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Patent- og
Varemærkestyrelsen

DESCRIPTION

Description

Cross-Reference to Related Applications

[0001] This application claims priority from U.S. provisional application No. 61/983,415, filed April 23, 2014.

Sequence Listing

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled 735042000240seqlist.txt, created April 22, 2015, which is 13 kilobytes in size.

Field

[0003] The present disclosure relates in some aspects to methods, cells, and compositions for preparing cells, and compositions for genetic engineering and cell therapy. Provided in some embodiments are streamlined cell preparation methods, e.g., for isolation, processing, incubation, and genetic engineering of cells and populations of cells. Also provided are cells and compositions produced by the methods and methods of their use. The cells can include immune cells, such as T cells, and generally include a plurality of isolated T cell populations or T cell sub-types. In some aspects, the methods are capable of preparing of a plurality of different cell populations for adoptive therapy using fewer steps and/or resources and/or reduced handling compared with other methods.

Background

[0004] Various methods are available for preparing cells for therapeutic use. For example, methods are available for isolating, processing, and engineering cells, including T cells and other immune cells. Methods are available to isolate such cells and to express genetically engineered antigen receptors, such as high affinity T cell receptors (TCRs) and chimeric antigen receptors (CARs). Methods are available to adoptively transfer such cells into subjects. Improved methods are needed for the preparation (e.g., isolation, processing, culturing, and engineering) of cells for 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.

[0005] Stemberger C, Dreher S, Tschulik C, Piossek C, Bet J, Yamamoto TN, et al. (2012), PLoS ONE 7(4): e35798, describes how novel serial positive enrichment technology enables clinical multiparameter cell sorting.
WO 2009/072003 A2 describes a sample processing system and methods.
WO 2013/011011 A2 describes a method of reversibly staining a target cell.
WO 2007/117602 A2 describes isolation and use of human regulatory T cells.

<u>Summary</u>

[0006] The present invention is defined in the appended set of claims.

[0007] In particular the present invention provides a composition comprising a dose of genetically engineered CD4+ and CD8+ T cells for use in a method of treating an autoimmune or inflammatory disease in a subject, wherein:

1. (i) the CD4+ and CD8+ T cells are at a ratio between 5:1 and 1:5;
2. (ii) the dose of CD4+ and CD8+ T cells is between 1×10^6 to 1×10^{11} cells;
3. (iii) the CD4+ and CD8+ T cells are genetically engineered with a chimeric antigen receptor (CAR), wherein the CAR:
    1. (a) recognizes an antigen expressed by cells of the autoimmune or inflammatory disease, wherein the antigen is CD19;
    2. (b) comprises an extracellular antigen recognition domain and an intracellular signaling domain comprising an ITAM-containing sequence and an intracellular signaling domain of a T cell costimulatory molecule; and
4. (iv) the CD4+ and CD8+ T cells are from a sample obtained from the subject with the autoimmune or inflammatory disease.

[0008] In some embodiments, the CD4+ and CD8+ T cells are at a ratio between 1:3 and 3:1.

[0009] In some embodiments, the CD4+ and CD8+ T cells are at a ratio between 2:1 and 1:5.

[0010] In some embodiments, the dose of CD4+ and CD8+ T cells is between 1×10^6 to 1×10^9 cells.

[0011] In some embodiments, the dose of CD4+ and CD8+ T cells is between 1×10^6 to 25×10^6 cells.

[0012] In some embodiments, the dose of CD4+ and CD8+ T cells is 5×10^6 cells.

[0013] In some embodiments, the dose of CD4+ and CD8+ T cells is 10×10^6 cells.

[0014] In some embodiments, the dose of CD4+ and CD8+ T cells is 20×10^6 cells.

[0015] In some embodiments, the sample is a blood or a blood-derived sample, optionally wherein the sample is a white blood cell sample, an apheresis sample, a leukapheresis sample, a peripheral blood mononuclear cell sample and whole blood.

[0016] In some embodiments, the sample is derived from an apheresis or a leukapheresis product.

[0017] In some embodiments, the autoimmune or inflammatory disease is selected from the group consisting of arthritis, Type I diabetes, systemic lupus erythematosus (SLE), inflammatory bowel disease, psoriasis, scleroderma, autoimmune thyroid disease, Grave's disease, Crohn's disease, multiple sclerosis, asthma, and a disease or condition associated with transplant.

[0018] In some embodiments, the autoimmune or inflammatory disease is SLE.

[0019] In some embodiments, the CAR comprises a single-chain antibody fragment (scFV) that binds CD19.

[0020] In some embodiments, the intracellular cellular signaling domain is derived from CD3zeta.

[0021] In some embodiments, the CAR includes a signaling domain and/or transmembrane portion of a costimulatory receptor, optionally selected from CD28, 4-1BB, OX40, DAP10, and ICOS.

[0022] The cells include CD4+ and CD8+ T cells, as referred to in the claims. The cells are present in combinations of multiple cell populations or cell types, which are included in the compositions at particular ratios or numbers of the cells or cell types, as defined in the claims.

[0023] In some embodiments, 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.

[0024] The genetically engineered antigen receptor is or includes a chimeric antigen receptor (CAR). In some aspects, the receptor specifically binds to an antigen expressed by cells of a disease or condition to be treated, as defined in the claims. The CAR comprises an extracellular antigen-recognition domain. The CAR comprises an intracellular signaling domain

comprising an ITAM-containing sequence and an intracellular signaling domain of a T cell costimulatory molecule.

Brief Description of the Drawings

[0025]

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 cell sample 5, a washing buffer reservoir 6, an elution buffer reservoir 7, a first Fab reservoir 18, a second Fab reservoir 19, and a pump 8, connected through a series of tubing and valves 13, to a first chromatography column 1 containing a first matrix 3 operably linked via a series of tubing lines to a second chromatography column 2 containing a second matrix 4. The second chromatography column 2 is operably linked to a removal chamber 9. The removal chamber 9 is operably linked to a valve 13, which directs cells and fluids to a waste container 10 or a culture vessel 12 via a series of tubing lines. The system is enclosed in an enclosure 14.

Figure 1B: 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 cell sample 5, a washing buffer reservoir 6, an elution buffer reservoir 7, a first Fab reservoir 18, a second Fab reservoir 19, a third Fab reservoir 20, and a pump 8, connected through a series of tubing to a first chromatography column 1 containing a first matrix 3. Valves 13 operably linked to the series of tubing direct fluids through the series of tubing. A valve 13 operably linked to the first chromatography column 1 directs cells and fluids to a second chromatography column 2 containing a second matrix 4, which is operably linked to a removal chamber 9. The valve 13 operably linked to the first chromatography column 1 also directs cells and fluids to a removal chamber 9, which is operably linked to a third chromatography column 15 containing a third matrix 16. The third chromatography column 15 is operably linked to a removal chamber 9. The removal chambers 9 are operably linked to a first waste container 10 or a culture vessel 12 via the series of tubing lines and valves 13. The system is enclosed in an enclosure 14.

Detailed Description

[0026] Embodiments of the invention are described in some of the instances disclosed herein. Any references herein to methods of treatment are intended to refer to products for use in said methods.

[0027] Unless defined otherwise, all terms of art, notations and other technical and scientific terms or terminology used herein are intended to have the same meaning as is commonly

understood by one of ordinary skill in the art to which the claimed subject matter pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art.

[0028] If a definition set forth herein is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications, the definition set forth herein prevails over the definition that referenced.

[0029] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

I. Methods and systems for isolating, culturing, and engineering cells for adoptive therapy

[0030] Disclosed are methods of preparing cells, e.g., T cells, for use in genetic engineering and therapeutic methods such as adoptive cell therapy. Specifically, in some instances, the methods use or generate compositions that contain a plurality of different cell populations or types of cells, such as isolated CD4$^+$ and CD8$^+$ T cell populations and sub-populations. In some instances, the methods include steps for isolating one or more cell populations, generally a plurality of cell populations. The cells generally are isolated from a sample derived from a subject.

[0031] In some instances, the methods are performed by employing simultaneous or sequential selections or enrichments in which a plurality of different cell populations, such as CD4+ or CD8+ cells, from a sample, such as a sample containing primary human T cells, are selected, enriched and/or isolated. In some instances, such methods are performed using a single processing stream in which a first population of cells, such as CD4+ or CD8+ cells, and a second population of cells, such as the other of the CD4+ or CD8+ cells, are selected, enriched and/or isolated without discarded any cells from the first population prior to performing the selection of the second population of cells. In some instances, the first and second selections can be performed simultaneously or sequentially. In some instances, the methods of selection are performed as a single process stream by performing a first selection to enrich for a first population of cells, such as one of CD4+ and CD8+ cells from a sample, such as a sample containing primary human T cells, and using the non-selected cells from the first selection as the source of cells for a second selection, such as for the other of the CD4+ or CD8+ cells from the sample.

[0032] In some instances, a further selection or selections can be performed of the first or second selected cells. In some instances, the further selection enriches for a sub-population of CD4+ or CD8+ cells expressing a marker on central memory T (T$_{CM}$) cells and/or enriches for a sub-population of cells expressing CD62L, CD45RA, CD45RO, CCR7, CD27, CD127, or

CD44.

[0033] In some instances, the methods of selection are performed in a closed system or apparatus. In some instances, within the closed system or apparatus, a composition, such as a culture-initiation composition, is generated containing the enriched or selected population of cells, such as both the enriched or selected CD4+ and CD8+ populations, in the same composition.

[0034] For example, in some instances, the one or more steps are carried out with the plurality of cell populations combined in the same composition, or present in the same vessel, e.g., in the same closed system or apparatus, or in the same vessel, unit, or chamber, such as the same column, e.g., magnetic separation column, tube, tubing set, culture or cultivation chamber, culture vessel, processing unit, cell separation vessel, centrifugation chamber, or using the same separation matrix, media, and/or reagents, such as the same magnetic or magnetically responsive matrix, particle, or bead, the same solid support, e.g., affinity-labeled solid support, and/or the same antibodies and/or other binding partners, such as fluorescently-labeled antibodies and binding partners, for the plurality of cell populations. In some instances, the one or more vessels, units or chambers, such as columns, are operably connected in the same closed system or apparatus, so that the methods of isolation, selection and/or enriching occurs in a single process stream in which a non-selected cell population from a first selection can be used as a source of cells for a second selection.

[0035] In some instances, the isolation of or enrichment for one or more specific populations or sub-populations of cells, e.g., CD4+ and CD8+ T cells to be cultured or engineered for adoptive therapy, provides one or more advantages. For example, engineering cells enriched for a plurality of different cell populations or types of cells, such as isolated $CD4^+$ and $CD8^+$ T cell populations and sub-populations, can improve efficacy of or reduce or avoid unwanted effects. In some instances, the isolation or enrichment increases the ability of cells ultimately administered to a subject to persist, expand, become activated, and/or engraft *in vivo* or upon administration to a subject. In some instances, it improves or increases one or more effector function or activation phenotype. For example, in some instances, enriching a T cell population, such as a CD8+ T cell population, for central memory (T_{CM}) cells can provide such advantages. In some instances, one or more of such advantages are disclosed by combining two or more isolated populations or sub-types, such as isolated populations or sub-populations of CD8+ and CD4+ T cells, such as a T_{CM}-enriched $CD8^+$ population and a $CD4^+$ population. For example, such advantages can be achieved in some instances by administering a CD4+ and a CD8+ population in comparison with a CD8+ population alone.

[0036] The methods in some instances include processing of a generated composition containing a plurality of the isolated or selected cell populations, such as a population of selected or enriched CD4+ cells and CD8+ cells. In one instance, processing of the generated composition includes incubating the cells under stimulating conditions, for example in some instances, to activate the cells for engineering or transduction or for cell expansion. The

methods, in some instances, include steps for engineering a plurality of cell types, such as CD4+ cells and CD8+ cells, such as those isolated and present in the incubated composition, such as the culture-initiation composition. In some instances, the engineering is carried out to introduce a genetically engineered antigen receptor into the cells, such as a TCR, e.g., a high-affinity TCR, or a functional non-TCR antigen receptor, such as a chimeric antigen receptor (CAR). In some instances, the methods include further processing, such as further incubation, for example at or about 37° C ± 2° C, and/or formulating of cells and compositions containing the same. In some instances, the processing produces a resulting output composition containing genetically engineered cells, such as genetically engineered CD4+ cells and CD8+ cells. In some instances, the resulting processed output composition can be used in methods for administering cells and compositions prepared by the methods to a patient, for example, in connection with adoptive cell therapy.

[0037] In certain instances, the isolation or separation is carried out using a system, device, or apparatus that carries out one or more of the isolation, cell preparation, separation, processing, incubation, culture, and/or formulation steps of the methods. In some instances, the system is used to carry out each of these steps in a closed or sterile environment, for example, to minimize error, user handling and/or contamination. In one example, the system is a system as described in International Patent Application, Publication Number WO2009/072003, or US 20110003380 A1. In some instances, the system is a closed apparatus or system, such as is depicted in Figure 1A or Figure 1B. In some instances, the system or apparatus is automated and/or carries out the selection steps to enrich cells according to the methods in an automated fashion.

[0038] In some instances, the system or apparatus carries out the isolation, such as selection or enrichments steps. In some instances, a further system or apparatus, such as a closed system or apparatus, can be used to carry out one or more of the other steps such as cells preparation, processing, incubation, culture and/or formulation steps of the method. In some instances, the system or apparatus carries out one or more, e.g., all, of the isolation, processing, engineering, and formulation steps in an integrated or self-contained system, and/or in an automated or programmable fashion. In some instances, the system or apparatus includes a computer and/or computer program in communication with the system or apparatus, which allows a user to program, control, assess the outcome of, and/or adjust various instances of the processing, isolation, engineering, and formulation steps.

[0039] In some instances, because the enriched composition, such as culture initiation composition, is generated by the disclosed methods to contain different cell populations, such as CD4+ and CD8+, the generated composition can be processed together and simultaneously under the same conditions, and in some instances, in a closed system. Thus, in some instances, the methods provide a process to produce and process a population of selected, enriched or isolated cells containing different populations of cells, such as CD4+ and CD8+ cells, in a single process stream. In some instances, this differs from prior art methods, including prior art methods that process cells for engineering for adoptive cell therapy, which typically include a separate process stream for each different cell population, such as at least

two process streams. For example, in some instances of existing methods, CD4+ T cells are separately isolated, enriched and/or selected and processed under stimulating conditions for genetic engineering, CD8+ T cells are separately isolated, enriched and/or selected and processed under stimulating conditions for genetic engineering, and the separate processed and engineered CD4+ and CD8+ T cells are re-combined prior to administration to a subject.

[0040] In some instances, the methods provide one or more advantages compared with other preparation, isolation, incubation, and engineering methods, such as cost, time, and/or resource savings. Such advantages can include the ability to isolate, process, e.g., incubate, and/or engineer the plurality of cell populations, present at or near a desired ratio, with increased efficiency and/or reduced complexity, time, cost, and/or use of resources, compared with other methods.

[0041] In some instances, such advantages are achieved by streamlining one or more of the method steps. For example, in some instances, isolation, culture, and/or engineering of the different populations is carried out using the same apparatus or equipment and/or simultaneously. In some instances, the isolation, culture, and/or engineering of the different populations is carried out based on the same starting composition. In some instances, such features of the methods reduce the amount of time, method steps, cost, complexity, and/or number of resources, compared to a method in which the cell populations are isolated, incubated, and/or engineered separately, in separate vessels, using separate equipment, at separate times, and/or beginning with different starting compositions.

[0042] In some instances, the methods of isolating, incubating and engineering cells results in an output composition in which the different cell populations or cell types, such as CD4+ cells and CD8+ cells, are present at a desired ratio, or within a certain degree of tolerated error of the desired ratio. Such ratios include those deemed optimal for the therapeutic use, e.g., output ratios deemed appropriate or optimal for administration to a patient in connection with adoptive cell therapy. Also disclosed are methods for administering cells and compositions prepared by the methods to a patient, for example, in connection with adoptive cell therapy

[0043] In some instances, the methods of isolation or selection are performed to achieve selection of cells at a chosen culture-initiation ratio of CD4+ cells to CD8+ cells or a sub-population thereof. In some instances, such ratios include ratios deemed optimal starting points for achieving an optimal ratio, such as a desired ratio in the output composition, at the completion of the method or one or more steps, such as following a culture, incubation, and/or engineering step. In some instances, providing the cells at a culture-initiation ratio, such as a ratio of CD4+ cells to CD8+ cells, accounts for differences in expansion of CD4+ and CD8+ T cells that can occur upon stimulation or activation with different reagents, in order to achieve a desired ratio in the output composition.

[0044] In some instances, the methods generate engineered cells or cells for engineering at or within a certain percentage of a desired output ratio, or do so at least a certain percentage of the time. The desired output ratio typically is a ratio that has been determined to be optimal for

administration to a patient via adoptive transfer. In some instances, the populations or sub-types of cells, such as CD8$^+$ and CD4$^+$ T cells are administered at or within a tolerated difference of a desired dose of total cells, such as a desired dose of T cells. In some instances, the desired dose is a desired number of cells or a desired number of cells per unit of body weight of the subject to whom the cells are administered, e.g., cells/kg. In some instances, the desired dose is at or above a minimum number of cells or minimum number of cells per unit of body weight. In some instances, among the total cells, administered at the desired dose, the individual populations or sub-types are present at or near a desired output ratio (such as CD4$^+$ to CD8$^+$ ratio), e.g., within a certain tolerated difference or error of such a ratio.

[0045] In some instances, the cells are administered at or within a tolerated difference of a desired dose of one or more of the individual populations or sub-types of cells, such as a desired dose of CD4+ cells and/or a desired dose of CD8+ cells. In some instances, the desired dose is a desired number of cells of the sub-type or population, or a desired number of such cells per unit of body weight of the subject to whom the cells are administered, e.g., cells/kg. In some instances, the desired dose is at or above a minimum number of cells of the population or sub-type, or minimum number of cells of the population or sub-type per unit of body weight.

[0046] Thus, in some instances, the dosage is based on a desired fixed dose of total cells and a desired ratio, and/or based on a desired fixed dose of one or more, e.g., each, of the individual sub-types or sub-populations. Thus, in some instances, the dosage is based on a desired fixed or minimum dose of T cells and a desired ratio of CD4$^+$ to CD8$^+$ cells, and/or is based on a desired fixed or minimum dose of CD4$^+$ and/or CD8$^+$ cells.

[0047] In some instances, disclosed are methods for determining such optimal output ratios and/or desired doses, and/or levels of variance from the desired output ratio or desired dose that would be tolerated, e.g., a tolerated difference. In some instances, in order to achieve the desired output ratio or dose(s) (or to achieve such a ratio a certain percentage of the time or within a certain tolerated difference), the cell populations are combined or incubated at ratios (e.g., culture-initiation ratios) designed to achieve the desired output ratio for adoptive transfer.

[0048] Also disclosed are methods for determining a culture-initiation ratio designed to achieve the desired output ratio or dose to do so within a certain tolerated difference and/or a certain percentage of the time. Also disclosed are methods for assessing interim ratios or numbers of the cell populations, such as at one or more periods of times over the course of the various method steps, e.g., during incubation. Also disclosed are methods for adjusting various conditions, such as culture conditions, based on such assessments. In some instances, the adjustments are carried out to ensure that a particular output ratio or dose is achieved or achieved within a tolerated difference.

[0049] In particular instances, disclosed are streamlined methods for preparing a composition having at or near a desired output ratio of a CD4$^+$ T cell population and a CD8$^+$ T cell

population (e.g., a CD8$^+$ population enriched for a sub-type of T cells such as central memory T cells), and/or a desired dose (e.g., number or number per unit of body weight) of T cells and/or of CD4$^+$ and CD8$^+$ T cells, for introduction of a genetically engineered antigen receptor for use in adoptive cell therapy, where the cell populations are isolated, incubated, and/or engineered in combination and the method is associated with increased efficiency and/or reduced complexity, time, cost, and/or use of resources compared to a method in which the populations are isolated, incubated, and/or engineered separately.

[0050] Also disclosed are cells and compositions prepared by the methods, including pharmaceutical compositions and formulations, and kits, systems, and devices for carrying out the methods. Also disclosed are methods for use of the cells and compositions prepared by the methods, including therapeutic methods, such as methods for adoptive cell therapy, and pharmaceutical compositions for administration to subjects.

A. Isolation, isolated cells, and other processing steps

[0051] Among the disclosed instances are methods for isolating a plurality of cells and populations of cells from a sample, as well as isolated, such as enriched, cells produced by such methods. The isolation can include one or more of various cell preparation and separation steps, including separation based on one or more properties, such as size, density, sensitivity or resistance to particular reagents, and/or affinity, e.g., immunoaffinity, to antibodies or other binding partners. In some instances, the isolation is carried out using the same apparatus or equipment sequentially in a single process stream and/or simultaneously. In some instances, 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.

[0052] In some instances, the plurality of cell populations are isolated in the same closed system or apparatus, and/or in the same vessel or set of vessels, e.g., same (or same set of) unit, chamber, column, e.g., magnetic separation column, tube, tubing set, culture or cultivation chamber, culture vessel, processing unit, cell separation vessel, centrifugation chamber. For example, in some cases, the isolation of a plurality of cell populations is carried out a system or apparatus employing a single or the same isolation or separation vessel or set of vessels, such as a single column or set of columns, and/or same tube, or tubing set, for example, without requirements to transfer the cell population, composition, or suspension from one vessel, e.g., tubing set, to another.

