure 1A**: provides a schematic representation of an embodiment of a closed system for use in embodiments of the provided methods. The depicted exemplary system includes a

CLAIMS

1. A method for enriching CD4+ or CD8+ T cells, the method comprising:

(a) performing a first selection in a closed system, said first selection comprising enriching for one of (i) CD4+ cells and (ii) CD8+ cells from a sample containing primary human T cells, the enrichment thereby generating a first selected population and a non-selected population; and

(b) performing a second selection in the closed system, said second selection comprising enriching for the other of (i) CD4+ cells and (ii) CD8+ cells from the non-selected population, the enrichment thereby generating a second selected population.

2. The method of claim 1, further comprising introducing a genetically engineered antigen receptor into cells of the first selected population and/or introducing a genetically engineered antigen receptor into cells of the second selected population.

3. The method of any of claims 1-2, further comprising incubating cells of the first selected population under stimulating conditions and/or incubating cells of the second selected population under stimulating conditions.

4. The method of claim 1, further comprising:

introducing a genetically engineered antigen receptor into cells of the first selected population and/or introducing a genetically engineered antigen receptor into cells of the second selected population; and

incubating cells of the first selected population under stimulating conditions and/or incubating cells of the second selected population under stimulating conditions,

wherein the incubation of cells of the first selected population and/or of cells of the second selected population is performed prior to, during and/or subsequent to introducing the genetically engineered antigen receptor.

5. The method of any of claims 1-4, further comprising (c) combining cells of the first selected population and cells of the second selected population, at a desired ratio of CD4+ cells to CD8+ cells.

6. The method of claim 5, wherein said combining is performed in the closed system.

7. The method of claim 5 or claim 6, wherein said desired ratio is between at or about 5:1 and at or about 1:5.

8. The method of any of claims 5-7, wherein the method thereby generates an output composition comprising the CD4+ and CD8+ T cells at the desired ratio, which is an output ratio.

9. A kit produced by the method of any of claims 1-7, comprising cells of the first selected population and cells of the second selected population at the desired CD4:CD8 ratio.

10. The kit of claim 9, further comprising instructions to administer cells in the kit to a subject, wherein the administration comprises administering the cells at the desired CD4+ to CD8+ ratio and/or within a tolerated error of said desired ratio.

11. The method or kit of any claims 5-10, wherein the desired CD4+ to CD8+ ratio is between 5:1 and 1:5, or between at or about 2:1 and at or about 1:2 or between 2:1 and 1:2.

12. The method or kit of claim 11, wherein the desired ratio is 1:1 or is about 1:1.

13. A method for producing genetically engineered T cells, the method comprising:

(a) performing a first selection in a closed system, said first selection comprising enriching for one of (i) $CD4^+$ cells and (ii) CD8+ cells from a sample containing primary human T cells, the enrichment thereby generating a first selected population and a non-selected population; and

(b) performing a second selection in the closed system, said second selection comprising enriching for the other of (i) CD4+ cells and (ii) CD8+ cells from the non-selected population, the enrichment thereby generating a second selected population;

(c) incubating a culture-initiating composition, which comprises cells of the first selected population and cells of the second selected population, in a culture vessel under stimulating conditions, thereby generating stimulated cells; and

(d) introducing a genetically engineered antigen receptor into stimulated cells generated in (c),

wherein the method thereby generates an output composition comprising $CD4^+$ T cells and $CD8^+$ T cells expressing the genetically engineered antigen receptor.

14. The method of claim 13, further comprising, prior to step (c), combining cells of the first and second selected cell populations to produce the culture-initiating composition and/or wherein the CD4+ and CD8+ cells in the culture-initiating composition are present at a culture-initiating ratio of CD4+ cells to CD8+ cells.

15. The method of claim 14, wherein said combining is performed in the closed system.

16. The method of any of claims 13-15, wherein the culture-initiating ratio of $CD4^+$ to $CD8^+$ cells is between at or about 10:1 and at or about 1:10, between at or about 5:1 and at or about 1:5, or between at or about 2:1 and at or about 1:2.

17. The method of any of claims 13-16, wherein the culture-initiating ratio of CD4+ to CD8+ cells is at or about 1:1.

18. The method of any of claims 5-15, wherein the sample is obtained from a human subject and:

the culture-initiating ratio or desired ratio of $CD4^+$ to $CD8^+$ cells is different than the ratio of $CD4^+$ to $CD8^+$ cells in the sample from the subject; and/or

the culture-initiating ratio or desired ratio of $CD4^+$ to $CD8^+$ cells is at least 10 %, at least 20 %, at least 30 %, at least 40 %, or at least 50 % greater or less than the ratio of $CD4^+$ to $CD8^+$ cells in the sample from the subject.