[0053] In some instances, such methods are achieved by a single process stream, such as in a closed system, by employing simultaneous or sequential selections in which a plurality of different cell populations, such as CD4+ or CD8+ cells, from a sample, such as a sample containing primary human T cells, are selected, enriched and/or isolated. In one instance, a sample containing cells is subjected to a selection by simultaneous enrichment of both the CD4+ and CD8+ populations. In some instances, carrying out the separation or isolation in the

same vessel or set of vessels, e.g., tubing set, is achieved by carrying out sequential positive and negative selection steps, the subsequent step subjecting the negative and/or positive fraction from the previous step to further selection, where the entire process is carried out in the same tube or tubing set. In one instance, a sample containing cells to be selected is subjected to a sequential selection in which a first selection is effected to enrich for one of the CD4+ or CD8+ populations, and the non-selected cells from the first selection is used as the source of cells for a second selection to enrich for the other of the CD4+ or CD8+ populations. In some instances, a further selection or selections can be effected to enrich for sub-populations of one or both of the CD4+ or CD8+ population, for example, central memory T (T_{CM}) cells.

[0054] In a particular instance, a first selection step is carried out using beads labeled with CD4-binding molecules, such as antibodies (or secondary reagents that recognize such molecules), and the positive and negative fractions from the first selection step are retained, followed by further positive or negative selection of the negative fraction to enrich for CD8+ cells, such as by using beads labeled with CD8-binding molecules, and optionally selection of a sub-population of cells from the CD8+ fraction, such as central memory $CD8^+$ T cells and/or cells expressing one or more markers CD62L, CD45RA, CD45RO, CCR7, CD27, CD127, or CD44. In some instances, the order of the selections can be reversed. In some instances, a CD4 selection is always performed first and, from the negative fraction (CD4-), the CD8+ fraction is enriched in a second selection, e.g., by negative selection for one or more of $CD45RA^+$ and CD14, and/or positive selection for one or more of CD62L, CCR7, and/or other markers expressed on central memory cells.

[0055] In some instances, a plurality of cell populations, e.g., a $CD4^+$ T cell population and a $CD8^+$ T cell population, is isolated, for example, to produce a culture-initiating composition containing the plurality of cell populations. The culture-initiating composition typically contains the cells at a culture-initiating ratio, which is designed to yield a particular desired output ratio of two or more cell types, such as a particular $CD4^+$ to $CD8^+$ ratio, following one or more incubation, culture, cultivation, and/or engineering steps. The desired output ratio in some instances is a ratio designed to be optimal for administration of the cells to a patient, e.g., in adoptive cell therapy.

[0056] In some instances, isolating the plurality of populations in a single or in the same isolation or separation vessel or set of vessels, such as a single column or set of columns, and/or same tube, or tubing set or using the same separation matrix or media or reagents, such as the same magnetic matrix, affinity-labeled solid support, or antibodies or other binding partners, include features that streamline the isolation, for example, resulting in reduced cost, time, complexity, need for handling of samples, use of resources, reagents, or equipment. In some instances, such features are advantageous in that they minimize cost, efficiency, time, and/or complexity associated with the methods, and/or avoid potential harm to the cell product, such as harm caused by infection, contamination, and/or changes in temperature.

[0057] In some instances the isolated cell populations obtained for use in the methods herein are sterile. Microbial contamination of cell separation products can in some cases lead to the infection of the recipient subject, such as an immunocompromised recipient patient unable to fight the infection. In some instances, the cells, cell populations, and compositions are produced under GMP (good manufacturing practice) conditions. In some instances, GMP conditions comprise stringent batch testing. In certain instances, tissue typing is performed prior to transplantation, e.g., to avoid human leukocyte antigen (HLA) mismatch and prevent problems such as graft-versus-host disease. In some instances, the disclosed methods reduce handling by individual users and automate various steps, which in some instances can increase consistency of the isolated cell populations and compositions and reduces error, thereby promoting consistency of the therapy and safety.

1. Cells and populations of cells

[0058] In some instances, the methods include performing a selection, isolation and/or enrichment of a cell sample, such as a primary human cell sample. The isolated cell populations typically include a plurality of cell populations, generally populations of blood or blood-derived cells, such as hematopoietic cells, leukocytes (white blood cells), peripheral blood mononuclear cells (PBMCs), and/or cells of the immune system, e.g., cells of the innate or adaptive immunity, such as myeloid or lymphoid cells, e.g., lymphocytes, typically T cells and/or NK cells. In some instances, the sample is an apheresis or leukapheresis sample. In some instances, the selection, isolation and/or enrichment can include positive or negative selection of cells from the sample.

[0059] In some instances, the sample is a sample containing primary human T cells, such as CD4+ and CD8+ T cells. In particular instances, the sample is one in which a plurality of T cell populations is isolated, such as a population of $CD4^+$ cells and a population of $CD8^+$ T cells. Thus, in some instances, the isolation includes positive selection for cells expressing CD4 or cells expressing CD8 and/or negative selection for cells expressing non-T cell markers, such as myeloid or B cell markers, for example, negative selection for cells expressing CD14, CD19, CD56, CD20, CD11b, and/or CD16.

[0060] In some instances, the sample is one containing a plurality of populations that includes a T cell population, such as a whole T cell or CD4+ population, and an NK cell population. Among the T cell populations that can be enriched, isolated and/or selected are populations of unfractionated T cells, unfractionated CD4+ cells, unfractionated CD8+ cells, and sub-populations of CD4+ and/or CD8+ T cells, including subpopulations of T cells generated by enrichment for or depletion of cells of a particular sub-type or based on a particular surface marker expression profile.

[0061] For example, among the sub-types of T cells (e.g., $CD4^+$ or $CD8^+$ T cells) that can be enriched, isolated and/or selected are those defined by function, activation state, maturity,

potential for differentiation, expansion, recirculation, localization, and/or persistence capacities, antigen-specificity, type of antigen receptor, presence in a particular organ or compartment, marker or cytokine secretion profile, and/or degree of differentiation.

[0062] Among the sub-types and subpopulations of T cells and/or of CD4+ and/or of CD8+ T cells that can be enriched, isolated and/or selected are naive T (TN) cells, effector T cells (TEFF), memory T cells and sub-types thereof, such as stem cell memory T (TSCM), central memory T (TCM), effector memory T (TEM), or terminally differentiated effector memory T cells, tumor-infiltrating lymphocytes (TIL), immature T cells, mature T cells, helper T cells, cytotoxic T cells, mucosa-associated invariant T (MAIT) cells, naturally occurring and adaptive regulatory T (Treg) cells, helper T cells, such as TH1 cells, TH2 cells, TH3 cells, TH17 cells, TH9 cells, TH22 cells, follicular helper T cells, alpha/beta T cells, and delta/gamma T cells.

[0063] In some instances, one or more of the T cell populations enriched, isolated and/or selected from a sample by the disclosed methods are cells that are positive for (marker+) or express high levels (markerhigh) of one or more particular markers, such as surface markers, or that are negative for (marker-) or express relatively low levels (markerlow) of one or more markers. In some cases, such markers are those that are absent or expressed at relatively low levels on certain populations of T cells (such as non-memory cells) but are present or expressed at relatively higher levels on certain other populations of T cells (such as memory cells). In one instance, the cells (such as the CD8+ cells or the T cells, e.g., CD3+ cells) are enriched for (i.e., positively selected for) cells that are positive or expressing high surface levels of CD45RO, CCR7, CD28, CD27, CD44, CD127, and/or CD62L and/or depleted of (e.g., negatively selected for) cells that are positive for or express high surface levels of CD45RA. In some instances, cells are enriched for or depleted of cells positive or expressing high surface levels of CD122, CD95, CD25, CD27, and/or IL7-Rα (CD127). In some examples, CD8+ T cells are enriched for cells positive for CD45RO (or negative for CD45RA) and for CD62L.

[0064] In some instances, a CD4+ T cell population and a CD8+ T cell sub-population, e.g., a sub-population enriched for central memory (TCM) cells.

[0065] In some instances, the cells are natural killer (NK) cells. In some instances, the cells are monocytes or granulocytes, e.g., myeloid cells, macrophages, neutrophils, dendritic cells, mast cells, eosinophils, and/or basophils.

2. Samples

[0066] The cells and cell populations typically are isolated from a sample, such as a biological sample, e.g., one obtained from or derived from a subject, such as one having a particular disease or condition or in need of a cell therapy or to which cell therapy will be administered. In some instances, the subject is a human, such as a subject who is a patient in need of a particular therapeutic intervention, such as the adoptive cell therapy for which cells are being isolated, processed, and/or engineered. Accordingly, the cells in some instances are primary

cells, e.g., primary human cells. The samples include tissue, fluid, and other samples taken directly from the subject, as well as samples resulting from one or more processing steps, such as separation, centrifugation, genetic engineering (e.g. transduction with viral vector), washing, and/or incubation. The biological sample can be a sample obtained directly from a biological source or a sample that is processed. Biological samples include, but are not limited to, body fluids, such as blood, plasma, serum, cerebrospinal fluid, synovial fluid, urine and sweat, tissue and organ samples, including processed samples derived therefrom.

[0067] In some instances, the sample is blood or a blood-derived sample, or is or is derived from an apheresis or leukapheresis product. Exemplary samples include whole blood, peripheral blood mononuclear cells (PBMCs), leukocytes, bone marrow, thymus, tissue biopsy, tumor, leukemia, lymphoma, lymph node, gut associated lymphoid tissue, mucosa associated lymphoid tissue, spleen, other lymphoid tissues, liver, lung, stomach, intestine, colon, kidney, pancreas, breast, bone, prostate, cervix, testes, ovaries, tonsil, or other organ, and/or cells derived therefrom. Samples include, in the context of cell therapy, e.g., adoptive cell therapy, samples from autologous and allogeneic sources.

[0068] In some instances, the cells are derived from cell lines, e.g., T cell lines. The cells in some instances are obtained from a xenogeneic source, for example, from mouse, rat, non-human primate, and pig.

3. Cell processing, preparation and non-affinity-based separation

[0069] In some instances, isolation of the cells or populations includes one or more preparation and/or non-affinity based cell separation steps. In some examples, cells are washed, centrifuged, and/or incubated in the presence of one or more reagents, for example, to remove unwanted components, enrich for desired components, lyse or remove cells sensitive to particular reagents. In some examples, cells are separated based on one or more property, such as density, adherent properties, size, sensitivity and/or resistance to particular components.

[0070] In some examples, cells from the circulating blood of a subject are obtained, e.g., by apheresis or leukapheresis. The samples, in some instances, contain lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and/or platelets, and in some instances contains cells other than red blood cells and platelets.

[0071] In some instances, the blood cells collected from the subject are washed, e.g., to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In some instances, the cells are washed with phosphate buffered saline (PBS). In some instances, the wash solution lacks calcium and/or magnesium and/or many or all divalent cations. In some instances, a washing step is accomplished a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor, Baxter) according to the manufacturer's instructions. In some instances, a washing step is

accomplished by tangential flow filtration (TFF) according to the manufacturer's instructions. In some instances, the cells are resuspended in a variety of biocompatible buffers after washing, such as, for example, Ca^{++}/Mg^{++} free PBS. In certain instances, components of a blood cell sample are removed and the cells directly resuspended in culture media.

[0072] In some instances, the methods include density-based cell separation methods, such as the preparation of white blood cells from peripheral blood by lysing the red blood cells and centrifugation through a Percoll or Ficoll gradient.

4. Separation based on affinity and/or marker profile

[0073] In some instances, the isolation methods include the separation of different cell types based on the expression or presence in the cell of one or more specific molecules, such as surface markers, e.g., surface proteins, intracellular markers, or nucleic acid. In some instances, any known method for separation based on such markers may be used. In some instances, the separation is affinity- or immunoaffinity-based separation. For example, the isolation in some instances includes separation of cells and cell populations based on the cells' expression or expression level of one or more markers, typically cell surface markers, for example, by incubation with an antibody or binding partner that specifically binds to such markers, followed generally by washing steps and separation of cells having bound the antibody or binding partner, from those cells having not bound to the antibody or binding partner.

[0074] Such separation steps can be based on positive selection, in which the cells having bound the reagents are retained for further use, and/or negative selection, in which the cells having not bound to the antibody or binding partner are retained. In some examples, both fractions are retained for further use. In some instances, negative selection can be particularly useful where no antibody is available that specifically identifies a cell type in a heterogeneous population, such that separation is best carried out based on markers expressed by cells other than the desired population.

[0075] The separation need not result in 100 % enrichment or removal of a particular cell population or cells expressing a particular marker. For example, positive selection of or enrichment for cells of a particular type, such as those expressing a marker, refers to increasing the number or percentage of such cells, but need not result in a complete absence of cells not expressing the marker. Likewise, negative selection, removal, or depletion of cells of a particular type, such as those expressing a marker, refers to decreasing the number or percentage of such cells, but need not result in a complete removal of all such cells. For example, in some instances, a selection of one of the CD4+ or CD8+ population enriches for said population, either the CD4+ or CD8+ population, but also can contain some residual or small percentage of other non-selected cells, which can, in some cases, include the other of the CD4 or CD8 population still being present in the enriched population.

[0076] In some examples, multiple rounds of separation steps are carried out, where the positively or negatively selected fraction from one step is subjected to another separation step, such as a subsequent positive or negative selection. In some examples, a single separation step can deplete cells expressing multiple markers simultaneously, such as by incubating cells with a plurality of antibodies or binding partners, each specific for a marker targeted for negative selection. Likewise, multiple cell types can simultaneously be positively selected by incubating cells with a plurality of antibodies or binding partners expressed on the various cell types.

[0077] For example, in some instances, specific subpopulations of T cells, such as cells positive or expressing high levels of one or more surface markers, e.g., $CD28^+$, $CD62L^+$, $CCR7^+$, $CD27^+$, $CD127^+$, $CD4^+$, $CD8^+$, $CD45RA^+$, and/or $CD45RO^+$ T cells, are isolated by positive or negative selection techniques.

[0078] For example, $CD3^+$, $CD28^+$ T cells can be positively selected using CD3/CD28 conjugated magnetic beads (e.g., DYNABEADS® M-450 CD3/CD28 T Cell Expander).

[0079] In some instances, isolation is carried out by enrichment for a particular cell population by positive selection, or depletion of a particular cell population, by negative selection. In some instances, positive or negative selection is accomplished by incubating cells with one or more antibodies or other binding agent that specifically bind to one or more surface markers expressed or expressed ($marker^+$) at a relatively higher level ($marker^{high}$) on the positively or negatively selected cells, respectively.

[0080] In some instances, T cells are separated from a PBMC sample by negative selection of markers expressed on non-T cells, such as B cells, monocytes, or other white blood cells, such as CD14. In some instances, the methods include isolation, selection and/or enrichment of CD4+ and CD8+ cells. In one example, to enrich for $CD4^+$ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16 and HLA-DR. In one example, to enrich for a $CD8^+$ population by negative selection is carried out by depletion of cells expressing CD14 and/or CD45RA. In some instances, a $CD4^+$ or $CD8^+$ selection step, such as positive selection for CD4 and positive selection for CD8, is used to separate $CD4^+$ helper and $CD8^+$ cytotoxic T cells. Such selections in some instances are carried out simultaneously and in other instances are carried out sequentially, in either order.

[0081] In some instances, the methods include a first positive selection for CD4+ cells in which the non-selected cells (CD4- cells) from the first selection are used as the source of cells for a second positive selection to enrich for CD8+ cells. In some instances, the methods include a first positive selection for CD8+ cells in which the non-selected cells (CD8- cells) from the first selection are used as the source of cells for a second position selection to enrich for CD4+ cells. Such $CD4^+$ and $CD8^+$ populations can be further sorted into sub-populations by positive

or negative selection for markers expressed or expressed to a relatively higher degree on one or more naive, memory, and/or effector T cell subpopulations.

[0082] In some instances, CD4+ cells are further enriched for or depleted of naive, central memory, effector memory and/or central memory stem cells, such as by positive or negative selection based on surface antigens associated with the respective population. $CD4^+$ T helper cells are sorted into naive, central memory, and effector cells by identifying cell populations that have cell surface antigens. $CD4^+$ lymphocytes can be obtained by standard methods. In some instances, naive $CD4^+$ T lymphocytes are $CD45RO^-$, $CD45RA^+$, $CD62L^+$, $CD4^+$ T cells. In some instances, central memory $CD4^+$ cells are $CD62L^+$ and $CD45RO^+$. In some instances, effector $CD4^+$ cells are $CD62L^-$ and CD45RO

[0083] In some instances, $CD8^+$ cells are further enriched for or depleted of naive, central memory, effector memory, and/or central memory stem cells, such as by positive or negative selection based on surface antigens associated with the respective subpopulation. In some instances, enrichment for central memory T (T_{CM}) cells is carried out to increase efficacy, such as to improve long-term survival, expansion, and/or engraftment following administration, which in some instances is particularly robust in such sub-populations. *See* Terakuraet al. (2012) Blood. 1:72-82; Wang et al. (2012) J Immunother. 35(9):689-701. In some instances, combining T_{CM}-enriched $CD8^+$ T cells and $CD4^+$ T cells further enhances efficacy.

[0084] In instances, memory T cells are present in both $CD62L^+$ and CD62L- subsets of $CD8^+$ peripheral blood lymphocytes. PBMC can be enriched for or depleted of CD62L-$CD8^+$ and/or $CD62L^+CD8^+$ fractions, such as using anti-CD8 and anti-CD62L antibodies.

[0085] In some instances, the enrichment for central memory T (T_{CM}) cells is based on positive or high surface expression of CD45RO, CD62L, CCR7, CD28, CD3, CD27 and/or CD 127; in some instances, it is based on negative selection for cells expressing or highly expressing CD45RA and/or granzyme B.

[0086] In some instances, the disclosed methods include isolation, selection and/or enrichment of CD8+ cells from a sample, such as by positive selection based on surface expression of CD8. In some instances, the methods can further include enriching for central memory T (T_{CM}) cells. In one instance, the enriched CD8+ cells can be further enriched for central memory T (T_{CM}) cells by selecting for one or more markers expressed on central memory T (T_{CM}) cells., such as one or more of CD45RO, CD62L, CCR7, CD28, CD3, CD27 and/or CD 127. The selection can be performed prior to or subsequent to isolation, selection and/or enrichment of CD4+ cells. Such selections in some instances are carried out simultaneously and in other instances are carried out sequentially, in either order.

[0087] In some instances, the methods include a first positive selection for CD4+ cells in which

the non-selected cells (CD4- cells) from the first selection are used as the source of cells for a second selection to enrich for CD8+ cells, and the enriched or selected CD8+ cells are used in a third selection to further enrich for cells expressing one or more markers expressed on central memory T (T_{CM}) cells., such as by a third selection to enrich for CD45RO+, CD62L+, CCR7+, CD28+, CD3+, CD27+ and/or CD127+ cells. In some instances, the methods include a first positive selection for CD8+ cells in which the non-selected cells (CD8- cells) from the first selection are used as the source of cells for the second selection to enrich for CD4+ cells, and the enriched or selected CD8+ cells from the first selection also are used in a third selection to further enrich for cells expressing one or more markers expressed on central memory T (T_{CM}) cells., such as by a third selection to enrich for CD45RO+, CD62L+, CCR7+, CD28+, CD3+, CD27+ and/or CD127+ cells.

[0088] In some instances, isolation of a CD8$^+$ population enriched for T_{CM} cells is carried out by depletion of cells expressing CD4, CD14, CD45RA, and positive selection or enrichment for cells expressing CD62L. In one instance, enrichment for central memory T (T_{CM}) cells is carried out starting with a negative fraction of cells selected based on CD4 expression, which is subjected to a negative selection based on expression of CD14 and CD45RA, and a positive selection based on CD62L. Such selections in some instances are carried out simultaneously and in other instances are carried out sequentially, in either order. In some instances, the same CD4 expression-based selection step used in preparing the CD8$^+$ cell population or subpopulation, also is used to generate the CD4$^+$ cell population or sub-population, such that both the positive and negative fractions from the CD4-based separation are retained and used in subsequent steps of the methods, optionally following one or more further positive or negative selection steps.

[0089] In a particular example, a sample of PBMCs or other white blood cell sample is subjected to selection of CD4$^+$ cells, where both the negative and positive fractions are retained. The negative fraction then is subjected to negative selection based on expression of CD14 and CD45RA or CD19, and positive selection based on a marker characteristic of central memory T cells, such as CD62L or CCR7, where the positive and negative selections are carried out in either order.

[0090] In some instances, the methods of isolating, selecting and/or enriching for cells, such as by positive or negative selection based on the expression of a cell surface marker or markers, for example by any of the methods described above, can include immunoaffinity-based selections. In some instances, the immunoaffinity-based selections include contacting a sample containing cells, such as primary human T cells containing CD4+ and CD8+ cells, with an antibody or binding partner that specifically binds to the cell surface marker or markers. In some instances, the antibody or binding partner is bound to a solid support or matrix, such as a sphere or bead, for example microbeads, nanobeads, including agarose, magnetic bead or paramagnetic beads, to allow for separation of cells for positive and/or negative selection. In some instances, the spheres or beads can be packed into a column to effect immunoaffinity chromatography, in which a sample containing cells, such as primary human T cells containing

CD4+ and CD8+ cells, is contacted with the matrix of the column and subsequently eluted or released therefrom.

a. Immunoaffinity Beads

[0091] For example, in some instances, the cells and cell populations are separated or isolated using immunomagnetic (or affinitymagnetic) separation techniques (reviewed in Methods in Molecular Medicine, vol. 58: Metastasis Research Protocols, Vol. 2: Cell Behavior In Vitro and In Vivo, p 17-25 Edited by: S. A. Brooks and U. Schumacher © Humana Press Inc., Totowa, NJ).

[0092] In some instances, the sample or composition of cells to be separated is incubated with small, magnetizable or magnetically responsive material, such as magnetically responsive particles or microparticles, such as paramagnetic beads . The magnetically responsive material, e.g., particle, generally is directly or indirectly attached to a binding partner, e.g., an antibody, that specifically binds to a molecule, e.g., surface marker, present on the cell, cells, or population of cells that it is desired to separate, e.g., that it is desired to negatively or positively select. Such beads are known and are commercially available from a variety of sources including, in some instances, Dynabeads® (Life Technologies, Carlsbad, CA), MACS® beads (Miltenyi Biotec, San Diego, CA) or Streptamer® bead reagents (IBA, Germany).

[0093] In some instances, the magnetic particle or bead comprises a magnetically responsive material bound to a specific binding member, such as an antibody or other binding partner. There are many well-known magnetically responsive materials used in magnetic separation methods. Suitable magnetic particles include those described in Molday, U.S. Pat. No. 4,452,773, and in European Patent Specification EP 452342 B. Colloidal sized particles, such as those described in Owen U.S. Pat. No. 4,795,698, and Liberti et al., U.S. Pat. No. 5,200,084 are other examples.

[0094] The incubation generally is carried out under conditions whereby the antibodies or binding partners, or molecules, such as secondary antibodies or other reagents, which specifically bind to such antibodies or binding partners, which are attached to the magnetic particle or bead, specifically bind to cell surface molecules if present on cells within the sample.

[0095] In some instances, the sample is placed in a magnetic field, and those cells having magnetically responsive or magnetizable particles attached thereto will be attracted to the magnet and separated from the unlabeled cells. For positive selection, cells that are attracted to the magnet are retained; for negative selection, cells that are not attracted (unlabeled cells) are retained. In some instances, a combination of positive and negative selection is performed during the same selection step, where the positive and negative fractions are retained and further processed or subject to further separation steps.

[0096] In certain instances, the magnetically responsive particles are coated in primary antibodies or other binding partners, secondary antibodies, lectins, enzymes, or streptavidin. In certain instances, the magnetic particles are attached to cells via a coating of primary antibodies specific for one or more markers. In certain instances, the cells, rather than the beads, are labeled with a primary antibody or binding partner, and then cell-type specific secondary antibody- or other binding partner (e.g., streptavidin)-coated magnetic particles, are added. In certain instances, streptavidin-coated magnetic particles are used in conjunction with biotinylated primary or secondary antibodies.

[0097] In some instances, the magnetically responsive particles are left attached to the cells that are to be subsequently incubated, cultured and/or engineered; in some instances, the particles are left attached to the cells for administration to a patient. In some instances, the magnetizable or magnetically responsive particles are removed from the cells. Methods for removing magnetizable particles from cells are known and include, e.g., the use of competing non-labeled antibodies, magnetizable particles or antibodies conjugated to cleavable linkers, etc. In some instances, the magnetizable particles are biodegradable.

[0098] In some instances, the affinity-based selection is via magnetic-activated cell sorting (MACS) (Miltenyi Biotech, Auburn, CA). Magnetic Activated Cell Sorting (MACS) systems are capable of high-purity selection of cells having magnetized particles attached thereto. In certain instances, MACS operates in a mode wherein the non-target and target species are sequentially eluted after the application of the external magnetic field. That is, the cells attached to magnetized particles are held in place while the unattached species are eluted. Then, after this first elution step is completed, the species that were trapped in the magnetic field and were prevented from being eluted are freed in some manner such that they can be eluted and recovered. In certain instances, the non-target cells are labelled and depleted from the heterogeneous population of cells.

[0099] In some instances, the affinity-based selection employs Streptamers®, which are magnetic beads, such as nanobeads or microbeads, for example 1-2 μM that, in some instances, are conjugated to a binding partner immunoaffinity reagent, such as an antibody via a streptavidin mutant, e.g. Strep-Tactin® or Strep-Tactin XT® (see e.g. U.S. Patent No. 6,103,493, International Published PCT Appl. Nos. WO/2013011011, WO 2014/076277). In some instances, the streptavidin mutant is functionalized, coated and/or immobilized on the bead.

[0100] In some instances, the streptavidin mutant exhibits a higher binding affinity for a peptide ligand containing the sequence of amino acids set forth in any of SEQ ID NOS:1-6, such as for example SEQ ID NO:5 and/or SEQ ID NO:6 (e.g. Strep-tag II®), than an unmodified or wild type streptavidin, such as an unmodified or wild type streptavidin set forth in SEQ ID NO: 11 or SEQ ID NO:14. In some instances, the streptavidin mutant exhibits a binding affinity as an affinity constant for such peptides that is greater than the binding affinity of wild type streptavidin for the same peptide by greater than 5-fold, 10-fold, 50-fold, 100-fold, 200-fold or

greater.

[0101] The streptavidin mutein contains one or more amino acid differences compared to an unmodified streptavidin, such as a wild type streptavidin or fragment thereof. The term "unmodified streptavidin" refers to a starting polypeptide to which one or more modifications are made. In some instances, the starting or unmodified polypeptide may be a wild type polypeptide set forth in SEQ ID NO:11. In some instances, the unmodified streptavidin is a fragment of wild type streptavidin, which is shortened at the N- and/or C-terminus. Such minimal streptavidins include any that begin N-terminally in the region of amino acid positions 10 to 16 of SEQ ID NO:11 and terminate C-terminally in the region of amino acid positions 133 to 142 of SEQ ID NO:11. In some instances, the unmodified streptavidin has the sequence of amino acids set forth in SEQ ID NO:14. In some instances, the unmodified streptavidin, such as set forth in SEQ ID NO:14, can further contain an N-terminal methionine at a position corresponding to Ala13 with numbering as set forth in SEQ ID NO:11. Reference to number of residues in streptavidin disclosed herein is with reference to numbering of residues in SEQ ID NO: 11.

[0102] The term "streptavidin mutein," "streptavidin mutant" or variations thereof, refers to a streptavidin protein that contains one or more amino acid differences compared to an unmodified or wild type streptavidin, such as a streptavidin set forth in SEQ ID NO: 11 or SEQ ID NO:14. The one or more amino acid differences can be amino acid mutations, such as one or more amino acid replacements (substitutions), insertions or deletions. In some instances, a streptavidin mutein can have at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid differences compared to a wild type or unmodified streptavidin. In some instances, the amino acid replacements (substitutions) are conservative or non-conservative mutations. The streptavidin mutein containing the one or more amino acid differences exhibits a binding affinity as an affinity constant that is greater than 2.7×10^4 M^{-1} for the peptide ligand (Trp Arg His Pro Gln Phe Gly Gly; also called Strep-tag®, set forth in SEQ ID NO:5). In some instances, the streptavidin mutant exhibits a binding affinity as an affinity constant that is greater than 1.4×10^4 M^{-1} for the peptide ligand (Trp Ser His Pro Gln Phe Glu Lys; also called Strep-tag® II, set forth in SEQ ID NO:6). In some instances, binding affinity can be determined by methods known in the art, such as any described below.

[0103] In some instances, the streptavidin mutein contains a mutation at one or more residues 44, 45, 46, and/or 47. In some instances, the streptavidin mutant contains residues Val44-Thr45-Ala46-Arg47, such as set forth in exemplary streptavidin muteins set forth in SEQ ID NO: 12 or SEQ ID NO:15. In some instances, the streptavidin mutein contains residues Ile44-Gly45-Ala-46-Arg47, such as set forth in exemplary streptavidin muteins set forth in SEQ ID NO: 13 or 16. In some instances, the streptavidin mutein exhibits the sequence of amino acids set forth in SEQ ID NO: 12, 13, 15 or 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 of amino acids set forth in SEQ ID NO:12, 13, 15 or 16, and exhibits a binding affinity that is greater than 2.7×10^4 M^{-1} for the peptide ligand (Trp Arg His Pro Gln Phe Gly Gly; also

called Strep-tag®, set forth in SEQ ID NO: 5) and/or greater than 1.4×10^4 M^{-1} for the peptide ligand (Trp Ser His Pro Gln Phe Glu Lys; also called Strep-tag® II, set forth in SEQ ID NO:6).

[0104] In some instance, the streptavidin mutein is a mutant as described in International Published PCT Appl. Nos. WO 2014/076277. In some instances, the streptavidin mutein contains at least two cysteine residues in the region of amino acid positions 44 to 53 with reference to amino acid positions set forth in SEQ ID NO:11. In some instances, the cysteine residues are present at positions 45 and 52 to create a disulfide bridge connecting these amino acids. In such an instance, amino acid 44 is typically glycine or alanine and amino acid 46 is typically alanine or glycine and amino acid 47 is typically arginine. In some instances, the streptavidin mutein contains at least one mutation or amino acid difference in the region of amino acids residues 115 to 121 with reference to amino acid positions set forth in SEQ ID NO:11. In some instances, the streptavidin mutein contains at least one mutation at amino acid position 117, 120 and 121 and/or a deletion of amino acids 118 and 119 and substitution of at least amino acid position 121.

[0105] In some instances, a streptavidin mutein can contain any of the above mutations in any combination, so long as the resulting streptavidin mutein exhibits a binding affinity that is greater than 2.7×10^4 M^{-1} for the peptide ligand (Trp Arg His Pro Gln Phe Gly Gly; also called Strep-tag®, set forth in SEQ ID NO:5) and/or greater than 1.4×10^4 M^{-1} for the peptide ligand (Trp Ser His Pro Gln Phe Glu Lys; also called Strep-tag® II, set forth in SEQ ID NO:6).

[0106] In some instances, the binding affinity of a streptavidin mutant for a peptide ligand binding reagent is greater than 5×10^4 M^{-1}, 1×10^5 M^{-1}, 5×10^5 M^{-1}, 1×10^6 M^{-1}, 5×10^6 M^{-1} or 1×10^7 M^{-1}, but generally is less than 1×10^{13} M^{-1}, 1×10^{12} M^{-1} or 1×10^{11} M^{-1}.

[0107] In some instances, the streptavidin mutant also exhibits binding to other streptavidin ligands, such as but not limited to, biotin, iminobiotin, lipoic acid, desthiobiotin, diaminobiotin, HABA (hydroxyazobenzene-benzoic acid) or/and dimethyl-HABA. In some instances, the streptavidin muteins exhibits a binding affinity for another streptavidin ligand, such as biotin or desthiobiotin, that is greater than the binding affinity of the streptavidin mutein for the peptide ligand (Trp Arg His Pro Gln Phe Gly Gly; also called Strep-tag®, set forth in SEQ ID NO:5) or the peptide ligand (Trp Ser His Pro Gln Phe Glu Lys; also called Strep-tag® II, set forth in SEQ ID NO: 6).

[0108] In some instances, the streptavidin mutein is a multimer. Multimers can be generated using any methods known in the art, such as any described in published U.S. Patent Application No. US2004/0082012. In some instances, oligomers or polymers of muteins can be prepared by the introduction of carboxyl residues into a polysaccharide, e.g. dextran. In some instances, streptavidin muteins then are coupled via primary amino groups of internal lysine residues and/or the free N-terminus to the carboxyl groups in the dextran backbone using conventional carbodiimide chemistry in a second step. In some instances, the coupling reaction

is performed at a molar ratio of about 60 moles streptavidin mutant per mole of dextran. In some instances, oligomers or polymers of can also be obtained by crosslinking via bifunctional linkers, such as glutardialdehyde or by other methods known in the art.

[0109] In some instances" an immunoaffinity bead, such as a Streptamer® or other immunoaffinity bead, can contain an antibody produced by or derived from a hybridoma as follows: OKT3 (αCD3), 13B8.2 (αCD4), OKT8 (αCD8), FRT5 (αCD25), DREG56 (αCD62L), MEM56 (αCD45RA). In some instances, any of the above antibodies can contain one or more mutations within the framework of heavy and light chain variable regions without targeting the highly variable CDR regions. Exemplary of such antibodies include, in some instances, anti-CD4 antibodies as described in U.S. Patent No. 7,482,000 and Bes et al. (2003) J. Biol. Chem., 278: 14265-14273. In some instances, an antigen-binding fragment, such as a Fab fragment, can be generated from such antibodies using methods known in the art, such as, in some instances, amplification of hypervariable sequences of heavy and light chains and cloning to allow combination with sequences coding for an appropriate constant domain. In some instances, the constant domain is of human subclass IgG1/κ. Such antibodies can be carboxy-terminally fused with a peptide streptavidin binding molecule, such as set forth in SEQ ID NO: 10. Exemplary of such antibodies are described in Stemberget et al. (2102) PLoS One, 7:35798 and International PCT Application No. WO2013/011011.

[0110] In some instances, the antibody specifically binding a cell surface marker associated with or coated on a bead or other surface is a full-length antibody or is an antigen-binding fragment thereof, including a (Fab) fragments, F(ab')$_2$ fragments, Fab' fragments, Fv fragments, variable heavy chain (V$_H$) regions capable of specifically binding the antigen, single chain antibody fragments, including single chain variable fragments (scFv), and single domain antibodies (e.g., sdAb, sdFv, nanobody) fragments. In some instances, the antibody is a Fab fragment. In some instances, the antibody can be monovalent, bivalent or multivalent. In some instances, the antibody, such as a Fab, is a multimer. In some instances, the antibody, such as a Fab multimer, forms a multivalent complex with the cell surface marker.

[0111] In some instances, the antibody, such as a Fab, associated with a Streptamer exhibits a particular kinetic measure of binding affinity (e.g. dissociation constant, K_D, association constant K_A, off-rate or other kinetic parameter of binding affinity). Such measurements can be determined using any binding assay known to a skilled artisan. In particular examples, an affinity-based biosensor technology is utilized as a measure of binding affinity. Exemplary biosensor technologies include, for example, Biacore technologies, BioRad ProteOn, Reichert, GWC Technologies, IBIS SPIR Imaging, Nomadics SensiQ, Akubio RAPid, ForteBio Octet, IAsys, Nanofilm and others (see e.g. Rich et al. (2009) Analytical Biochemistry, 386:194-216). In some instances, binding affinity is determined by fluorescence titration or titration calorimetry.

[0112] In some instances, the antibody, such as a Fab, exhibits a k_{off} rate (also called dissociation rate constant) for the binding to a cell surface marker on a cell that is greater than

about 0.5×10^{-4} sec^{-1}, about 1×10^{-4} sec^{-1}, about 2×10^{-4} sec^{-1}, about 3×10^{-4} sec^{-1}, about 4×10^{-4} sec^{-1}, about 5×10^{-4} sec^{-1}, about 1×10^{-3} sec^{-1}, about 1.5×10^{-3} sec^{-1}, about 2×10^{-3} sec^{-1}, about 3×10^{-3} sec^{-1}, about 4×10^{-3} sec^{-1}, about 5×10^{-3} sec^{-1}, about 1×10^{-2} sec^{-1}, or about 5×10^{-1} sec^{-1} or greater. The particular k_{Off} rate can determine the rate at which the antibody reagent can dissociate from the its interaction with a cell via its binding to a cell surface marker (see e.g. International Published PCT Appl. No. WO/2013011011). For example, in some instances, the k_{Off} range can be chosen within a range, depending, for example, on the particular application or use of a selected or enriched cell, including factors such as the desire to remove the bound antibody from the cell surface, the time of the cells in culture or incubation, the sensitivity to the cell and other factors. In one instance, an antibody has a high k_{Off} rate of, for example, greater than 4.0×10^{-4} sec^{-1}, so that, after the disruption of the multivalent binding complexes, most of the antibody can be removed within one hour, since, in some instances taking into account the half-life T1/2 of the complex, within 56 minutes the concentration of the complexes is reduced to 25 % of the original concentration, assuming that rebinding effects can be neglected due to sufficient dilution). In another instance, an antibody with a lower kOff rate of, for example, 1.0×10^{-4} sec^{-1}, the dissociation may take longer, for example about or approximately 212 min or about 3 and a half hours to remove 75% of the antibody from the surface.

[0113] In some instances, the antibody, such as a Fab, exhibits a dissociation constant (Ka) for the binding to a cell surface marker on a cell that can be the range of about 10^{-2} M to about 10^{-8} M, or of about 10^{-2} M to about 10^{-9} M, or of about 10^{-2} M to about 0.8×10^{-9}M, or of about 10^{-2} M to about 0.6×10^{-9}M, or of about 10^{-2} M to about 0.4×10^{-9} M, or of about 10^{-2} M to about 0.3×10^{-9} M, or of about 10^{-2} M to about 0.2×10^{-9}, or of about 10^{-2} M to about 0.15×10^{-9}M, or of about 10^{-2} to about 10^{-10}, In some instances, the dissociation constant (Ka) for the binding a cell surface marker on a cell can be in the range of about 10^{-7} M to about 10^{-10} M, or of about 10^{-7} M to about 0.8×10^{-9} M, or of about 10^{-7} M to about 0.6×10^{-9} M, of about 10^{-7} M to about 0.3×10^{-9} M, of 1.1×10^{-7} M to about 10^{-10} M, or of about 1.1×10^{-7} M to about 0.15×10^{-9}M, or of about 1.1×10^{-7} M to about 0.3×10^{-9} M, or of about 1.1×10^{-7} M to about 0.6×10^{-9} M, or of about 1.1×10^{-7} M to about 0.8×10^{-9} M.

[0114] In some instances, the immunoaffinity reagent, such as antibody, for example a Fab, is linked, directly or indirectly, to a peptide ligand, such as a peptide ligand capable of binding to a streptavidin mutant (see e.g. U.S. Patent No. 5,506,121). In some instances, such as peptide contains the sequence of amino acids set forth in any of SEQ ID NOS:1-6. In some instances, the immunoaffinity reagent, such as antibody, for example a Fab, is linked, directly or indirectly, to a peptide ligand containing the sequence of amino acids set forth in SEQ ID NO:6.

[0115] In some instances, the immunoaffinity reagent, such as antibody, for example a Fab, is fused directly or indirectly with a peptide sequence that contains a sequential arrangement of

at least two streptavidin-binding modules, wherein the distance between the two modules is at least 0 and not greater than 50 amino acids, wherein one binding module has 3 to 8 amino acids and contains at least the sequence His-Pro-Xaa (SEQ ID NO: 1), where Xaa is glutamine, asparagine, or methionine, and wherein the other binding module has the sequence of the same or different streptavidin peptide ligand, such as set forth in SEQ ID NO:3 (see e.g. International Published PCT Appl. No. WO02/077018; U.S. Patent No. 7,981,632). In some instances, the peptide ligand fused, directly or indirectly, to the immunoaffinity reagent, such as antibody, for example Fab, contains a sequence having the formula set forth in any of SEQ ID NO: 7 or 8. In some instances, the peptide ligand has the sequence of amino acids set forth in any of SEQ ID NOS: 9, 10 or 17-19.

[0116] Alternatively, other streptavidin-binding peptides known in the art may be used, e.g. as described by Wilson et al. (Proc.Natl.Acad.Sci.USA 98 (2001), 3750-3755). In some instances, the peptide is fused to the N- and/or C-terminus of the protein.

[0117] In some instances, the antibody, such as a Fab, fused to a peptide ligand capable of binding a streptavidin mutant, is contacted with streptavidin-mutant containing beads to coat the beads with antibody. In some instances, the coated beads can be used in enrichment and selection methods as described herein by contacting such beads with a sample containing cells to be enriched or selected.

[0118] In some instances, the bond between the peptide ligand binding partner and streptavidin mutein binding reagent is reversible. In some instances, the bond between the peptide ligand binding partner and streptavidin mutein binding reagent is high, such as described above, but is less than the binding affinity of the streptavidin binding reagent for biotin or a biotin analog. Hence, in some instances, biotin (Vitamin H) or a biotin analog can be added to compete for binding to disrupt the binding interaction between the streptavidin mutein binding reagent on the bead and the peptide ligand binding partner associated with the antibody specifically bound to a cell marker on the surface. In some instances, the interaction can be reversed in the presence of low concentrations of biotin or analog, such as in the presence of 0.1 mM to 10 mM, 0.5 mM to 5 mM or 1 mM to 3 mM, such as generally at least or about at least 1 mM or at least 2 mM, for example at or about 2.5 mM. In some instances, incubation in the presence of a competing agent, such as a biotin or biotin analog, releases the bead from the selected cell.

b. Immunoaffinity Chromatography

[0119] In some instances, the affinity-based selection employs immunoaffinity chromatography. Immunoaffinity chromatography methods include, in some instances, one or more chromatography matrix as described in U.S. Published Patent Appl. No. US2015/0024411. In some instances, the chromatographic method is a fluid chromatography, typically a liquid chromatography. In some instances, the chromatography can be carried out in a flow through mode in which a fluid sample containing the cells to be isolated is applied, for example, by

gravity flow or by a pump on one end of a column containing the chromatography matrix and in which the fluid sample exits the column at the other end of the column. In addition, in some instances, the chromatography can be carried out in an "up and down" mode in which a fluid sample containing the cells to be isolated is applied, for example, by a pipette on one end of a column containing the chromatography matrix packed within a pipette tip and in which the fluid sample enters and exits the chromatography matrix/pipette tip at the other end of the column. In some instances, the chromatography can also be carried out in a batch mode in which the chromatography material (stationary phase) is incubated with the sample that contains the cells, for example, under shaking, rotating or repeated contacting and removal of the fluid sample, for example, by means of a pipette.