19. The method of any of claims 1-18, wherein the enriching cells in the first and/or second selection comprises immunoaffinity-based selection.

20. The method of claim 19, wherein the first and/or second immununoaffinity-based selection comprises:

contacting cells with an antibody capable of specifically binding to a cell surface marker, wherein the antibody is immobilized on a magnetic particle; and

recovering cells bound to the antibody, thereby effecting positive selection, or recovering cells not bound to the antibody, thereby effecting negative selection, wherein the recovered cells are enriched for the CD4+ cells or the CD8+ cells.

21. The method of any of claims 1-20, wherein the first selection and second selection are carried out in separate separation vessels, which are operably connected.

22. The method of any of claims 19-21, wherein the immunoaffinity-based selection in the first and/or second selection comprises: contacting cells with an antibody immobilized on or attached to an affinity chromatography matrix, said antibody capable of specifically binding to a cell surface marker to effect positive or negative selection of CD4+ or CD8+ cells; and

recovering cells from the affinity chromatography matrix enriched for the CD4+ cells or the CD8+ cells.

23. The method of claim 22, wherein:

the antibody comprises one or more binding partners capable of forming a reversible bond with a binding reagent immobilized on the affinity chromatography matrix, whereby the antibody is reversibly bound to said matrix during said contacting; and

cells expressing a cell surface marker specifically bound by the antibody on said affinity chromatography matrix are capable of being recovered from the affinity chromatography matrix by disrupting the reversible binding between the binding reagent and binding partner.

24. The method of any of claim 22 or 23, wherein the affinity chromatography matrix of the first and/or second selection is packed in a separation vessel, which is a column.

25. The method of any of claim 22-24, wherein the affinity chromatography matrix adsorbs and/or is capable of selecting at least or at least about 50 x 10^6 cells/mL, 100 x 10^6 cells/mL, 200 x 10^6 cells/mL or 400 x 10^6 cells/mL.

26. The method of any of claims 1-25, wherein the enriching for the CD4+ cells comprises positive selection based on surface expression of CD4 and/or the enriching for the CD8+ cells comprises positive selection based on surface expression of CD8.

27. The method of any of claims 1-26, wherein the one of the first and second selection that comprises enriching for the CD8+ cells further comprises enriching for central memory T (T_{CM}) cells and/or enriching for cells expressing a marker selected from among CD28, CD62L, CCR7, CD127 and CD27.

28. A method for enriching CD4+ and CD8+ T cells, the method comprising contacting cells of a sample containing primary human T cells with a first immunoaffinity reagent that specifically binds to CD4 and a second immunoaffinity reagent that specifically binds to CD8 in an incubation composition, under conditions whereby the immunoaffinity reagents specifically bind to CD4 and CD8 molecules, respectively, on the surface of cells in the sample; and

recovering cells bound to the first and/or the second immunoaffinity reagent, thereby generating an enriched composition comprising CD4+ cells and CD8+ cells at a desired ratio, wherein:

the first and/or second immunoaffinity reagent are present in the incubation composition at a sub-optimal yield concentration, whereby the enriched composition contains less than 70% of the total CD4+ cells in the incubation composition and/or less than 70% of the CD8+ cells in the incubation composition.

29. A method for producing genetically engineered T cells, the method comprising:

(a) enriching a sample for primary human T cells , the enriching comprising:

contacting cells of said sample with a first immunoaffinity reagent that specifically binds to CD4 and a second immunoaffinity reagent that specifically binds to CD8 in an incubation composition, under conditions whereby the immunoaffinity reagents specifically bind to CD4 and CD8 molecules, respectively, on the surface of cells in the sample; and

recovering cells bound to the first and/or the second immunoaffinity reagent, thereby generating an enriched composition comprising CD4+ cells and CD8+ cells at a culture-initiating ratio, wherein:

the first and/or second immunoaffinity reagent are present in the incubation composition at a sub-optimal yield concentration, whereby the enriched composition contains less than 70% of the total CD4+ cells in the incubation composition and/or less than 70% of the CD8+ cells in the incubation composition; and

(b) incubating cells of the enriched composition at or substantially at the culture-initiation ratio in a culture-initiation composition in a culture vessel under stimulating conditions; and

(c) introducing a genetically engineered antigen receptor into cells stimulated in (b), thereby generating an output composition comprising $CD4^+$ T cells and $CD8^+$ T cells expressing the genetically engineered antigen receptor.