[0120] In some instances, the chromatography matrix is a stationary phase. In some instances, the chromatography is column chromatography. In some instances, any suitable chromatography material can be used. In some instances, the chromatography matrix has the form of a solid or semi-solid phase. In some instances, the chromatography matrix can include a polymeric resin or a metal oxide or a metalloid oxide. In some instances, the chromatography matrix is a non-magnetic material or non-magnetizable material. In some instances, the chromatography matrix is a derivatized silica or a crosslinked gel, such as in the form of a natural polymer, for example a polysaccharide. In some instances, the chromatography matrix is an agarose gel. Agarose gel for use in a chromatography matrix are known in the art and include, in some instances, Superflow™ agarose or a Sepharose material such as Superflow™ Sepharose®, which are commercially available in different bead and pore sizes. In some instances, the chromatography matrix is a particular cross-linked agarose matrix to which dextran is covalently bonded, such as any known in the art, for example in some instances, Sephadex®, Superdex® or Sephacryl®, which are available in different bead and pore sizes.

[0121] In some instances, a chromatography matrix is made of a synthetic polymer, such as polyacrylamide, a styrene-divinylbenzene gel, a copolymer of an acrylate and a diol or of an acrylamide and a diol, a co-polymer of a polysacchardie and agarose, e.g. a polyacrylamide/agarose composite, a polysaccharide and N, N'-methylenebiscarylamide, or a derivatized silica coupled to a synthetic or natural polymer.

[0122] In some instances, the chromatography matrix, such as agarose beads or other matrix, has a size of at least or about at least 50 µm, 60 µm, 70 µm, 80 µm, 90 µm, 100 µm, 120 µm or 150 µm or more. The exclusion limit of the size exclusion chromatography matrix is selected to be below the maximal width of the target cell in a sample, e.g. T cells. In some instances, the volume of the matrix is at least 0.5 mL, 1 mL, 1.5 mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL, 7 mL, 8 mL, 9 mL, 10 mL or more. In some instances, the chromatography matrix is packed into a column.

[0123] In some instances, the chromatography matrix, which is an immunoaffinity chromatography matrix, includes an affinity reagent, such as an antibody or antigen-binding fragment, such as Fab, immobilized thereto. The antibody or antigen-binding fragment, such

as a Fab, can be any as described above, including, in some instances, known antibodies in the art, antibodies having a particular k_{Off} rate and/or antibodies having a particular dissociation constant (Ka).

[0124] In some instances, the affinity reagent, such as antibody or antigen-binding fragment, such as a Fab, is immobilized. In some instances, the immunoaffinity reagent, such as an antibody or antigen-binding fragment, such as a Fab, is fused or linked to a binding partner that interacts with a binding reagent immobilized on the matrix. In some instances, the binding capacity of the chromatography matrix is sufficient to adsorb or is capable of adsorbing at least 1×10^7 cells/mL, 5×10^7 cells/mL, 1×10^8 cells/mL, 5×10^8 cells/mL, 1×10^9 cells/ml or more, in which said cells are cells expressing a cell surface marker specifically recognized by the affinity reagent, such as antibody or Fab.

[0125] In some instances, the interaction between the binding reagent and binding partner forms a reversible bond, so that binding of the antibody to the matrix is reversible. In some instances, the reversible binding can be mediated by a streptavidin mutant binding partner and a binding reagent immobilized on the matrix that is streptavidin, a streptavidin analog or mutein, avidin or an avidin analog or mutein.

[0126] In some instances, reversible binding of the affinity reagent, such as antibody or antigen-binding fragment, such as Fab is via a peptide ligand binding reagent and streptavidin mutein interaction, as described above with respect to immunoaffinity beads. In instances of the chromatography matrix, the matrix, such as agarose beads or other matrix, is functionalized or conjugated with a streptavidin mutein, such as any described above, for example any set forth in SEQ ID NOS: 12, 13, 15 or 16. In some instances, the antibody or antigen-binding fragment, such as a Fab, is fused or linked, directly or indirectly, to a peptide ligand capable of binding to a streptavidin mutant, such as any described above. In some instances, the peptide ligand is any as described above, such as a peptide containing the sequence of amino acids set forth in any of SEQ ID NOS:1-10 or 17-19. In some instances, the chromatography matrix column is contacted with such an affinity reagent, such as an antibody or antigen-binding fragment, such as a Fab to immobilize or reversibly bind the affinity reagent to the column.

[0127] In some instances, the immunoaffinity chromatography matrix can be used in enrichment and selection methods as described herein by contacting said matrix with a sample containing cells to be enriched or selected. In some instances, the selected cells are eluted or released from the matrix by disrupting the interaction of the binding partner/binding reagent. In some instances, binding partner/binding reagents is mediated by a peptide ligand and streptavidin mutant interaction, and the release or selected cells can be effected due to the presence of a reversible bond. For example, in some instances, the bond between the peptide ligand binding partner and streptavidin mutein binding reagent is high, such as described above, but is less than the binding affinity of the streptavidin binding reagent for biotin or a biotin analog. Hence, in some instances, biotin (Vitamin H) or a biotin analog can be added to compete for binding to disrupt the binding interaction between the streptavidin mutein binding

reagent on the matrix and the peptide ligand binding partner associated with the antibody specifically bound to a cell marker on the surface. In some instances, the interaction can be reversed in the presence of low concentrations of biotin or analog, such as in the presence of 0.1 mM to 10 mM , 0.5 mM to 5 mM or 1 mM to 3 mM, such as generally at least or about at least 1 mM or at least 2 mM, for example at or about 2.5 mM. In some instances, elution in the presence of a competing agent, such as a biotin or biotin analog, releases the selected cell from the matrix.

[0128] In some instances, immunoaffinity chromatography in the disclosed methods is performed using at least two chromatography matrix columns that are operably connected, whereby an affinity or binding agent to one of CD4 or CD8, such as an antibody, e.g. a Fab, is coupled to a first chromatography matrix in a first selection column and an affinity or binding agent to the other of CD4 or CD8, such as an antibody, e.g. a Fab, is coupled to a second chromatography matrix in a second selection column. In some instances, the at least two chromatography matrix columns are present in a closed system or apparatus, such as a closed system or apparatus that is sterile.

[0129] In some instances, also disclosed herein is a closed system or apparatus containing at least two chromatography matrix columns that are operably connected, whereby an affinity or binding agent to one of CD4 or CD8, such as an antibody, e.g. a Fab, is coupled to a first chromatography matrix in a first selection column and an affinity or binding agent to the other of CD4 or CD8, such as an antibody, e.g. a Fab, is coupled to a second chromatography matrix in a second selection column. Exemplary of such systems and methods are depicted in Figure 1A an Figure 1B, and in Examples.

[0130] In some instances, the closed system is automated. In some instances, components associated with the system can include an integrated microcomputer, peristaltic pump, and various valves, such as pinch valves or stop cocks, to control flow of fluid between the various parts of the system. The integrated computer in some instances controls all components of the instrument and directs the system to perform repeated procedures in a standardized sequence. In some instances, the peristaltic pump controls the flow rate throughout the tubing set and, together with the pinch valves, ensures the controlled flow of buffer through the system.

[0131] With reference to Figure 1A and Figure 1B, in some instances, the first affinity matrix **3** containing a first affinity or binding agent in a first selection column **1** is i) operably coupled to the second affinity matrix **4** containing a second affinity or binding agent in a second selection column **2** via tubing and a valve **13** so that cells having passed through the first affinity chromatography matrix and not being bound to a first affinity or binding agent thereon are capable of being passed into the second affinity matrix and ii) is operably coupled to an output container, such as a culture vessel **12,** to collect selected cells from the first affinity matrix that bound to the first affinity or binding agent thereon, such as after elution and release of such cells from the first affinity matrix. In some instances, the second affinity matrix **4** containing a second affinity or binding agent in a second selection column **2** also is operably coupled to the

output container, such as a culture vessel **12,** to collect selected cells from the second affinity matrix that bound to the second affinity or binding agent thereon, such as after elution and release of such cells from the second affinity matrix. In some instances, the second selection column **2** is operably connected to the output container, such as culture vessel **12,** through a removal chamber **9** that contains a binding reagent, such as a streptavidin mutant, that is able to bind with high affinity, such as greater than 10' ^{10} M^{-1}, to an elution reagent, such as biotin.

[0132] In some instances, the size, e.g. length and/or diameter, of the first selection column **1** and second selection column **2** can be the same or different. In some instances, the size, e.g. length and/or diameter, of one of the first column **1** or second column **2** is larger than the size of the other of the columns by at least 1.2 times, 1.5 times, 2 times, 3 times, 4 times, 5 times, 5 times, 7 times, 8 times, 9 times, 10 times or more.

[0133] In some instances, the first affinity matrix **3** containing a first affinity or binding agent in a first selection column **1** also is operably connected to a third affinity matrix **17** containing a third affinity or binding agent in a third selection column **15** via tubing and a valve **13,** so that selected cells from the first affinity matrix that bound to the first affinity or binding agent thereon are capable of being passed into the third affinity matrix, such as after elution and release of such cells from the first affinity matrix. In some instances, the first selection column **1** is operably connected to the third selection column **15** through a removal chamber **9** that contains a binding reagent, such as a streptavidin mutant, that is able to bind with high affinity, such as greater than 10' ^{10} M^{-1}, to an elution reagent, such as biotin. In some instances, the third affinity matrix **17** containing a third affinity or binding agent in a third selection column **15** also is operably coupled to the output container, such as a culture vessel **12,** to collect selected cells from the third affinity matrix having bound to the third affinity or binding agent thereon (and previously having bound to the first affinity or binding agent), such as after elution and release of such cells from the third affinity matrix (and previously from the first affinity matrix). In some instances, the third selection column **15** is operably connected to the output container, such as culture vessel **12,** through a removal chamber **9** that contains a binding reagent, such as a streptavidin mutant, that is able to bind with high affinity, such as greater than 10^{-10} M^{-1}, to an elution reagent, such as biotin.

[0134] In some instances, the size, e.g. length and/or diameter, of the first selection column **1** and third selection column **15** can be the same or different. In some instances, the size, e.g. length and/or diameter, of one of the first column **1** or third column **15** is larger than the size of the other of the columns by at least 1.2 times, 1.5 times, 2 times, 3 times, 4 times, 5 times, 5 times, 7 times, 8 times, 9 times, 10 times or more.

[0135] In some instances, the first selection column **1** is operably coupled to a storage reservoir containing cell sample **5,** such as via tubing, valves and a pump **8,** in order to provide a cell sample into the first selection column.

[0136] In some instances, the first selection column **1** also is operably coupled to a washing

reservoir 6 containing wash buffer and/or an elution reservoir 7 containing an eluent, such as through tubing and valves, in order to permit passage of a washing buffer or an elution buffer, respectively, into the first chromatography matrix in the first selection column. In some instances, due to the operable connection between the first and second selection column, the washing buffer and/or elution buffer can operably pass through into the second column. In some instances, due to the operable connection between the first and third selection column, the washing buffer and/or elution buffer can operably pass through into the third selection column.

[0137] In some instances, the second selection column **2** also is operably coupled to a washing reservoir **6** containing wash buffer and/or an elution reservoir **7** containing an eluent, such as through tubing and valves, in order to permit passage of a washing buffer or an elution buffer, respectively, into the first chromatography matrix in the first selection column.

[0138] The washing buffer can be any physiological buffer that is compatible with cells, such as phosphate buffered saline. In some instances, the washing buffer contains bovine serum albumin, human serum albumin, or recombinant human serum albumin, such as at a concentration of 0.1% to 5% or 0.2% to 1%, such as or at about 0.5%. In some instances, the eluent is biotin or a biotin analog, such as desbiotin, for example in an amount that is or is about at least 0.5 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 4 mM, or 5 mM.

[0139] In some instances, the first affinity matrix **3** in the first selection column **1** is operably connected through tubing and valves to a first affinity reagent reservoir (e.g. Fab reservoir) **18** containing the first affinity or binding agent, such as an antibody, e.g. a Fab, such as for immobilization onto the first affinity matrix. In some instances, the second affinity matrix 4 in the second column **2** is operably connected through tubing and valves to a second affinity or binding agent reservoir (e.g. Fab reservoir) **19** containing the second affinity or binding agent, such as an antibody, e.g. a Fab, such as for immobilization onto the second affinity matrix. In some instances, the third affinity matrix **17** in the third selection column **15** is operably connected through tubing and valves to a third affinity or binding agent reservoir (e.g. Fab reservoir) **20** containing the third affinity or binding agent, such as an antibody, e.g. a Fab, such as for immobilization onto the second affinity matrix.

[0140] In some instances, the first and/or second affinity reagent specifically binds CD4 or CD8, where the first and second affinity reagent are not the same. In some instances, the third affinity reagent specifically binds a marker on naive, resting or central memory T cells or specifically binds a marker that is CD45RO, CD62L, CCR7, CD28, CD3, CD27 and/or CD127.

5. Enrichments and Ratios of Generated Compositions

[0141] In some instances, performing a first and second selection using methods as described above enriches from a sample a first population of cells expressing a first cell surface marker and a second population of cells expressing a second cell surface marker, respectively. In

particular examples, the first and/or second population of enriched cells can be a population of cells enriched for CD4+ cells, and the other of the enriched population of cells, i.e. the other of the first or second population of cells, can be a population enriched for CD8+. As described above, in some instance, a third, fourth or subsequent selection can be performed to enrich for a further sub-population of cells from a population of cells previously enriched in the first, second or subsequent enrichments, such as a sub-population of CD4+ cells and/or a sub-population of CD8+ cells..

[0142] In some instances, the method produces an enriched composition of cells containing the first and second population of enriched cells, such as a population of cells enriched for CD4+ cells and a population of cells enriched for CD8+ cells. In some instances, the enriched composition of cells is designated a culture initiation composition and is used in subsequent processing steps, such as subsequent processing steps involving incubation, stimulation, activation, engineering and/or formulation of the enriched cells. In some instances, subsequent to the further processing steps, such as processing steps involving incubation, stimulation, activation, engineering and/or formulation, and output composition is generated that, in some instances, can contain genetically engineered cells containing CD4+ cells and CD8+ cells expressing a genetically engineered antigen receptor.

[0143] In some instances, the enriched compositions of cells are enriched cells from a starting sample as describe above, in which the number of cells in the starting sample is at least greater than the desired number of cells in an enriched composition, such as a culture-initiation composition. In some instances, the number of cells in the starting sample is greater by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 500%, 1000%, 5000% or more greater than the desired number of cells in the enriched composition. In some examples, the desired number of cells in the enriched population, including enriched CD4+ cells, CD8+ cells or sub-populations thereof, is at least 1×10^6 cells, 2×10^6 cells, 4×10^6 cells, , 6×10^6 cells 8×10^6 cells, 1×10^7 cells, 2×10^7 cells, 4×10^7 cells, 6×10^7 cells, 8×10^7 cell, 1×10^8 cells, 2×10^8 cells, 4×10^8 cells, 6×10^8 cells, 8×10^8 cells, 1×10^9 cells or greater. In some instances, the number of cells in the starting sample, is at least 1×10^8 cells, 5×10^8 cells, 1×10^9 cells, 2×10^9 cells, 3×10^9 cells, 4×10^9 cells, 5×10^9 cells, 6×10^9 cells, 7×10^9 cells, 8×10^9 cells, 9×10^9 cells, 1×10^{10} cells or more.

[0144] In some instances, the yield of the first and/or second population or sub-population thereof, in the enriched composition, i.e. the number of enriched cells in the population or sub-population compared to the number of the same population or sub-population of cells in the starting sample, is 10% to 100%, such as 20% to 80%, 20% to 60%, 20% to 40%, 40% to 80%, 40% to 60%, or 60%, to 80%,. In some instances, the yield of the first and/or second population of cells or sub-population thereof is less than 70%, less than 60%, less than 50%, less than 40%, less than 30% or less than 20%.

[0145] In some instances, the purity of the first and/or second population of cells or sub-population of cells thereof in the enriched composition, i.e. the percentage of cells positive for

the selected cell surface marker versus total cells in the population of enriched cells, is at least 90%, 91%, 92%, 93%, 94%, and is generally at least 95%, 96%, 97%, 98%, 99% or greater.

[0146] In some instances, the enriched composition of cells, such as a culture-initiation composition, contains a ratio of CD4+ cells to CD8+ cells at a culture-initiation ratio. The culture-initiation ratio is the ratio or number of cells at which two types of cells or isolated cell populations are included in a culture-initiating composition, designed to result in the desired output ratio or dose, e.g., ratio or dose for administration to a patient, or within a tolerated error rate or difference thereof, at the completion of the incubation and/or engineering step or other processing steps and/or upon thaw and/or just prior to administration to a subject. In instances of the methods disclosed herein, the first and/or second selections, or selections for sub-populations thereof, can be performed in a manner to result in a chosen culture-initiation ratio. Exemplary of such methods are described below and in Examples.

a. Culture-initiation ratios and numbers

[0147] In some instances, the culture-initiating ratio of CD4$^+$ or sub-populations thereof to CD8$^+$ cells or sub-populations thereof 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. In some instances, the culture-initiating ratio of CD4+ cells or sub-populations thereof to CD8+ cells or sub-populations thereof is at or about 1:1.

[0148] In some instances, the culture-initiating ratio of CD4+ to CD8+ cells, or sub-populations thereof, is different than the ratio of CD4+ to CD8+ cells or the sub-populations thereof in the sample from the subject. In some instances, it is reported that the ratio of CD4+ to CD8+ T cells in a sample from subjects, such as a blood sample, is between 1:1 and 14:1 CD4+:CD8+ cells, and generally is between about 1.5:1 and about 2.5:1 CD4+:CD8+ cells. In some instances, the ratio of CD4+ to CD8+ T cells in a sample, such as a blood sample, is about 2:1 CD4+: CD8+ cells In some instances, the ratio of CD4+ to CD8+ T cells in a sample, such as a blood sample, is about 1:1. (*See e.g.* Amadori, A et al., Nature Med. 1: 1279-1283, 1995; Chakravarti, A., Nature Med. 1: 1240-1241, 1995; Clementi, M., et al., Hum. Genet. 105: 337-342, 1999.) In some instances, a subject ratio is less than 1:1 CD4+:CD8+ cells. (*See e.g.* Muhonen, T. J Immunother Emphasis Tumor Immunol. 1994 Jan;15(1):67-73.). In some instances, the culture-initiating ratio of CD4+ to CD8+ cells is at least 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 125%, at least 150, at least 200%, at least 300%, at least 400%, or at least 500% greater or less than the ratio of CD4+ to CD8+ cells in the sample from the subject.

[0149] In some instances, prior to performing the first and/or second selection, the ratio of CD4+ to CD8+ T cells in the sample from the subject is determined. Based on the particular ratio of the CD4+ to CD8+ T cells in the subject, which can vary among subject, the particular mode of selection can be individualized to the subject, for example by sizing of

chromatography columns or selection of amount or concentration of immunoaffinity reagents, to achieve the desired or chosen culture-initiating ratio. The relative level or frequency of various cell populations in a subject can be determined based on assessing surface expression of a marker or markers present on such populations or sub-populations. A number of well-known methods for assessing expression level of surface markers or proteins may be used, such as detection by affinity-based methods, e.g., immunoaffinity-based methods, e.g., in the context of cell surface proteins, such as by flow cytometry.

[0150] In some contexts, the appropriate culture-initiating ratio for particular cell types can vary depending on context, e.g., for example, for a particular disease, condition, or prior treatment of a subject from which cells are derived, and/or a particular antigen-specificity of the cells, relative representation among cells of a particular type (e.g., $CD8^+$ T cells) of various subpopulations, e.g., effector versus memory versus naive cells, and/or one or more conditions under which cells will be incubated, such as medium, stimulating agents, time of culture, buffers, oxygen content carbon dioxide content, antigen, cytokine, antibodies, and other components. Thus, it may be that a cell type which typically or in general is known to proliferate or expand more rapidly than another will not always have such a property in every context. Thus, in some instances, the culture-initiation ratio is determined based on known capacities of cell types in a normal or typical context, coupled with assessment of phenotypes or states of the cells or subject from which the cells are derived, and/or empirical evidence.

[0151] In some instances, the culture-initiation ratios are based on knowledge of one or more of these features for a particular type of cell known or determined to be in the concentration. In some instances, the ratio of $CD4^+$ to $CD8^+$ cells in the culture-initiating composition is greater than or less than 1.5 times, 2, times, 3 times, 4 times, 5 times, 6 times, 7 times, 8 times, 9 times, 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times more or less, respectively, than the desired output ratio of $CD4^+$ to $CD8^+$

[0152] In some instances, for example, CD4+ cells in some contexts are known to proliferate or expand to a lesser degree or less rapidly compared with CD8+ cells, when incubated under certain stimulating conditions. *See,* e.g., Foulds et al. (2002) J Immunol. 168(4): 1528-1532; Caggiari et al. (2001) Cytometry. 46(4) 233-237; Hoffman, et al. (2002) Transplantation. 74(6): 836-845; and Rabenstein et al. (2014) J Immunol. Published online before print March 17, 2014, doi: 10.4049/jimmunol. 1302725. Thus, in some examples, the ratio of $CD4^+$ to $CD8^+$ cells in the culture-initiating composition is 10 times or 100 times the desired output ratio. For example, if a 1:1 (or 50%/50%) CD4/CD8 output ratio is desired, the $CD4^+$ and $CD8^+$ populations may in one example be included in the culture-initiating composition at a ratio of 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 20:1, 30:1, 40:1, 50:1, 60:1, 70:1, 80:1, 90:1, or 100:1, for example, to account for differences in expansion rates over a particular period of time. Depending on the differences in rates or expansion or proliferation of one of the CD4+ or CD8+ cells, or sub-populations thereof, from the other, a skilled artisan can empirically determine the culture-initiation ratio to achieve a desired output ratio following under particular conditions of stimulation or activation.

[0153] The culture-initiation ratio will not necessarily be identical, or even approximate, the desired output ratio. For example, if it is desired to administer CD4$^+$ and CD8$^+$ engineered (e.g., CAR-expressing) T cells at or within a certain error of 1:1, the culture-initiation ratio of CD4$^+$ and CD8$^+$ cells often is not 1:1. In some instances, the culture initiation ratio that results in the desired output ratio varies depending, e.g., on the source of the cells, the cell types to be cultured, the patient to whom the cells are to be administered, the subject or subjects from whom the cells have been isolated or derived, such as what diseases or conditions such a subject has, the disease to be treated, culture conditions, and other parameters.

[0154] In some instances, the culture initiation ratio is based on the composition of each subpopulation of cells. In certain instances, the culture initiation ratio is based on the length of time the populations of cells are culture prior to their being genetically engineered. In some instances, the culture initiation ratio is based on the proliferation rate of each population of cells.

[0155] In some instances, the methods further include determining a culture-initiation ratio or number. In some instances, the disclosed methods include methods and steps for determining appropriate ratios, doses, and numbers of cells, cell types, and populations of cells. For example, disclosed are methods for determining ratios of CD4$^+$/CD8$^+$ cells, populations, and/or sub-populations and determining appropriate doses of such cells and sub-types. In some instances, disclosed are methods for determining appropriate ratios or numbers of cell types or cell populations to be included in a composition, such as a culture-initiating composition, to achieve a desired outcome. In some instances, such ratios or numbers are designed for use in an incubation or engineering step to achieve a desired output ratio or dose. In some instances, disclosed are methods for determining desired ratio of the cells, types, or populations, and/or cell numbers thereof, for administration to a subject or patient.

[0156] In some instances, the chosen culture initiation ratio is based on the relative capacity of the different cell types or populations for survival and/or proliferation or expansion rate of each population of cells or type of cell (such as CD4$^+$ versus CD8$^+$ cells) in culture when incubated under such conditions. Thus, in some instances, proliferation rate, survival, and/or output ratios are measured or assessed following test incubations, for example, at particular point or points in time following incubation, and/or following a cryopreservation or freeze step and/or following a thaw after such procedure, such as just prior to administration, e.g., at the bedside, in order to determine the optimal ratios for culture initiation. Any of a number of well-known methods for determination of *in vitro* or *ex vivo* cell proliferation rates or survival include flow-cytometry methods such as labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE) or similar fluorescent dye prior to incubation, followed by assessment of fluorescent intensity by flow cytometry, and/or assessment of binding of cells to Annexin V or other compound recognizing markers on or in apoptotic cells and/or uptake of DNA interchelating agents such as propidium iodide or 7AAD, and assessment of uptake and/or cell cycle stages as a measure of proliferation or apoptosis by flow cytometry.

[0157] In some instances, the culture-initiation ratio is determined by incubating two isolated populations, e.g., subpopulations, of cells at a range of different ratios in test compositions, under certain test conditions, and assessing one or more outcomes, such as the output ratio achieved after a certain period. In some instances, the conditions are stimulatory conditions, such as those approximating conditions under which the culture-initiating compositions are to be incubated for culture and/or engineering steps. For example, the test compositions in some instances are administered in the presence of one or more, e.g., each, of the same stimulating agents, media, buffers, gas content, and/or in the same type of container or vessel, and/or for the same or approximately the same amount of time as the parameters to be used in the incubation and/or engineering steps that are to be used in preparing or producing the ultimate composition, such as the engineered composition for administration.

[0158] Exemplary test ratios for the test culture-initiating compositions can include at or about 90%/10%, 80%/20%, 70%/30%, 60/40%, 50/50%, 40/60%, 30/70%, 20%/80%, and 10%/90%, or 0.1:1, 0.5:1, 0.7:1, 0.8:1, 0.9:1, 1:1, 1:1.1, 1:1.2., 1:1.3, 1:1.4, 1:1.5, or more than at or about 1:1.5, or at or about 1:0.1, 1:0.4, 1:0.7, 1:0.8, 1:0.8, 1.1:1, 1.2:1, 1.3:1, 1.4:1, or 1.5;1, or more.

[0159] In some contexts, the appropriate culture-initiation ratio of CD4$^+$ versus CD8$^+$ cells to achieve a desired CD4: CD8 ratio at the end of production is determined based on the sub-population of the CD4$^+$ and/or CD8$^+$ fractions in a particular isolated cell product, such as the presence and/or percentage of naive, effector, and various memory compartments are represented in a particular isolated composition. Such assessment can be by determining the presence or level of various surface markers on the cells, such as by flow cytometry.

[0160] In some instances, the culture initiation ratio is based on the phenotype of each population of cells. In certain instances, the culture initiation ratio is based on the culture conditions (e.g., such as the composition of the media, the presence and/or absence of growth factors, stimulants, and/or other agents, temperature, aeration conditions, etc.).

[0161] In some instances of the methods described herein, the culture initiation ratio produces an output composition that comprises a ratio of CD4$^+$: CD8$^+$ cells or a number of such sub-types or total cell number that is within about 10%, about 15%, about 20%, about 25%, about 30% about 35%, about 40%, about 45% or about 50% of the desired ratio or dose, including any range in between these values. In some instances of the methods described herein, the culture initiation ratio produces an output composition having the desired ratio of CD4$^+$:CD8$^+$ cells or dose of such cells at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or more than 95% of the time, including any range between these values. In certain instances of the methods, the culture initiation ratio produces a ratio of CD4$^+$ to CD8$^+$ cells in the output composition that is within 20 % of the desired output ratio at least 80 % of the time. In some instances of the methods described herein, the output composition comprises a ratio of CD4$^+$ to CD8$^+$ cells that

is within a tolerated difference of the desired output ratio, said tolerated difference having been determined as described above, e.g., by administering CD4$^+$ and CD8$^+$ cells to one or more subjects at a plurality of ratios.

b. Output ratios and doses

[0162] In some instances, the method results an output composition following one or more processing steps of the culture-initiation composition, such as incubation, stimulation, activation, engineering and/or formulation of cells. In some instances, the method is performed to achieve or result in a desired ratio of CD4$^+$ to CD8$^+$ cells, or sub-populations thereof, that is between at or about 2: 1 and at or about 1:5, at or about 2:1 and at or about 1:2, at or about 1.5:1 and at or about 1:5, at or about 1.5: 1 and at or about 1:2, or at or about 1:1 and at or about 1:2. In some instances, the desired output ratio of CD4$^+$ to CD8$^+$ cells in the output composition is 1:1 or is about 1: 1.

[0163] In some instances, the method produces or generates an output composition, such as a composition containing genetically engineered CD4+ T cells and CD8+ T cells or sub-populations thereof, in which the output ratio of CD4+ to CD8+ cells in the composition, or sub-populations thereof, is between at or about 2:1 and at or about 1:5, at or about 2:1 and at or about 1:2, at or about 1.5:1 and at or about 1:5, at or about 1.5:1 and at or about 1:2, or at or about 1:1 and at or about 1:2. In some instances, the method produces or generates an output composition containing an output ratio of CD4$^+$ to CD8$^+$ cells that is 1: 1 or is about 1:1.

[0164] In some instances, the output ratio of CD4+ to CD8+ T cells is a ratio that is desired as part of a dosage of T cells for immunotherapy, such as in connection with methods of adoptive immunotherapy.

[0165] In some instances, desired dosages, such as desired cell numbers and/or desired output ratios of the cell types or populations (e.g., ratios optimal for therapeutic administration to a patient), are determined. In some instances, the methods include steps for determining tolerated error or tolerated difference from a desired output or administration ratio or dose, i.e., the margin of error by which the ratio in a given composition, e.g., engineered composition, can vary from a desired output ratio, and still achieve a desired outcome, such as an acceptable degree of safety in a subject or patient, or efficacy in treating a particular disease or condition or other therapeutic effect.

[0166] In some instances, the desired dose, ratio and/or the tolerated error depends on the disease or condition to be treated, subject, source of cells, such as whether the cells are from a subject having a particular condition or disease and whether the cells are for autologous or allogeneic transplant, e.g., whether they are isolated from a subject who also is to receive the cells in adoptive cell therapy and/or whether the subject has received or is receiving another treatment and/or the identity of such treatment. In some instances, the ratio, number, and/or

tolerated difference or error depends on one or more other property of the cells, such as proliferation rate, survival capacity, expression of particular markers or secretion of factors, such as cytokines, or particular sub-populations isolated prior to incubation and engineering steps. In some examples, the desired ratio and/or the tolerated error or difference can vary depending on the age, sex, health, and/or weight of the subject, on biomarkers as an indication of disease trait, on treatments to be co-administered or having previously been administered to the subject.

[0167] In some instances, the desired ratio, dose, and/or tolerated error is determined by administering various test compositions, each containing the cell types or populations of interest at different ratios or different numbers to a test subject, followed by assessment of one or more outcome or parameter, such as a parameter indicative of safety, therapeutic efficacy, *in vivo* concentration or localization of the cells, and/or other desired outcome.

[0168] The test subject in some instances is a non-human animal, such as a normal animal or an animal model for disease, such as the disease or condition to be treated by administration of the cells. In some instances, the various test ratios for administration to test subjects are at or about 90%/10%, 80%/20%, 70%/30%, 60/40%, 50/50%, 40/60%, 30/70%, 20%/80%, and 10%/90%, e.g., expressed as percentage of one cell type or sub-type (e.g., CD4$^+$ T cell or sub-type thereof) and another cell type (e.g., CD8$^+$ T cell or NK cell or sub-type thereof), and interim values. The ratios can be expressed as relative percentages or in any other format, such as ratios of at or about 0.1:1, 0.5:1, 0.7:1, 0.8:1, 0.9:1, 1:1, 1:1.1, 1:1.2., 1:1.3, 1:1.4, 1:1.5, or more than at or about 1:1.5, or at or about 1:0.1, 1:0.4, 1:0.7, 1:0.8, 1:0.8, 1.1:1, 1.2:1, 1.3:1, 1.4:1, or 1.5;1, or more, including any range in between these values. In some instances, the test subject is a human. In some instances, the desired ratio is the average, mean, or median ratio among test subjects with a particular optimal effect. In some instances, the desired ratio is a ratio that achieved some optimal balance of safety versus efficacy. In some instances, the desired ratio or dose is a ratio or dose that achieves the highest efficacy of all test ratios or doses, while still maintaining a threshold degree of safety. In some instances, the desired ratio or dose is a ratio or dose that achieves the highest degree of safety while maintaining a threshold degree of efficacy or within a range of efficacy. In some cases, the optimal ratio or dose is expressed as a range, such as between 1:1 and 1:2 of one cell type to another or between 10^4 and 10^9 or between 10^5 and 10^6 cells per kg body weight.

[0169] In some instances, the tolerated error is determined based on the deviation from the desired average test subjects are monitored to assess the therapeutic efficacy and/or safety of each percentage combination. In some instances, the tolerated error will be 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, including any value in between these ranges.

6. Exemplary Methods of Selecting or Enriching Cells

a. Single Process Stream and/or Simultaneous Selection Using Immunomagnetic Beads

[0170] In some instances, the separation and/or steps is carried out using immunomagnetic beads. In some instances, a cell sample containing CD4+ and CD8+ cells, such as a primary human T cell sample, is contacted with magnetic beads containing a first immunoaffinity reagent that binds to CD4 or CD8 and magnetic beads containing a second immunoaffinity reagent that binds to the other of the CD4 or CD8. The separation and/or steps can occur simultaneously and/or sequentially.

[0171] In some instances, contacting the cells with the magnetic beads is performed simultaneously, whereby enrichment of cells containing the surface markers CD4 and CD8 also is performed simultaneously. In some such instances, 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 including CD4+ cells and CD8+ cells at a culture-initiating ratio.

[0172] In some instances, 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.

[0173] In some instances, the suboptimal yield concentration of the affinity reagent is a concentration below a concentration used or required to achieve an optimal or maximal yield of bound cells in a given selection or enrichment involving incubating cells with the reagent and recovering or separating cells having bound to the reagent ("yield," for example, being the number of the cells so-recovered or selected compared to the total number of cells in the incubation that are targeted by the reagent or to which the reagent is specific or that have a marker for which the reagent is specific and capable of binding). The suboptimal yield concentration generally is a concentration or amount of the reagent that in such process or step achieves no more than 70 % yield of bound cells, upon recovery of the cells having bound to the reagent. In some instances, no more than at or about 50 %, 45 %, 40 %, 30 %, or 25 % yield is achieved by the suboptimal concentration. The concentration may be expressed in terms of number or mass of particles or surfaces per cell and/or number of mass or molecules of agent (e.g., antibody, such as antibody fragment) per cell.

[0174] For example, in some instances, the suboptimal yield concentration is less than at or about 30 µM agent (e.g., antibody) per 1×10^9, per 2×10^9 cells, per 3×10^9 cells, per $4 \times$

10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition. In some instances, the suboptimal yield concentration is less than at or about 30 µM agent (e.g., antibody) per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 20 µM agent (e.g., antibody) per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 10 µM agent (e.g., antibody) per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 15 µM agent (e.g., antibody) per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 10 µM agent (e.g., antibody) per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 5 µM agent (e.g., antibody) per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 1 µm agent (e.g., antibody) per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 0.5 µM agent (e.g., antibody) per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 0.2 µM agent (e.g., antibody) per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition.

[0175] In some instances, the suboptimal yield concentration is less than at or about 15 mg beads, particles, surface, or total regent, per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 10 mg beads, particles, surface, or total regent, per 1 × 10^9, per 2 × 10^9 cells, per 3 × 10^9cells, per 4 × 10^9cells, per 5 × 10^9 cells, per 10 × 10^9 cells, per 15 × 10^9 cells, or per 20 × 10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 5 mg beads, particles, surface, or total regent, per 1 × 10^9, per 2 × 10^9

cells, per 3×10^9cells, per 4×10^9cells, per 5×10^9 cells, per 10×10^9 cells, per 15×10^9 cells, or per 20×10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 4 mg beads, particles, surface, or total regent, per 1×10^9, per 2×10^9 cells, per 3×10^9cells, per 4×10^9cells, per 5×10^9 cells, per 10×10^9 cells, per 15×10^9 cells, or per 20×10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 3 mg beads, particles, surface, or total regent, per 1×10^9, per 2×10^9 cells, per 3×10^9cells, per 4×10^9cells, per 5×10^9 cells, per 10×10^9 cells, per 15×10^9 cells, or per 20×10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 2 mg beads, particles, surface, or total regent, per 1×10^9, per 2×10^9 cells, per 3×10^9cells, per 4×10^9cells, per 5×10^9 cells, per 10×10^9 cells, per 15×10^9 cells, or per 20×10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 1 mg beads, particles, surface, or total regent, per 1×10^9, per 2×10^9 cells, per 3×10^9cells, per 4×10^9cells, per 5×10^9 cells, per 10×10^9 cells, per 15×10^9 cells, or per 20×10^9cells in the incubation composition; in some instances, the suboptimal yield concentration is less than at or about 0.5 mg beads, particles, surface, or total regent, per 1×10^9, per 2×10^9 cells, per 3×10^9cells, per 4×10^9cells, per 5×10^9 cells, per 10×10^9 cells, per 15×10^9 cells, or per 20×10^9cells in the incubation composition.

[0176] In some instances, e.g., when operating in a suboptimal yield concentration for each or one or more of two or more selection reagents with affinity to two or more markers or cells, one or more of such reagents is used at a concentration that is higher than one or more of the other such reagent(s), in order to bias the ratio of the cell type recognized by that reagent as compared to the cell type(s) recognized by the other(s). For example, the reagent specifically binding to the marker for which it is desired to bias the ratio may be included at a concentration (e.g., agent or mass per cells) that is increased by half, 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, or more, compared to other(s), depending on how much it is desired to increase the ratio.

[0177] In some instances, employing suboptimal yield concentration to bias one or both populations of cells can achieve a desired or chosen culture-initiation ratio. In some instances, the selection is performed from a sample, which is a sample containing CD4+ and CD8+ cells, containing high numbers of cells, such as at least 1×10^9 cells, 2×10^9 cells, 3×10^9 cells, 4×10^9 cells, 5×10^9 cells, 6×10^9 cells, 7×10^9 cells, 8×10^9 cells, 1×10^{10} cells, 2×10^{10} cells, 3×10^{10} cells, 4×10^{10} cells, 5×10^{10} cells or more. In some instances, the high number of cells is sufficient to ensure saturation of the immunoaffinity reagents in the sample to cells expressing a marker, such as CD4 or CD8, in which the reagent specifically binds.

[0178] In some instances, when operating in the suboptimal range and/or with enough cells to achieve saturation of reagents, the amount of immunoaffinity reagent is proportional to the approximate yield of enriched cells. In one instance, to achieve a culture-initiation ratio of about

or approximately 1:1 of CD4+ cells to CD8+ cells, selection of CD4+ cells and CD8+ cells can be performed with the same, or about the same, suboptimal yield concentration of immunoaffinity reagents to CD4 and CD8, respectively. In another exemplary instance, to achieve a culture-initiation ratio of about or approximately 2:1 of CD4+ cells to CD8+ cells, selection of CD4+ cells can be performed with a suboptimal yield concentration of an immunoaffinity reagent to CD4 that is about or approximately two times greater than the suboptimal yield concentration of an immunoaffinity reagent to CD8. It is within the level of a skilled artisan to empirically select or choose an appropriate amount or concentration of immunoaffinity reagents depending on the desired or chosen culture-initiating ratio of the generated composition containing enriched or selected cells in view of the above exemplification.

[0179] In some instances, the separation and/or steps is carried out using magnetic beads in which immunoaffinity reagents are reversibly bound, such as via a peptide ligand interaction with a streptavidin mutein as described above. Exemplary of such magnetic beads are Streptamers®. In some instances, the separation and/or steps is carried out using magnetic beads, such as those commercially available from Miltenyi Biotec.

[0180] In some instances, the separation and/or other steps is carried out for automated separation of cells on a clinical-scale level in a closed and sterile system. Components can include an integrated microcomputer, magnetic separation unit, peristaltic pump, and various pinch valves. The integrated computer in some instances controls all components of the instrument and directs the system to perform repeated procedures in a standardized sequence. The magnetic separation unit in some instances includes a movable permanent magnet and a holder for the selection column. The peristaltic pump controls the flow rate throughout the tubing set and, together with the pinch valves, ensures the controlled flow of buffer through the system and continual suspension of cells. In some instances, the separation and/or other steps is carried out using CliniMACS system (Miltenyi Biotic).

[0181] In some instances, the automated separation, such as using the CliniMACS system, in some instances uses antibody-coupled magnetizable particles that are supplied in a sterile, non-pyrogenic solution. In some instances, after labelling of cells with magnetic particles the cells are washed to remove excess particles. A cell preparation bag is then connected to the tubing set, which in turn is connected to a bag containing buffer and a cell collection bag. The tubing set consists of pre-assembled sterile tubing, including a pre-column and a separation column, and are for single use only. After initiation of the separation program, the system automatically applies the cell sample onto the separation column. Labelled cells are retained within the column, while unlabeled cells are removed by a series of washing steps. In some instances, the cell populations for use with the methods described herein are unlabeled and are not retained in the column. In some instances, the cell populations for use with the methods described herein are labeled and are retained in the column. In some instances, the cell populations for use with the methods described herein are eluted from the column after removal of the magnetic field, and are collected within the cell collection bag.

[0182] In certain instances, separation and/or other steps are carried out using a system equipped with a cell processing unit that permits automated washing and fractionation of cells by centrifugation. In some instances, the separation and/or other steps are carried out using the CliniMACS Prodigy system (Miltenyi Biotec). A system with a cell processing unit can also include an onboard camera and image recognition software that determines the optimal cell fractionation endpoint by discerning the macroscopic layers of the source cell product. For example, peripheral blood is automatically separated into erythrocytes, white blood cells and plasma layers. A cell processing system, such as the CliniMACS Prodigy system, can also include an integrated cell cultivation chamber which accomplishes cell culture protocols such as, e.g., cell differentiation and expansion, antigen loading, and long-term cell culture. Input ports can allow for the sterile removal and replenishment of media and cells can be monitored using an integrated microscope. See, e.g., Klebanoff et al.(2012) J Immunother. 35(9): 651-660, Terakuraet al. (2012) Blood.1:72-82, and Wang et al. (2012) J Immunother. 35(9):689-701.

[0183] In some instances, a cell population described herein is collected and enriched (or depleted) via flow cytometry, in which cells stained for multiple cell surface markers are carried in a fluidic stream. In some instances, a cell population described herein is collected and enriched (or depleted) via preparative scale (FACS)-sorting. In certain instances, a cell population described herein is collected and enriched (or depleted) by use of microelectromechanical systems (MEMS) chips in combination with a FACS-based detection system (see, e.g., WO 2010/033140, Cho et al. (2010) Lab Chip 10, 1567-1573; and Godin et al. (2008) J Biophoton. 1(5):355-376. In both cases, cells can be labeled with multiple markers, allowing for the isolation of well-defined T cell subsets at high purity.