30. The method of claim 28 or claim 29, wherein the enriching in (a) or the contacting and/or the recovering is performed in a closed system.

31. The method of claim any of claims 28-30, wherein the sample comprises at least 1 x 10^9 CD3+ T cells.

32. The method of any of claims 28-31, wherein the concentration of one of the first and second immunoaffinity reagent in the incubation composition is greater than that of the other, whereby the greater concentration effects a higher yield in the enriched composition of CD4+ cells or CD8+ cells, respectively, compared to the yield of the other of the CD4+ or CD8+ cells, thereby producing the culture-initiating ratio in the enriched composition.

33. The method of any of claims 28-32, wherein the desired and/or culture-initiating ratio of $CD4^+$ to $CD8^+$ cells is between at or about 10:1 and at or about 1:10, between at or about 5:1 and at or about 1:5 or is between at or about 2:1 and at or about 1:2.

34. The method of any of claims 28-33, wherein the desired and/or culture-initiating ratio of CD4+ to CD8+ cells is at or about 1:1.

35. The method of any of claims 28-34, wherein:

the desired and/or culture-initiating ratio of $CD4^+$ to $CD8^+$ cells is different than the ratio of $CD4^+$ to $CD8^+$ cells in the sample from the subject; and/or

the desired and/or culture-initiating ratio of $CD4^+$ to $CD8^+$ cells is at least 10 %, at least 20 %, at least 30 %, at least 40 %, or at least 50 % greater or less than the ratio of $CD4^+$ to $CD8^+$ cells in the sample from the subject.

36. The method of any of claims 28-35, wherein each of the immunoaffinity reagents comprises an antibody.

37. The method of claim 36, wherein the antibody is immobilized on the outside surface of a particle or bead.

38. The method of claim 37, wherein the bead is a magnetic bead.

39. The method of claim 37 or claim 38, wherein:

the antibody comprises one or more binding partners capable of forming a reversible bond with a binding reagent immobilized on the surface, whereby the antibody is reversibly immobilized to said surface; and

the method comprises after contacting cells in the sample to the first and second immunoaffinity reagent, applying a competition reagent to disrupt the bond between the binding partner and binding reagent, thereby recovering the selected cells bound to the antibody.

40. The method of claim 23 or 39, wherein:

the binding partner is selected from among biotin, a biotin analog, or a peptide capable of binding to the binding reagent; and

the binding reagent is selected from among streptavidin, a streptavidin analog or mutein that binds biotin and/or a streptavidin binding peptide, avidin, and an avidin analog or mutein that binds biotin and/or a streptavidin binding peptide.

41. The method of claim 40, wherein:

the binding partner comprises a peptide comprising the sequence of amino acids set forth in SEQ ID NO:6-10 and/or

the binding reagent is streptavidin mutein comprising the sequence of amino acids set forth in SEQ ID NO: 12, 13, 15 or 16.

42. The method of any of claims 39-41, wherein the competition reagent is biotin or a biotin analog.

43. The method of any of claims 2-8 and 14-32, comprising:

prior to performing the first selection and/or second selection, determining the ratio of CD4+ to CD8+ T cells in the sample; and

based on the ratio of CD4+ to CD8+ T cells in the sample, adjusting the first and/or second selection to produce the composition comprising CD4+ cells and CD8+ cells at the desired or culture-initiating ratio.

44. The method of claim 43, wherein:

the first and/or second selection comprises immunoaffinity-based selection comprising an affinity chromatography matrix; and

adjusting the first and/or second selection comprises choosing the amount of affinity chromatography matrix in the first and/or second selection sufficient to achieve the desired or culture-initiation ratio.

45. The method of any of claims 29-42, comprising:

prior to contacting cells of the sample with the first and second immunoaffinity reagent, determining the ratio of CD4+ to CD8+ T cells in the sample; and

based on the ratio of CD4+ to CD8+ T cells in the sample, choosing the concentration of the first and/or second immunoaffinity reagent to produce the enriched composition comprising CD4+ cells and CD8+ cells at the culture-initiating ratio.

46. The method of claim 13, wherein the method results in an output composition or kit comprising a ratio of $CD4^+$ to $CD8^+$ cells that is between at or about 2:1 and at or about 1:5.