[0184] In some instances, the antibodies or binding partners are labeled with one or more detectable marker, to facilitate separation for positive and/or negative selection. For example, separation may be based on binding to fluorescently labeled antibodies. In some examples, separation of cells based on binding of antibodies or other binding partners specific for one or more cell surface markers are carried in a fluidic stream, such as by fluorescence-activated cell sorting (FACS), including preparative scale (FACS) and/or microelectromechanical systems (MEMS) chips, e.g., in combination with a flow-cytometric detection system. Such methods allow for positive and negative selection based on multiple markers simultaneously.

b. Single Process Stream and/or Sequential Selections Using Immunoaffinity Chromatography

[0185] In some instances, the first selection or enrichment of a population of cells and the second selection and/or enrichment of a population of cells are performed using immunoaffinity-based reagents that include at least a first and second affinity chromatography matrix, respectively, having immobilized thereon an antibody. In some instances, one or both of the first and/or second selection can employ a plurality of affinity chromatography matrices and/or antibodies, whereby the plurality of matrices and/or antibodies employed for the same

selection, i.e. the first selection or the second selection, are serially connected. In some instances, the affinity chromatography matrix or matrices employed in a first and/or second selection adsorbs or is capable of selecting or enriching at least about 50×10^6 cells/mL, 100×10^6 cells/mL, 200×10^6 cells/mL or 400×10^6 cells/mL. In some instances, the adsorption capacity can be modulated based on the diameter and/or length of the column. In some instances, the culture-initiating ratio of the selected or enriched composition is achieved by choosing a sufficient amount of matrix and/or at a sufficient relative amount to achieve the culture-initiating ratio assuming based on, for example, the adsorption capacity of the column or columns for selecting cells.

[0186] In one exemplary instance, the adsorption capacity of the matrix or matrices is the same between the first and second selection, e.g. is or is about 1×10^8 cells/mL for both, whereby enrichment or selection of cells in the first selection and second selection results in a composition containing a CD4+ cells to CD8+ cells at a culture-initiating ratio of or about 1:1. In another exemplary instance, the adsorption capacity of the matrix or matrices used in one of the first selection or second selection is at least 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold, 5.0-fold, 6.0-fold, 7.0-fold, 8.0-fold, 9.0-fold, 10.0-fold or greater than the adsorption capacity of the matrix or matrices used in the other of the first selection or second selection, thereby resulting in a culture-initiation ratio in which cells selected with the greater adsorption capacity, e.g. CD4+ cells or CD8+ cells, are present in the culture initiating ratio in an amount that is at least 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold, 5.0-fold, 6.0-fold, 7.0-fold, 8.0-fold, 9.0-fold, 10.0-fold or greater than the other cell population. It is within the level of a skilled artisan to select or choose an appropriate volume, diameter or number of affinity matrix chromatography columns for the first and/or second selection depending on the desired or chosen culture-initiating ratio of the generated composition containing enriched or selected cells.

[0187] Exemplary processes for carrying out selections by the disclosed methods are set forth in Example 2. In some instances, such processes achieve a desired culture-initiation ratio in an enriched or generated composition.

[0188] In some instances, the first and/or second selection in the disclosed methods includes first enriching for one of CD4+ or CD8+ cells, and then enriching for a sub-population of cells based on, for example, surface expression of a marker expressed on resting, naive or central memory T cells, e.g. a marker that is CD28, CD62L, CCR7, CD127 or CD27. In some instances, the first and/or second selection includes enriching for CD8+ cells, said selection further comprises enriching for central memory T (T_{CM}) cells, where the other of the first and/or second selection includes enriching for CD4+ cells. In some instance, the methods are performed to enrich or select for CD4+ cells and to enrich or select for a sub-population of cells that are CD8+/CD28+, CD8+/CD62L+, CD8+/CCR7+, CD8+/CD127+ or CD8+/CD27+. In some instances, the first selection includes enriching for CD8+ cells and the second selection includes enriching for CD4+ cells, where the first selection, which includes cells enriched for CD8+ cells, further includes enriching for central memory T (T_{CM}) cells or enriching for cells that expresses a marker that is CD28, CD62L, CCR7, CD127 or CD27, thereby generating a

composition, such as a culture-initiation composition, containing CD4+ cells and CD8+ enriched for central memory T (T_{CM}) cells or a cell expressing a marker that is CD28, CD62L, CCR7, CD127 or CD27. In some instances, the first selection includes enriching for CD4+ cells and the second selection includes enriching for CD8+ cells, wherein the second selection, which includes cells enriched for CD8+ cells, further includes enriching for central memory T (T_{CM}) cells or enriching for cells that expresses a marker that is CD28, CD62L, CCR7, CD127 or CD27, thereby generating a composition, such as a culture-initiation composition, containing CD4+ cells and CD8+ enriched for central memory T (T_{CM}) cells or a cell expressing a marker that is CD28, CD62L, CCR7, CD127 or CD27.

[0189] In some such instances involving a further enrichment of a sub-population of cells, to achieve the culture-initiating ratio of CD4+ cells or a sub-population thereof to CD8+ cells or a sub-population thereof, the adsorption capacity of a column matrix or matrices is adjusted to account for differences in the frequency of a sub-population, i.e. CD4+ or CD8+ cells enriched for resting, naive, central memory cells or cells expressing a marker that is CD28, CD62L, CCR7, CD127 or CD27, compared to the frequency of cells of the respective CD4+ or CD8+ parent population in the starting sample from the subject. The relative level or frequency of various cell populations in a subject can be determined based on assessing surface expression of a marker or markers present on such populations or sub-populations. A number of well-known methods for assessing expression level of surface markers or proteins may be used, such as detection by affinity-based methods, e.g., immunoaffinity-based methods, e.g., in the context of cell surface proteins, such as by flow cytometry.

[0190] In an exemplary instance, a sample is enriched for CD4+ and CD8+/CD62L+ cells to yield a CD4+ to CD8+ ratio of 1:1. In this exemplary instance, the first selection can include enriching for CD8+ cells using a column with an adsorption capacity adjusted for the relative frequencies of CD4+ cells to CD8+/CD62L+ cells known to be present in the sample, or using ratios generally estimated to be in such samples. For example, the CD62L+ subpopulation of CD8+ cells collected from a human subject can sometimes be about 25% of the total CD8+ T cell fraction. *See e.g.* Maldonado, Arthritis Res Ther. 2003; 5(2): R91-R96. In such an instance, the columns can be arrayed to collect 4-fold more CD8+ cells than CD4+ cells in order to generate a 1:1 culture initiation ratio of the CD4+ cells and the CD8+ sub-population further containing CD62L+ cells. Thus, assuming a similar adsorption capacity and efficiency for each selection column, the CD8+ column can be about or approximately 4-fold larger than the CD4 selection column or the CD62L selection column. The size of the columns can also be adjusted for expected yield. For example, if each column is only 80% efficient, the size of each column can be adjusted to account for the efficiency of each subsequent selection.

[0191] For example a desired or chosen culture initiation composition can be one that contains 200×10^6 CD4+ cells and 200×10^6 CD8+/CD62L+ cells. In this example, assuming the ratios presented above, the sample to be enriched can contain at least or about 200×10^6 CD4+ cells and at least or about 800×10^6 CD8+ cells, approximately 25% of which (200×10^6) may also be CD62L+. Assuming that each 2 mL of selection matrix can enrich for about or

approximately 200 × 10^6 cells, a CD8 selection column with 8 mL of selection matrix can bind about or approximately 800 × 10^6 CD8+ cells while the flow-through passes to the CD4 selection column. A CD4 selection column with 2 mL of selection matrix can bind about or approximately 200 × 10^6 CD4+ cells. The CD8+ cells can be further enriched for CD62L by eluting the CD8+ cells into a CD62L selection column. In this exemplary instance, the CD62L selection column can contain 2 mL of selection matrix, thus enriching for about or approximately 200 × 10^6 CD8+/CD62L+ cells. The CD4 and CD8/CD62L columns can be eluted into a culture vessel, yielding the initiation culture composition or a composition having about or approximately a 1:1 culture-initiation ratio.

[0192] In another exemplary instance, a sample is enriched for CD4+ and CD8+/CCR7+ cells to yield a CD4+ to CD8+ ratio of 1:1. In this exemplary instance, the first selection can include enriching for CD8+ cells using a column with an adsorption capacity adjusted for the relative frequencies of CD4+ cells to CD8+/CCR7+ cells known to be present in the sample, or using ratios generally estimated to be in such samples. For example, the CCR7+ subpopulation of CD8+ cells collected from a human subject can sometimes be about 60% of the total CD8+ T cell fraction. *See e.g.* Chen, Blood. 2001 Jul 1;98(1):156-64. The columns can be arrayed to collect 3⅓-fold more CD8+ cells than CD4+ cells in order to generate a 1:1 culture initiation ratio. Assuming a similar adsorption capacity and efficiency for each selection column, the CD8+ column can be about or approximately 3⅓-fold larger than the CD4 selection column or the CCR7 selection column. The size of the columns can also be adjusted for expected yield. For example, if each column is only 80% efficient, the size of each column can be adjusted to account for the efficiency of each subsequent selection.

[0193] For example, a desired initiation culture can contain 200 × 10^6 CD4+ cells and 200 × 10^6 CD8+/CCR7+ cells. In this example, assuming the ratios presented above, the sample to be enriched can contain at least 200 × 10^6 CD4+ cells and at least or about 6.6 × 10^6 CD8+ cells, approximately 60% of which (200 × 10^6) may also be CCR7+. Assuming that each 2 mL of selection matrix can enrich for 200 × 10^6 cells, a CD8 selection column with 3⅓mL of selection matrix can bind about or approximately 6.6×10^6 CD8+ cells while the flow-through passes to the CD4 selection column. A CD4 selection column with 2 mL of selection matrix can bind about or approximately 200 × 10^6 CD4+ cells. The CD8+ cells can be further enriched for CD62L by eluting the CD8+ cells into a CCR7 selection column. In this exemplary instance, the CCR7 selection column can contain 1mL of selection matrix, thus enriching for about or approximately 200 × 10^6 CD8+/CCR7+ cells. The CD4 and CCCR7 columns can be eluted into a culture vessel, yielding the initiation culture containing about or approximately 200 × 10^6 CD4+ cells and about or approximately 200 × 10^6 CD8+/CCR7+ cells, or a 1:1 culture-initiation ratio.

[0194] It is within the level of a skilled artisan to empirically select or choose an appropriate volume, diameter or number of affinity matrix chromatography columns for the first and/or

second and/or third selection depending on the desired or chosen culture-initiating ratio of the generated composition containing enriched or selected cells, the expected frequency of each sub-population, the varying efficiencies of each selection column and other factors within the level of the skilled artisan and in view of the above exemplification.

B. Incubation of isolated cells

[0195] In some instances, the disclosed methods include one or more of various steps for incubating isolated cells and cell populations, such as populations isolated according to the methods herein, such as steps for incubating an isolated CD4+ T cell population, e.g., unfractionated CD4+ T cell population or subpopulation(s) thereof, and an isolated CD8+ T cell population, e.g., isolated unfractionated CD8+ T cell population or subpopulation(s) thereof. In some instances, the cell populations are incubated in a culture-initiating composition.

[0196] The plurality of isolated cell populations, e.g., the CD4+ and CD8+ cell populations (e.g., unfractionated or subpopulations thereof) are generally incubated with the cell populations combined in a culture-initiating composition in the same culture vessel, such as the same unit, chamber, well, column, tube, tubing set, valve, vial, culture dish, bag, or other container for culture or cultivating cells.

[0197] In some instances, the cell populations or cell types are present in the culture-initiating composition at a culture-initiating ratio, e.g., ratio of $CD4^+$ and $CD8^+$ cells, designed to achieve a particular desired output ratio, or a ratio that is within a certain range of tolerated error of such a desired output ratio, following the incubation and/or engineering steps, or designed to do so a certain percentage of the time. The output ratio, for example, can be a ratio optimal for achieving one or more therapeutic effects upon administration to a patient, e.g., via adoptive cell therapy. In some instances, the culture-initiating ratio is determined empirically, e.g., using a determination method as described herein, for example, to determine the optimal culture-initiating ratio for achieving a desired output ratio in a particular context.

[0198] The incubation steps can include culture, cultivation, stimulation, activation, propagation, including by incubation in the presence of stimulating conditions, for example, conditions designed to induce proliferation, expansion, activation, and/or survival of cells in the population, to mimic antigen exposure, and/or to prime the cells for genetic engineering, such as for the introduction of a genetically engineered antigen receptor.

[0199] The conditions can include one or more of particular media, temperature, oxygen content, carbon dioxide content, time, agents, e.g., nutrients, amino acids, antibiotics, ions, and/or stimulatory factors, such as cytokines, chemokines, antigens, binding partners, fusion proteins, recombinant soluble receptors, and any other agents designed to activate the cells. In one example, the stimulating conditions include one or more agent, e.g., ligand, which turns on or initiates TCR/CD3 intracellular signaling cascade in a T cell. Such agents can include

antibodies, such as those specific for a TCR component and/or costimulatory receptor, e.g., anti-CD3, anti-CD28, anti-4-1BB, for example, bound to solid support such as a bead, and/or one or more cytokines. Optionally, the expansion method may further comprise the step of adding anti-CD3 and/or anti CD28 antibody to the culture medium (e.g., at a concentration of at least about 0.5 ng/ml). Optionally, the expansion method may further comprise the step of adding IL-2 and/or IL-15 and/or IL-7 and/or IL-21 to the culture medium (e.g., wherein the concentration of IL-2 is at least about 10 units/m l).

[0200] In some instances, incubation is carried out in accordance with techniques such as those described in US Patent No. 6,040,1 77 to Riddell et al., Klebanoff et al.(2012) J Immunother. 35(9): 651-660, Terakuraet al. (2012) Blood.1:72-82, and/or Wang et al. (2012) J Immunother. 35(9):689-701.

[0201] In some instances, the cell populations, such as CD4$^+$ and CD8$^+$ populations or subpopulations, are expanded by adding to the culture-initiating composition feeder cells, such as non-dividing peripheral blood mononuclear cells (PBMC), (e.g., such that the resulting population of cells contains at least about 5, 10, 20, or 40 or more PBMC feeder cells for each T lymphocyte in the initial population to be expanded); and incubating the culture (e.g. for a time sufficient to expand the numbers of T cells). In some instances, the non-dividing feeder cells can comprise gamma- irradiated PBMC feeder cells. In some instances, the PBMC are irradiated with gamma rays in the range of about 3000 to 3600 rads to prevent cell division. In some instances, the feeder cells are added to culture medium prior to the addition of the populations of T cells.

[0202] In some instances, the stimulating conditions include temperature suitable for the growth of human T lymphocytes, for example, at least about 25 degrees Celsius, generally at least about 30 degrees, and generally at or about 37 degrees Celsius. In some instances, a temperature shift is effected during culture, such as from 37 degrees Celsius to 35 degrees Celsius. Optionally, the incubation may further comprise adding non-dividing EBV-transformed lymphoblastoid cells (LCL) as feeder cells. LCL can be irradiated with gamma rays in the range of about 6000 to 10,000 rads. The LCL feeder cells in some instances is disclosed in any suitable amount, such as a ratio of LCL feeder cells to initial T lymphocytes of at least about 10:1.

[0203] In instances, populations of CD4$^+$ and CD8$^+$ that are antigen specific can be obtained by stimulating naive or antigen specific T lymphocytes with antigen. For example, antigen-specific T cell lines or clones can be generated to cytomegalovirus antigens by isolating T cells from infected subjects and stimulating the cells in vitro with the same antigen. Naive T cells may also be used.

Interim assessment and adjustment

[0204] In some instances, the methods include assessment and/or adjustment of the cells or composition containing the cells, at a time subsequent to the initiation of the incubation or culture, such as at a time during the incubation. Assessment can include taking one or more measurements of a composition or vessel containing the cells, such as assessing cells for proliferation rate, degree of survival, phenotype, e.g., expression of one or more surface or intracellular markers, such as proteins or polynucleotides, and/or assessing the composition or vessel for temperature, media component(s), oxygen or carbon dioxide content, and/or presence or absence or amount or relative amount of one or more factors, agents, components, and/or cell types, including subtypes. Assessment in some instances includes determining an intermediate ratio of a plurality, e.g., two cell types, such as $CD4^+$ and $CD8^+$ T cells, in the composition or vessel being incubated. In some instances, the assessment is performed in an automated fashion, for example, using a device as described herein, and/or is set ahead of time to be carried out at certain time-points during incubation. In some instances, the outcome of the assessment, such as a determined interim ratio of two types of cells, indicates that an adjustment should be made, such as addition or removal of one or more cell types.

[0205] Adjustment can include adjusting any cell culture factor or parameter, such as temperature, length (time) for which incubation or a step thereof will be carried out (duration of incubation), replenishment, addition and/or removal of one or more components in the composition being incubated, e.g., media or buffer or components thereof, agents, e.g., nutrients, amino acids, antibiotics, ions, and/or stimulatory factors, such as cytokines, chemokines, antigens, binding partners, fusion proteins, recombinant soluble receptors, or cells or cell types or populations of cells. In some instances, the removal or addition of various components or other adjustment is carried out in an automated fashion, for example, using a device or system as described herein. In some instances, the system is programmed such that an adjustment is automatically initiated based on a certain readout from an interim assessment. For example, in some cases, a system or device is programmed to carry out one or more assessments at a particular time; the system or device in such cases can be further programmed such that a particular outcome of such an assessment, such as a particular ratio of one cell type to another, initiates a particular adjustment, such as addition of one or more of the cell types.

[0206] In some instances, the adjustment is carried out by addition or removal in a way that does not disrupt a closed environment containing the cells and compositions, such as by input and/or removal valves, designed to add or remove components while maintaining sterility, such as in one or more device or system as described herein.

[0207] In a particular instance, an interim ratio of $CD4^+$ to $CD8^+$ T cells is assessed during the incubation period. In some instances, the assessment is carried out after 1, 2, 3, 4, 5, 6, or 7 days, such as between 3 and 5 days, and/or at a time point in which all cells are in or suspected of being in cell cycle. In some instances, the interim ratio so-determined indicates that $CD4^+$ or $CD8^+$ T cells, such as cells of the isolated population of $CD4^+$ T cells or isolated

population of CD8$^+$ T cells (e.g., sub-population, such as central memory CD8$^+$ T cells), should be added to or enriched in the culture vessel or to the composition being incubated. Thus, in some instances, the assessment is followed by such an addition or removal, typically an addition. In some instances, multiple assessments and possible adjustments are carried out over the course of incubation, e.g., in an iterative fashion.

[0208] In some instances, where cells are engineered, e.g., to introduce a genetically engineered antigen receptor, the incubation in the presence of one or more stimulating agents continues during the engineering phase.

[0209] In some instances, the cells are incubated for at or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days, either in total or prior to engineering.

C. Engineering, engineered antigen receptors, and engineered cells

[0210] In some instances, the methods include genetic engineering of the isolated and/or incubated cells, such as to introduce into the cells recombinant genes for expression of molecules, such as receptors, e.g., antigen receptors, useful in the context of adoptive therapy.

[0211] Among the genes for introduction are those to improve the efficacy of therapy, such as by promoting viability and/or function of transferred cells; genes to provide a genetic marker for selection and/or evaluation of the cells, such as to assess *in vivo* survival or localization; genes to improve safety, for example, by making the cell susceptible to negative selection in vivo as described by Lupton S. D. et al., Mol. and Cell Biol., 11:6 (1991); and Riddell et al., Human Gene Therapy 3:319-338 (1992); see also the publications of PCT/US91/08442 and PCT/US94/05601 by Lupton et al. describing the use of bifunctional selectable fusion genes derived from fusing a dominant positive selectable marker with a negative selectable marker. This can be carried out in accordance with known techniques *(see,* e.g., Riddell et al., US Patent No. 6,040,177, at columns 14-17) or variations thereof that will be apparent to those skilled in the art based upon the present disclosure.

[0212] The engineering generally includes introduction of gene or genes for expression of a genetically engineered antigen receptor. Among such antigen receptors are genetically engineered T cell receptors (TCRs) and components thereof, and functional non-TCR antigen receptors, such as chimeric antigen receptors (CAR).

[0213] The antigen receptor in some instances specifically binds to a ligand on a cell or disease to be targeted, such as a cancer or other disease or condition, including those described herein for targeting with the disclosed methods and compositions. Exemplary antigens are orphan tyrosine kinase receptor ROR1, tEGFR, Her2, LI-CAM, CD19, CD20, CD22, mesothelin, CEA, and hepatitis B surface antigen, anti-folate receptor, CD23, CD24, CD30, CD33, CD38, CD44, EGFR, EGP-2, EGP-4, 0EPHa2, ErbB2, 3, or 4, FBP, fetal acethycholine e receptor, GD2, GD3, HMW-MAA, IL-22R-alpha, IL-13R-alpha2, kdr, kappa

light chain, Lewis Y, L1-cell adhesion molecule, MAGE-A1, mesothelin, MUC1, MUC16, PSCA, NKG2D Ligands, NY-ESO-1, MART-1, gp100, oncofetal antigen, ROR1, TAG72, VEGF-R2, carcinoembryonic antigen (CEA), prostate specific antigen, PSMA, Her2/neu, estrogen receptor, progesterone receptor, ephrinB2, CD123, CS-1, c-Met, GD-2, and MAGE A3 and/or biotinylated molecules, and/or molecules expressed by HIV, HCV, HBV or other pathogens.

Antigen Receptors

[0214] In one instance, the engineered antigen receptors are CARs. The CARs generally include genetically engineered receptors including an extracellular ligand binding domain linked to one or more intracellular signaling components. Such molecules typically mimic or approximate a signal through a natural antigen receptor and/or signal through such a receptor in combination with a costimulatory receptor.

[0215] In some instances, CARs are constructed with specificity for a particular marker, such as a marker expressed in a particular cell type to be targeted by adoptive therapy, e.g., a cancer marker. This is achieved in some instances by inclusion in the extracellular portion of the CAR one or more antigen binding molecule, such as one or more antigen-binding fragment, domain, or portion, or one or more antibody variable domains, and/or antibody molecules. In some instances, the CAR includes an antigen-binding portion or portions of an antibody molecule, such as a single-chain antibody fragment (scFv) derived from the variable heavy (VH) and variable light (VL) chains of a monoclonal antibody (mAb).

[0216] In some instances, the CAR comprises an antibody heavy chain domain that specifically binds a cell surface antigen of a cell or disease to be targeted, such as a tumor cell or a cancer cell, such as any of the target antigens described herein or known in the art.

[0217] In some instances, the tumor antigen or cell surface molecule is a polypeptide. In some instances, the tumor antigen or cell surface molecule is selectively expressed or overexpressed on tumor cells as compared to non-tumor cells of the same tissue.

[0218] In some instances, the CAR binds a pathogen-specific antigen. In some instances, the CAR is are specific for viral antigens (such as HIV, HCV, HBV, etc.), bacterial antigens, and/or parasitic antigens.

[0219] In some instances, the antigen-specific binding, or recognition component is linked to one or more transmembrane and intracellular signaling domains. In some instances, the CAR includes a transmembrane domain fused to the extracellular domain of the CAR. In one instance, the transmembrane domain that naturally is associated with one of the domains in the CAR is used. In some instances, the transmembrane domain is selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

[0220] The transmembrane domain in some instances is derived either from a natural or from a synthetic source. Where the source is natural, the domain in some instances is derived from any membrane-bound or transmembrane protein. Transmembrane regions include those derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD 16, CD22, CD33, CD37, CD64, CD80, CD86, CD 134, CD137, CD 154. Alternatively the transmembrane domain in some instances is synthetic. In some instances, the synthetic transmembrane domain comprises predominantly hydrophobic residues such as leucine and valine. In some instances, a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain.

[0221] In some instances, a short oligo- or polypeptide linker, for example, a linker of between 2 and 10 amino acids in length, such as one containing glycines and serines, e.g., glycine-serine doublet, is present and forms a linkage between the transmembrane domain and the cytoplasmic signaling domain of the CAR.

[0222] The CAR generally includes intracellular signaling component or components. In some instances, the CAR includes an intracellular component of the TCR complex, such as a TCR $CD3^+$ chain that mediates T-cell activation and cytotoxicity, e.g., CD3 zeta chain. Thus, in some instances, the antigen binding molecule is linked to one or more cell signaling modules. In some instances, cell signaling modules include CD3 transmembrane domain, CD3 intracellular signaling domains, and/or other CD transmembrane domains. In some instances, the CAR further includes a portion of one or more additional molecules such as Fc receptor γ, CD8, CD4, CD25, or CD16. For example, in some instances, the CAR includes a chimeric molecule between CD3-zeta (CD3-ζ) or Fc receptor γ and CD8, CD4, CD25 or CD16.

[0223] In some instances, upon ligation of the CAR, the cytoplasmic domain or intracellular signaling domain of the CAR activates at least one of the normal effector functions of the immune cell, e.g., T cell engineered to express the cell. For example, in some contexts, the CAR induces a function of a T cell such as cytolytic activity or T-helper activity, such as secretion of cytokines or other factors. In some instances, a truncated portion of an intracellular signaling domain of an antigen receptor component or costimulatory molecule. Such truncated portion in some instances is used in place of an intact immunostimulatory chain, for example, if it transduces the effector function signal. In some instances, the intracellular signaling domain or domains include the cytoplasmic sequences of the T cell receptor (TCR), and in some instances also those of co-receptors that in the natural context act in concert with such receptor to initiate signal transduction following antigen receptor engagement, and/or any derivative or variant of such molecules, and/or any synthetic sequence that has the same functional capability.

[0224] In the context of a natural TCR, full activation generally requires not only signaling through the TCR, but also a costimulatory signal. Thus, in some instances, to promote full activation, a component for generating secondary or co-stimulatory signal is also included in

the CAR. T cell activation is in some instances described as being mediated by two classes of cytoplasmic signaling sequences: those that initiate antigen- dependent primary activation through the TCR (primary cytoplasmic signaling sequences), and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal (secondary cytoplasmic signaling sequences). In some instances, the CAR includes one or both of such signaling components.

[0225] Primary cytoplasmic signaling sequences can in some instances regulate primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary cytoplasmic signaling sequences that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs. Examples of ITAM containing primary cytoplasmic signaling sequences include those derived from TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CDS, CD22, CD79a, CD79b, and CD66d. In some instances, cytoplasmic signaling molecule(s) in the CAR contain(s) a cytoplasmic signaling domain, portion thereof, or sequence derived from CD3 zeta.

[0226] In some instances, the CAR includes a signaling domain and/or transmembrane portion of a costimulatory receptor, such as CD28, 4-1BB, OX40, DAP10, and ICOS.

[0227] In certain instances, the intracellular signaling domain comprises a CD28 transmembrane and signaling domain linked to a CD3 intracellular domain. In some instances, the intracellular signaling domain comprises a chimeric CD28 and CD137 co-stimulatory domains, linked to a CD3 intracellular domain. In some instances, a CAR can also include a transduction marker (e.g., tEGFR). In some instances, the intracellular signaling domain of the $CD8^+$ cytotoxic T cells is the same as the intracellular signaling domain of the $CD4^+$ helper T cells. In some instances, the intracellular signaling domain of the $CD8^+$ cytotoxic T cells is different than the intracellular signaling domain of the $CD4^+$ helper T cells.

[0228] In some instances, the CAR encompasses two or more costimulatory domain combined with an activation domain, e.g., primary activation domain, in the cytoplasmic portion. One example is a receptor including intracellular components of CD3-zeta, CD28, and 4-1BB.

[0229] CARs and production and introduction thereof can include those described, for example, by published patent disclosures WO200014257, US6451995, US2002131960, US7446190, US8252592, EP2537416, US2013287748, and WO2013126726, and/or those described by Sadelain et al., Cancer Discov. 2013 April; 3(4): 388-398; Davila et al. (2013) PLoS ONE 8(4): e61338; Turtle et al., Curr. Opin. Immunol., 2012 October; 24(5): 633-39; Wu et al., Cancer, 2012 March 18(2): 160-75.

[0230] In some instances, the T cells are modified with a recombinant T cell receptor. In some instances, the recombinant TCR is specific for an antigen, generally an antigen present on a target cell, such as a tumor-specific antigen, an antigen expressed on a particular cell type

associated with an autoimmune or inflammatory disease, or an antigen derived from a viral pathogen or a bacterial pathogen.

[0231] In some instances, the T cells are engineered to express T-cell receptors (TCRs) cloned from naturally occurring T cells. In some instances, a high-affinity T cell clone for a target antigen (e.g., a cancer antigen) is identified, isolated from a patient, and introduced into the cells. In some instances, the TCR clone for a target antigen has been generated in transgenic mice engineered with human immune system genes (e.g., the human leukocyte antigen system, or HLA). *See,* e.g., tumor antigens (see, e.g., Parkhurst et al. (2009) Clin Cancer Res. 15: 169-180 and Cohen et al. (2005) J Immunol. 175:5799-5808. In some instances, phage display is used to isolate TCRs against a target antigen (see, e.g., Varela-Rohena et al. (2008) Nat Med. 14:1390-1395 and Li (2005) Nat Biotechnol. 23:349-354.

[0232] In some instances, after the T-cell clone is obtained, the TCR alpha and beta chains are isolated and cloned into a gene expression vector. In some instances, the TCR alpha and beta genes are linked via a picornavirus 2A ribosomal skip peptide so that both chains are coexpression. In some instances, genetic transfer of the TCR is accomplished via retroviral or lentiviral vectors, or via transposons (see, e.g., Baum et al. (2006) Molecular Therapy: The Journal of the American Society of Gene Therapy. 13:1050-1063; Frecha et al. (2010) Molecular Therapy: The Journal of the American Society of Gene Therapy. 18:1748-1757; an Hackett et al. (2010) Molecular Therapy: The Journal of the American Society of Gene Therapy. 18:674-683.

[0233] In some instances, gene transfer is accomplished by first stimulating T cell growth and the activated cells are then transduced and expanded in culture to numbers sufficient for clinical applications.

[0234] In some contexts, overexpression of a stimulatory factor (for example, a lymphokine or a cytokine) may be toxic to a subject. Thus, in some contexts, the engineered cells include gene segments that cause the cells to be susceptible to negative selection *in vivo,* such as upon administration in adoptive immunotherapy. For example in some instances, the cells are engineered so that they can be eliminated as a result of a change in the *in vivo* condition of the patient to which they are administered. The negative selectable phenotype may result from the insertion of a gene that confers sensitivity to an administered agent, for example, a compound. Negative selectable genes include the Herpes simplex virus type I thymidine kinase (HSV-I TK) gene (Wigler et al., Cell II :223, I977) which confers ganciclovir sensitivity; the cellular hypoxanthine phosphribosyltransferase (HPRT)gene, the cellular adenine phosphoribosyltransferase (APRT) gene, bacterial cytosine deaminase, (Mullen et al., Proc. Natl. Acad. Sci. USA. 89:33 (1992)).

[0235] In some instances, the cells further are engineered to promote expression of cytokines, such as proinflammatory cytokines, e.g., IL-2, IL-12, IL-7, IL-15, IL-21.

Introduction of the genetically engineered components

[0236] Various methods for the introduction of genetically engineered components, e.g., antigen receptors, e.g., CARs, are well known and may be used with the disclosed methods and compositions. Exemplary methods include those for transfer of nucleic acids encoding the receptors, including via viral, e.g., retroviral or lentiviral, transduction, transposons, and electroporation.

[0237] In some instances, recombinant nucleic acids are transferred into cells using recombinant infectious virus particles, such as, e.g., vectors derived from simian virus 40 (SV40), adenoviruses, adeno-associated virus (AAV). In some instances, recombinant nucleic acids are transferred into T cells using recombinant lentiviral vectors or retroviral vectors, such as gamma-retroviral vectors (see, e.g., Koste et al. (2014) Gene Therapy 2014 Apr 3. doi: 10.1038/gt.2014.25; Carlens et al. (2000) Exp Hematol 28(10): 1137-46; Alonso-Camino et al. (2013) Mol Ther Nucl Acids 2, e93; Park et al., Trends Biotechnol. 2011 November; 29(11): 550-557.

[0238] In some instances, the retroviral vector has a long terminal repeat sequence (LTR), e.g., a retroviral vector derived from the Moloney murine leukemia virus (MoMl,V), myeloproliferative sarcoma virus (MPSV), murine embryonic stem cell virus (MESV), murine stem cell virus (MSCV), spleen focus forming virus (SFFV), or adeno-associated virus (AAV). Most retroviral vectors are derived from murine retroviruses. In some instances, the retroviruses include those derived from any avian or mammalian cell source. The retroviruses typically are amphotropic, meaning that they are capable of infecting host cells of several species, including humans. In one instance, the gene to be expressed replaces the retroviral gag, pol and/or env sequences. A number of illustrative retroviral systems have been described (e.g., U.S. Pat. Nos. 5,219,740; 6,207,453; 5,219,740; Miller and Rosman (1989) BioTechniques 7:980-990; Miller, A. D. (1990) Human Gene Therapy 1:5-14; Scarpa et al. (1991) Virology 180:849-852; Burns et al. (1993) Proc. Natl. Acad. Sci. USA 90:8033-8037; and Boris-Lawrie and Temin (1993) Cur. Opin. Genet. Develop. 3:102-109.

[0239] Methods of lentiviral transduction are known. Exemplary methods are described in, e.g., Wang et al. (2012) J. Immunother. 35(9): 689-701; Cooper et al. (2003) Blood. 101:1637-1644; Verhoeyen et al. (2009) Methods Mol Biol. 506: 97-114; and Cavalieri et al. (2003) Blood. 102(2): 497-505.

[0240] In some instances, recombinant nucleic acids are transferred into T cells via electroporation (see, e.g., Chicaybam et al, (2013) PLoS ONE 8(3): e60298 and Van Tedeloo et al. (2000) Gene Therapy 7(16): 1431-1437). In some instances, recombinant nucleic acids are transferred into T cells via transposition (see, e.g., Manuri et al. (2010) Hum Gene Ther 21(4): 427-437; Sharma et al. (2013) Molec Ther Nucl Acids 2, e74; and Huang et al. (2009) Methods Mol Biol 506: 115-126). Other methods of introducing and expressing genetic material in immune cells include calcium phosphate transfection (e.g., as described in Current Protocols in Molecular Biology, John Wiley & Sons, New York. N.Y.), protoplast fusion, cationic liposome-

mediated transfection; tungsten particle-facilitated microparticle bombardment (Johnston, Nature, 346: 776-777 (1990)); and strontium phosphate DNA co- precipitation (Brash et al., Mol. Cell Biol., 7: 2031-2034 (1987)).

[0241] In some instances, the same CAR is introduced into each of CD4$^+$ and CD8$^+$ T lymphocytes. In some instances, a different CAR is introduced into each of CD4$^+$ and CD8$^+$ T lymphocytes. In some instances, the CAR in each of these populations has an antigen binding molecule that specifically binds to the same antigen. In some instances, the CAR in each of these populations binds to a different molecule. In some instances, the CAR in each of these populations has cellular signaling modules that differ. In some instances each of the CD4$^+$ or CD8$^+$ T lymphocytes have been sorted in to naive, central memory, effector memory or effector cells prior to transduction.

[0242] In other instances, the cells, e.g., T cells, are not engineered to express recombinant receptors, but rather include naturally occurring antigen receptors specific for desired antigens, such as tumor-infiltrating lymphocytes and/or T cells cultured *in vitro* or *ex vivo,* e.g., during the incubation step(s), to promote expansion of cells having particular antigen specificity. For example, in some instances, the cells are produced for adoptive cell therapy by isolation of tumor-specific T cells, e.g. autologous tumor infiltrating lymphocytes (TIL). The direct targeting of human tumors using autologous tumor infiltrating lymphocytes can in some cases mediate tumor regression (see Rosenberg SA, et al.(1988) N Engl JMed. 319:1676-1680). In some instances, lymphocytes are extracted from resected tumors . In some instances, such lymphocytes are expanded in vitro. In some instances, such lymphocytes are cultured with lymphokines (e.g., IL-2). In some instances, such lymphocytes mediate specific lysis of autologous tumor cells but not allogeneic tumor or autologous normal cells.

[0243] In some instances, the incubation and/or engineering steps and/or the methods generally result in a desired output ratio (or a ratio that is within a tolerated error or difference from such a ratio), or do so within a certain percentage of the time that the methods are performed.

D. Cryopreservation

[0244] In some instances, the disclosed methods include steps for freezing, e.g., cryopreserving, the cells, either before or after isolation, incubation, and/or engineering. In some instances, the freeze and subsequent thaw step removes granulocytes and, to some extent, monocytes in the cell population. In some instances, the cells are suspended in a freezing solution, e.g., following a washing step to remove plasma and platelets. Any of a variety of known freezing solutions and parameters in some instances may be used. One example involves using PBS containing 20% DMSO and 8% human serum albumin (HSA), or other suitable cell freezing media. This is then diluted 1:1 with media so that the final concentration of DMSO and HSA are 10% and 4%, respectively. The cells are then frozen to

-80° C. at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank.

II. Kits and systems

[0245] Also disclosed are systems, apparatuses, and kits useful in performing the disclosed methods. In one example, a single system is disclosed which carries out one or more of the isolation, cell preparation, separation, e.g., separation based on density, affinity, sensitivity to one or more components, or other property, washing, processing, incubation, culture, and/or formulation steps of the methods. In some instances, the system is used to carry out each of these steps in a closed or sterile environment, for example, to minimize error, user handling and/or contamination. In one example, the system is a system as described in International Patent Application, Publication Number WO2009/072003, or US 20110003380 A1.

[0246] In some instances, the system or apparatus carries out one or more, e.g., all, of the isolation, processing, engineering, and formulation steps in an integrated or self-contained system, and/or in an automated or programmable fashion. In some instances, the system or apparatus includes a computer and/or computer program in communication with the system or apparatus, which allows a user to program, control, assess the outcome of, and/or adjust various instances of the processing, isolation, engineering, and formulation steps.

[0247] Also disclosed are kits for carrying out the disclosed methods. In some instances, the kits include antibodies or other binding partners, generally coupled to solid supports, for the isolation, e.g., for immunoaffinity-based separation steps, of the methods.

[0248] In some instances, the kit comprises antibodies for positive and negative selection, bound to magnetic beads. In one instance, the kit comprises instructions to carry out selection starting with a sample, such as a PBMC sample, by selecting based on expression of a first surface marker, recognized by one or more of the antibodies disclosed with the kit, retaining both positive and negative fractions. In some instances, the instructions further include instructions to carry out one or more additional selection steps, starting with the positive and/or negative fractions derived therefrom, for example, while maintaining the compositions in a contained environment and/or in the same separation vessel.

[0249] In one instance, a kit comprises anti-CD4, anti-CD14, anti-CD45RA, anti-CD14, and anti-CD62L antibodies, bound to magnetic beads. In one instance, the kit comprises instructions to carry out selection starting with a sample, such as a PBMC sample, by selecting based on CD4 expression, retaining both positive and negative fractions, and on the negative fraction, further subjecting the fraction to a negative selection using the anti-CD14, anti-CD45RA antibodies, and a positive selection using the anti-CD62L antibody, in either order. Alternatively, the components and instructions are adjusted according to any of the separation instances described herein.

[0250] In some instances, the kit further includes instructions to transfer the cells of the populations isolated by the selection steps to a culture, cultivation, or processing vessel, while maintaining the cells in a self-contained system. In some instances, the kit includes instructions to transfer the different isolated cells at a particular ratio.

III. Cells, compositions, and methods of administration

[0251] Also disclosed are cells, cell populations, and compositions (including pharmaceutical and therapeutic compositions) containing the cells and populations, produced by the disclosed methods. Also disclosed are methods, e.g., therapeutic methods for administrating the cells and compositions to subjects, e.g., patients.

[0252] Disclosed are methods of administering the cells, populations, and compositions, and uses of such cells, populations, and compositions to treat or prevent diseases, conditions, and disorders, including cancers. In some instances, the cells, populations, and compositions are administered to a subject or patient having the particular disease or condition to be treated, e.g., via adoptive cell therapy, such as adoptive T cell therapy. In some instances, cells and compositions prepared by the disclosed methods, such as engineered compositions and end-of-production compositions following incubation and/or other processing steps, are administered to a subject, such as a subject having or at risk for the disease or condition. In some instances, the methods thereby treat, e.g., ameliorate one or more symptom of, the disease or condition, such as by lessening tumor burden in a cancer expressing an antigen recognized by an engineered T cell.

[0253] Methods for administration of cells for adoptive cell therapy are known and may be used in connection with the disclosed methods and compositions. For example, adoptive T cell therapy methods are described, e.g., in US Patent Application Publication No. 2003/0170238 to Gruenberg et al; US Patent No. 4,690,915 to Rosenberg; Rosenberg (2011) Nat Rev Clin Oncol. 8(10):577-85). See, e.g., Themeli et al. (2013) Nat Biotechnol. 31(10): 928-933; Tsukahara et al. (2013) Biochem Biophys Res Commun 438(1): 84-9; Davila et al. (2013) PLoS ONE 8(4): e61338.

[0254] In some instances, the cell therapy, e.g., adoptive T cell therapy, is carried out by autologous transfer, in which the cells are isolated and/or otherwise prepared from the subject who is to receive the cell therapy, or from a sample derived from such a subject. Thus, in some instances, the cells are derived from a subject, e.g., patient, in need of a treatment and the cells, following isolation and processing are administered to the same subject.

[0255] In some instances, the cell therapy, e.g., adoptive T cell therapy, is carried out by allogeneic transfer, in which the cells are isolated and/or otherwise prepared from a subject other than a subject who is to receive or who ultimately receives the cell therapy, e.g., a first subject. In such instances, the cells then are administered to a different subject, e.g., a second subject, of the same species. In some instances, the first and second subjects are genetically

identical. In some instances, the first and second subjects are genetically similar. In some instances, the second subject expresses the same HLA class or supertype as the first subject.

[0256] In some instances, the subject, e.g., patient, to whom the cells, cell populations, or compositions are administered is a mammal, typically a primate, such as a human. In some instances, the primate is a monkey or an ape. The subject can be male or female and can be any suitable age, including infant, juvenile, adolescent, adult, and geriatric subjects. In some instances, the subject is a non-primate mammal, such as a rodent.

[0257] Also disclosed are pharmaceutical compositions for use in such methods.

[0258] Among the diseases, conditions, and disorders for treatment with the disclosed compositions, cells, methods and uses are tumors, including solid tumors, hematologic malignancies, and melanomas, and infectious diseases, such as infection with a virus or other pathogen, e.g., HIV, HCV, HBV, CMV, and parasitic disease. In some instances, the disease or condition is a tumor, cancer, malignancy, neoplasm, or other proliferative disease. Such diseases include but are not limited to 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.

[0259] In some instances, the disease or condition is an infectious disease or condition, such as, but not limited to, viral, retroviral, bacterial, and protozoal infections, immunodeficiency, Cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus, BK polyomavirus. In some instances, the disease or condition is an autoimmune or inflammatory disease or condition, such as arthritis, e.g., rheumatoid arthritis (RA), Type I diabetes, systemic lupus erythematosus (SLE), inflammatory bowel disease, psoriasis, scleroderma, autoimmune thyroid disease, Grave's disease, Crohn's disease multiple sclerosis, asthma, and/or a disease or condition associated with transplant.