47. The method of claim 46, wherein the ratio of $CD4^+$ to $CD8^+$ cells in the output composition or kit is 1:1 or is about 1:1.

48. The method of any of claims 1-47, wherein the sample is obtained from a subject.

49. The method of any of claims 1, wherein the sample is an apheresis, peripheral blood mononuclear cell (PBMC), or leukapheresis sample.

50. The method of any of claims 13-27 or 29-49, wherein incubating the composition in a culture vessel under stimulating conditions is performed prior to, during and/or subsequent to introducing a genetically engineered antigen receptor.

51. The method of any of claims 13-27 or 29-50, wherein: the stimulating condition comprises an agent capable of activating one or more intracellular signaling domains of one or more components of a TCR complex; and/or comprises the presence of an anti-CD3 antibody, and anti-CD28 antibody, anti-4-1BB antibody, and/or a cytokine.

52. The method of any of claims 13-27 or 29-51, wherein: the genetically engineered antigen receptor comprises a T cell receptor (TCR) or a functional non-TCR antigen receptor; or the genetically engineered antigen receptor comprises a chimeric antigen receptor (CAR).

53. A composition or kit comprising cells produced by the method of any of claims 1-52.

54. The composition or kit of any one of claims 9-12 and claim 53, further comprising a pharmaceutically acceptable carrier.

55. The composition or kit of any one of claims 9-12 and claim 53 or 54 for use in treating a disease or condition in a subject.

56. Use of a composition of claim 53 or 54 for the manufacture of a medicament for treating a disease or condition in a subject.

57. The composition of claim 55 or use of claim 56, wherein the genetically engineered antigen receptor specifically binds to an antigen associated with the disease or condition.

58. The composition or use of any of claims 55-57, wherein the disease or condition is a cancer.

59. A closed apparatus system for purification of target cells, comprising:

a) a first affinity chromatography matrix comprising a first binding agent immobilized thereon, which binding agent specifically binds to a first cell surface marker present on a first cell type, said first affinity matrix not comprising a second binding agent that specifically binds to a second cell surface marker, wherein:

the first affinity chromatography matrix is operably connected to a storage reservoir comprising a cell sample; and

is operably connected to an output vessel;

b) a second affinity chromatography matrix comprising a second binding agent immobilized thereon, which second binding agent specifically binds to a second cell surface marker present on a second cell type, wherein:

the first affinity chromatography matrix is operably connected to the second affinity chromatography matrix; and

the second affinity chromatography matrix is operably connected to the output vessel, and

is operably linked to a waste vessel;

c) the output vessel; and

d) the waste vessel,

wherein the system is configured to permit, within the closed system, collection of, in the output vessel in a single composition, (i) cells having bound and been recovered from the first affinity chromatography matrix and (ii) cells having passed through and not bound to the first affinity chromatography matrix and having bound and been recovered from the second chromatography matrix.

60. The closed apparatus system of claim 59, wherein:

the first affinity chromatography matrix is one of a CD4+ affinity chromatography matrix or a CD8+ affinity chromatography matrix; and

the second affinity chromatography matrix is the other of the CD4+ affinity chromatography matrix or the CD8+ affinity chromatography matrix.

61. The closed apparatus system of claim 59 or 60, further comprising:

e) a third affinity chromatography matrix comprising a third binding agent immobilized thereon, which binding agent specifically binds to a third cell surface marker, whereby the third binding agent is capable of binding cells expressing the third cell surface marker.

62. The closed apparatus system of any of claims 59-61,

wherein the third affinity chromatography matrix is operably connected to the first matrix, the output container and/or the waste container.

63. The closed apparatus system of claim 62, wherein:

the first affinity chromatography matrix comprises a binding agent that specifically binds CD8;

the second affinity chromatography matrix comprises a binding agent that specifically binds CD4; and

the third affinity chromatography matrix comprises a binding agent that specifically binds a marker expressed on central memory T (T_{CM}) cells.

64. The closed apparatus system of any of claims 59-63, wherein:

one or more or all of the affinity chromatography matrices are further operably connected to an elution buffer reservoir comprising one or more competition reagents; and/or the first, second and/or third affinity chromatography matrix is operably connected to a competition agent removal chamber.

65. The closed apparatus system of any of claims 59-64, wherein one or more or all of the binding agents is an antibody.

66. The closed apparatus system of claim 65, wherein the antibody is a Fab.

67. The closed apparatus system of any of claims 59-66, wherein the one or more or all of the binding agents is reversibly bound to the affinity chromatography matrix.