[0260] In some instances, the antigen associated with the disease or disorder is selected from the group consisting of orphan tyrosine kinase receptor ROR1, tEGFR, Her2, Ll-CAM, CD19, CD20, CD22, mesothelin, CEA, and hepatitis B surface antigen, anti-folate receptor, CD23, CD24, CD30, CD33, CD38, CD44, EGFR, EGP-2, EGP-4, 0EPHa2, ErbB2, 3, or 4, FBP, fetal acethycholine e receptor, GD2, GD3, HMW-MAA, IL-22R-alpha, IL-13R-alpha2, kdr, kappa light chain, Lewis Y, L1-cell adhesion molecule, MAGE-A1, mesothelin, MUC1, MUC16, PSCA, NKG2D Ligands, NY-ESO-1, MART-1, gp100, oncofetal antigen, ROR1, TAG72, VEGF-R2, carcinoembryonic antigen (CEA), prostate specific antigen, PSMA, Her2/neu, estrogen receptor, progesterone receptor, ephrinB2, CD123, CS-1, c-Met, GD-2, and MAGE A3 and/or biotinylated molecules, and/or molecules expressed by HIV, HCV, HBV or other pathogens.

[0261] In some instances, the cells and compositions are administered to a subject in the form of a pharmaceutical composition, such as a composition comprising the cells or cell populations and a pharmaceutically acceptable carrier or excipient. The pharmaceutical compositions in some instances additionally comprise other pharmaceutically active agents or drugs, such as chemotherapeutic agents, e.g., asparaginase, busulfan, carboplatin, cisplatin, daunorubicin, doxorubicin, fluorouracil, gemcitabine, hydroxyurea, methotrexate, paclitaxel, rituximab, vinblastine, vincristine, etc. In some instances, the agents are administered in the form of a salt, e.g., a pharmaceutically acceptable salt. Suitable pharmaceutically acceptable acid addition salts include those derived from mineral acids, such as hydrochloric, hydrobromic, phosphoric, metaphosphoric, nitric, and sulphuric acids, and organic acids, such as tartaric, acetic, citric, malic, lactic, fumaric, benzoic, glycolic, gluconic, succinic, and arylsulphonic acids, for example, p-toluenesulphonic acid.

[0262] The choice of carrier will in the pharmaceutical composition is determined in part by the particular engineered CAR or TCR, vector, or cells expressing the CAR or TCR, as well as by the particular method used to administer the vector or host cells expressing the CAR. Accordingly, there are a variety of suitable formulations. For example, the pharmaceutical composition can contain preservatives. Suitable preservatives may include, for example, methylparaben, propylparaben, sodium benzoate, and benzalkonium chloride. In some instances, a mixture of two or more preservatives is used. The preservative or mixtures thereof are typically present in an amount of about 0.0001% to about 2% by weight of the total composition.

[0263] In addition, buffering agents in some instances are included in the composition. Suitable buffering agents include, for example, citric acid, sodium citrate, phosphoric acid, potassium phosphate, and various other acids and salts. In some instances, a mixture of two or more buffering agents is used. The buffering agent or mixtures thereof are typically present in an amount of about 0.001 % to about 4% by weight of the total composition. Methods for preparing administrable pharmaceutical compositions are known. Exemplary methods are described in more detail in, for example, Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins; 21st ed. (May 1 , 2005).

[0264] In certain instances, the pharmaceutical composition is formulated as an inclusion complex, such as cyclodextrin inclusion complex, or as a liposome. Liposomes can serve to target the host cells (e.g., T-cells or NK cells) to a particular tissue. Many methods are available for preparing liposomes, such as those described in, for example, Szoka et al., Ann. Rev. Biophys. Bioeng., 9: 467 (1980), and U.S. Patents 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

[0265] In some instances, the pharmaceutical composition employs time-released, delayed release, and/or sustained release delivery systems, such that the delivery of the composition occurs prior to, and with sufficient time to cause, sensitization of the site to be treated. Many types of release delivery systems are available and known to those of ordinary skill in the art.

Such systems in some instances can avoid repeated administrations of the composition, thereby increasing convenience to the subject and the physician.

[0266] In some instances, the he pharmaceutical composition comprises the cells or cell populations in an amount that is effective to treat or prevent the disease or condition, such as a therapeutically effective or prophylactically effective amount. Thus, in some instances, the methods of administration include administration of the cells and populations at effective amounts. Therapeutic or prophylactic efficacy in some instances is monitored by periodic assessment of treated subjects. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful and can be determined. The desired dosage can be delivered by a single bolus administration of the composition, by multiple bolus administrations of the composition, or by continuous infusion administration of the composition.

[0267] In some instances, the cells are administered at a desired dosage, which in some instances includes a desired dose or number of cells or cell type(s) and/or a desired ratio of cell types. Thus, the dosage of cells in some instances is based on a total number of cells (or number per kg body weight) and a desired ratio of the individual populations or sub-types, such as the CD4+ to CD8+ ratio. In some instances, the dosage of cells is based on a desired total number (or number per kg of body weight) of cells in the individual populations or of individual cell types. In some instances, the dosage is based on a combination of such features, such as a desired number of total cells, desired ratio, and desired total number of cells in the individual populations.

[0268] In some instances, the populations or sub-types of cells, such as $CD8^+$ and $CD4^+$ T cells, are administered at or within a tolerated difference of a desired dose of total cells, such as a desired dose of T cells. In some instances, the desired dose is a desired number of cells or a desired number of cells per unit of body weight of the subject to whom the cells are administered, e.g., cells/kg. In some instances, the desired dose is at or above a minimum number of cells or minimum number of cells per unit of body weight. In some instances, among the total cells, administered at the desired dose, the individual populations or sub-types are present at or near a desired output ratio (such as $CD4^+$ to $CD8^+$ ratio), e.g., within a certain tolerated difference or error of such a ratio.

[0269] In some instances, the cells are administered at or within a tolerated difference of a desired dose of one or more of the individual populations or sub-types of cells, such as a desired dose of CD4+ cells and/or a desired dose of CD8+ cells. In some instances, the desired dose is a desired number of cells of the sub-type or population, or a desired number of such cells per unit of body weight of the subject to whom the cells are administered, e.g., cells/kg. In some instances, the desired dose is at or above a minimum number of cells of the population or sub-type, or minimum number of cells of the population or sub-type per unit of body weight.

[0270] Thus, in some instances, the dosage is based on a desired fixed dose of total cells and a desired ratio, and/or based on a desired fixed dose of one or more, e.g., each, of the individual sub-types or sub-populations. Thus, in some instances, the dosage is based on a desired fixed or minimum dose of T cells and a desired ratio of CD4$^+$ to CD8$^+$ cells, and/or is based on a desired fixed or minimum dose of CD4$^+$ and/or CD8$^+$ cells.

[0271] In certain instances, the cells, or individual populations of sub-types of cells, are administered to the subject at a range of about one million to about 100 billion cells, such as, e.g., 1 million to about 50 billion cells (e.g., about 5 million cells, about 25 million cells, about 500 million cells, about 1 billion cells, about 5 billion cells, about 20 billion cells, about 30 billion cells, about 40 billion cells, or a range defined by any two of the foregoing values), such as about 10 million to about 100 billion cells (e.g., about 20 million cells, about 30 million cells, about 40 million cells, about 60 million cells, about 70 million cells, about 80 million cells, about 90 million cells, about 10 billion cells, about 25 billion cells, about 50 billion cells, about 75 billion cells, about 90 billion cells, or a range defined by any two of the foregoing values), and in some cases about 100 million cells to about 50 billion cells (e.g., about 120 million cells, about 250 million cells, about 350 million cells, about 450 million cells, about 650 million cells, about 800 million cells, about 900 million cells, about 3 billion cells, about 30 billion cells, about 45 billion cells) or any value in between these ranges.

[0272] In some instances, the dose of total cells and/or dose of individual sub-populations of cells is within a range of between at or about 10^4 and at or about 10^9 cells/kilograms (kg) body weight, such as between 10^5 and 10^6 cells / kg body weight, for example, at or about 1×10^5 cells/kg, 1.5×10^5 cells/kg, 2×10^5 cells/kg, or 1×10^6 cells/kg body weight. For example, in some instances, the cells are administered at, or within a certain range of error of, between at or about 10^4 and at or about 10^9 T cells/kilograms (kg) body weight, such as between 10^5 and 10^6 T cells / kg body weight, for example, at or about 1×10^5 T cells/kg, 1.5×10^5 T cells/kg, 2×10^5 T cells/kg, or 1×10^6 T cells/kg body weight.

[0273] In some instances, the cells are administered at or within a certain range of error of between at or about 10^4 and at or about 10^9 CD4$^+$ and/or CD8$^+$ cells/kilograms (kg) body weight, such as between 10^5 and 10^6 CD4$^+$ and/or CD8$^+$cells / kg body weight, for example, at or about 1×10^5 CD4$^+$ and/or CD8$^+$ cells/kg, 1.5×10^5 CD4$^+$ and/or CD8$^+$ cells/kg, 2×10^5 CD4$^+$ and/or CD8$^+$ cells/kg, or 1×10^6 CD4$^+$ and/or CD8$^+$ cells/kg body weight.

[0274] In some instances, the cells are administered at or within a certain range of error of, greater than, and/or at least about 1×10^6, about 2.5×10^6, about 5×10^6, about 7.5×10^6, or about 9×10^6 CD4$^+$ cells, and/or at least about 1×10^6, about 2.5×10^6, about 5×10^6, about 7.5×10^6, or about 9×10^6 CD8+ cells, and/or at least about 1×10^6, about 2.5×10^6, about 5×10^6, about 7.5×10^6, or about 9×10^6 T cells. In some instances, the cells are administered

at or within a certain range of error of between about 10^8 and 10^{12} or between about 10^{10} and 10^{11} T cells, between about 10^8 and 10^{12} or between about 10^{10} and 10^{11} CD4$^+$ cells, and/or between about 10^8 and 10^{12} or between about 10^{10} and 10^{11} CD8$^+$ cells.

[0275] In some instances, the cells are administered at or within a tolerated range of a desired output ratio of multiple cell populations or sub-types, such as CD4+ and CD8+ cells or sub-types. In some instances, the desired ratio can be a specific ratio or can be a range of ratios. for example, in some instances, the desired ratio (e.g., ratio of CD4$^+$ to CD8$^+$ cells) is between at or about 5:1 and at or about 5:1 (or greater than about 1:5 and less than about 5:1), or 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, such as at or about 5:1, 4.5:1, 4:1, 3.5:1, 3:1, 2.5:1, 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. In some instances, 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, including any value in between these ranges.

[0276] The cell populations and compositions in some instances are administered to a subject using standard administration techniques, including oral, intravenous, intraperitoneal, subcutaneous, pulmonary, transdermal, intramuscular, intranasal, buccal, sublingual, or suppository administration. In some instances, the cell populations are administered parenterally. The term "parenteral," as used herein, includes intravenous, intramuscular, subcutaneous, rectal, vaginal, and intraperitoneal administration. In some instances, the cell populations are administered to a subject using peripheral systemic delivery by intravenous, intraperitoneal, or subcutaneous injection.

[0277] The cell populations obtained using the methods described herein in some instances are co-administered with one or more additional therapeutic agents or in connection with another therapeutic intervention, either simultaneously or sequentially in any order. In some contexts, the cells are co-administered with another therapy sufficiently close in time such that the cell populations enhance the effect of one or more additional therapeutic agents, or vice versa. In some instances, the cell populations are administered prior to the one or more additional therapeutic agents. In some instances, the cell populations are administered after to the one or more additional therapeutic agents.

[0278] Following administration of the cells, the biological activity of the engineered cell populations in some instances is measured, e.g., by any of a number of known methods. Parameters to assess include specific binding of an engineered or natural T cell or other immune cell to antigen, in vivo, e.g., by imaging, or ex vivo, e.g., by ELISA or flow cytometry. In certain instances, the ability of the engineered cells to destroy target cells can be measured using any suitable method known in the art, such as cytotoxicity assays described in, for example, Kochenderfer et al., J. Immunotherapy, 32(7): 689-702 (2009), and Herman et al. J.

Immunological Methods, 285(1): 25-40 (2004). In certain instances, the biological activity of the cells is measured by assaying expression and/or secretion of one or more cytokines, such as CD 107a, IFNγ, IL-2, and TNF. In some instances the biological activity is measured by assessing clinical outcome, such as reduction in tumor burden or load.

[0279] In certain instances, the engineered cells are further modified in any number of ways, such that their therapeutic or prophylactic efficacy is increased. For example, the engineered CAR or TCR expressed by the population can be conjugated either directly or indirectly through a linker to a targeting moiety. The practice of conjugating compounds, e.g., the CAR or TCR, to targeting moieties is known in the art. See, for instance, Wadwa et al., J. Drug Targeting 3: 1 1 1 (1995), and U.S. Patent 5,087,616.

IV. Definitions

[0280] As used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, "a" or "an" means "at least one" or "one or more."

[0281] Throughout this disclosure, various aspects of the claimed subject matter are presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the claimed subject matter. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the claimed subject matter. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the claimed subject matter, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the claimed subject matter. This applies regardless of the breadth of the range.

[0282] The term "about" as used herein refers to the usual error range for the respective value readily known to the skilled person in this technical field. Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se.*

[0283] As used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, "a" or "an" means "at least one" or "one or more."

[0284] Throughout this disclosure, various aspects of the claimed subject matter are presented in a range format. It should be understood that the description in range format is merely for

convenience and brevity and should not be construed as an inflexible limitation on the scope of the claimed subject matter. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the claimed subject matter. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the claimed subject matter, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the claimed subject matter. This applies regardless of the breadth of the range.

[0285] As used herein, "percent (%) amino acid sequence identity" and "percent identity" when used with respect to an amino acid sequence (reference polypeptide sequence) is defined as the percentage of amino acid residues in a candidate sequence (e.g., a streptavidin mutein) that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[0286] An amino acid substitution may include replacement of one amino acid in a polypeptide with another amino acid. Amino acids generally can be grouped according to the following common side-chain properties:

1. (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
2. (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
3. (3) acidic: Asp, Glu;
4. (4) basic: His, Lys, Arg;
5. (5) residues that influence chain orientation: Gly, Pro;
6. (6) aromatic: Trp, Tyr, Phe.

[0287] Non-conservative amino acid substitutions will involve exchanging a member of one of these classes for another class.

[0288] As used herein, a subject includes any living organism, such as humans and other mammals. Mammals include, but are not limited to, humans, and non-human animals, including farm animals, sport animals, rodents and pets.

[0289] As used herein, a composition refers to any mixture of two or more products, substances, or compounds, including cells. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

[0290] As used herein, "depleting" when referring to one or more particular cell type or cell population, refers to decreasing the number or percentage of the cell type or population, e.g., compared to the total number of cells in or volume of the composition, or relative to other cell types, such as by negative selection based on markers expressed by the population or cell, or by positive selection based on a marker not present on the cell population or cell to be depleted. The term does not require complete removal of the cell, cell type, or population from the composition.

[0291] As used herein, "enriching" when referring to one or more particular cell type or cell population, refers to increasing the number or percentage of the cell type or population, e.g., compared to the total number of cells in or volume of the composition, or relative to other cell types, such as by positive selection based on markers expressed by the population or cell, or by negative selection based on a marker not present on the cell population or cell to be depleted. The term does not require complete removal of other cells, cell type, or populations from the composition and does not require that the cells so enriched be present at or even near 100 % in the enriched composition.

[0292] As used herein, the terms "treatment," "treat," and "treating," refer to complete or partial amelioration or reduction of a disease or condition or disorder, or a symptom, adverse effect or outcome, or phenotype associated therewith. In certain embodiments, the effect is therapeutic, such that it partially or completely cures a disease or condition or adverse symptom attributable thereto.

[0293] As used herein, a "therapeutically effective amount" of a compound or composition or combination refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result, such as for treatment of a disease, condition, or disorder, and/or pharmacokinetic or pharmacodynamic effect of the treatment. The therapeutically effective amount may vary according to factors such as the disease state, age, sex, and weight of the subject, and the populations of cells administered.

[0294] As used herein, a statement that a cell or population of cells is "positive" for a particular marker refers to the detectable presence on or in the cell of a particular marker, typically a surface marker. When referring to a surface marker, the term refers to the presence of surface expression as detected by flow cytometry, for example, by staining with an antibody that specifically binds to the marker and detecting said antibody, wherein the staining is detectable by flow cytometry at a level substantially above the staining detected carrying out the same procedure with an isotype-matched control or fluorescence minus one (FMO) gating control under otherwise identical conditions and/or at a level substantially similar to that for cell known to be positive for the marker, and/or at a level substantially higher than that for a cell known to be negative for the marker.

[0295] As used herein, a statement that a cell or population of cells is "negative" for a particular marker refers to the absence of substantial detectable presence on or in the cell of a particular marker, typically a surface marker. When referring to a surface marker, the term refers to the absence of surface expression as detected by flow cytometry, for example, by staining with an antibody that specifically binds to the marker and detecting said antibody, wherein the staining is not detected by flow cytometry at a level substantially above the staining detected carrying out the same procedure with an isotype-matched control or fluorescence minus one (FMO) gating control under otherwise identical conditions, and/or at a level substantially lower than that for cell known to be positive for the marker, and/or at a level substantially similar as compared to that for a cell known to be negative for the marker.

[0296] In some embodiments, a decrease in expression of one or markers refers to loss of 1 $\log^{10}$ in the mean fluorescence intensity and/or decrease of percentage of cells that exhibit the marker of at least about 20% of the cells, 25% of-the cells, 30% of the cells, 35% of the cells, 40% of the cells, 45% of the cells, 50% of the cells, 55% of the cells, 60% of the cells, 65% of the cells, 70% of the cells, 75% of the cells, 80% of the cells, 85% of the cells, 90% of the cell, 95% of the cells, and 100% of the cells and any % between 20 and 100% when compared to a reference cell population. In some embodiments, a cell population positive for one or markers refers to a percentage of cells that exhibit the marker of at least about 50% of the cells, 55% of the cells, 60% of the cells, 65% of the cells, 70% of the cells, 75% of the cells, 80% of the cells, 85% of the cells, 90% of the cell, 95% of the cells, and 100% of the cells and any % between 50 and 100% when compared to a reference cell population.

VI. EXAMPLES

[0297] The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1: Generation of a CD4+ and CD8+ T cell Composition Using a Single Process Stream By Immunomagnetic Separation For Genetic Engineering And Adoptive Cell Therapy

[0298] In an exemplary method, a CD4$^+$ T cell population and a CD8$^+$ T cell population, enriched for central memory T cells, are isolated from an apheresis product sample, and subsequently incubated and engineered, followed by administration to a subject. The isolation procedure is carried out using a single process stream by immunomagnetic separation using the CliniMACS$^®$ Prodigy system. The process is streamlined as compared to other methods, in which CD4$^+$ cells are separated from a first apheresis fraction and CD8$^+$ cells are separated and further depleted/enriched, from a second apheresis fraction, using a CliniMACS$^®$ Prodigy

device and three separate tubing sets.

[0299] The streamlined process is performed using one tubing set for isolation of the CD4$^+$ and CD8$^+$ cell populations, without transferring the cell populations from one vessel (e.g., tubing set) to another.

[0300] The CD4$^+$ cell population is isolated by incubating an apheresis sample with a CliniMACS$^®$ CD4 reagent. The cells are then separated by the CliniMACS$^®$ Prodigy device set to run an enrichment program, and both cell fractions (i.e., the immunomagnetically selected enriched CD4$^+$ cell fraction and the flow-through cell fraction) are retained. The enriched (positive) fraction is an isolated CD4$^+$ T cell population. A population of CD8$^+$ T cells is isolated by incubating the flow-through (negative) fraction with a CliniMACS$^®$ CD14 reagent and a CliniMACS$^®$ CD45RA reagent or CD19 reagent. The CliniMACS$^®$ Prodigy device is set to run a depletion program. The cell/reagent mixture then is separated by the CliniMACS$^®$ Prodigy device using the same tubing set used in the first separation step. The flow-through (negative) fraction from which CD14$^+$/CD45RA$^+$ or CD14+/CD19+ cells have been depleted is incubated with CliniMACS$^®$ CD62L reagent. The cell/reagent mixture is separated using the CliniMACS$^®$ Prodigy device running an enrichment program using the same tubing set. The positive fraction is an isolated CD8$^+$ cell population enriched for central memory cells.

[0301] The isolated CD4+ and CD8+ populations are combined in the same culture vessel in a culture-initiating composition at a culture initiation ratio. The culture-initiation ratio is designed to achieve a particular desired output ratio, or a ratio that is within a certain range of tolerated error of such a desired output ratio, following the incubation and/or engineering steps, or designed to do so a certain percentage of the time. The cells are incubated under stimulating conditions, such as using anti-CD3/anti-CD28 beads in the presence IL-2 (100 IU/mL) for 72 hours at 37 °C, within the Prodigy system.

[0302] The cell composition is optionally periodically assessed and/or adjusted at one or more times subsequent to the initiation of the incubation or culture, such as at a time during the incubation. Assessment includes measuring proliferation rate, measuring degree of survival, determining phenotype, e.g., expression of one or more surface or intracellular markers, such as proteins or polynucleotides, and/or adjusting the composition or vessel for temperature, media component(s), oxygen or carbon dioxide content, and/or presence or absence or amount or relative amount of one or more factors, agents, components, and/or cell types, including subtypes.

[0303] Cells so-incubated then are genetically engineered, by introducing into the cells recombinant genes for expression of recombinant antigen receptors, such as chimeric antigen receptors (CARs) or recombinant TCRs. The introduction is performed in the contained environment within the CliniMACS$^®$ Prodigy device, with CD4$^+$ and CD8$^+$ cells present in the

same composition and vessel. The method results in an output composition with engineered CD4$^+$ and CD8$^+$ T cells.

Example 2: Generation of a CD4+ and CD8+ T cell Composition by Sequential Purification in a Closed System

[0304] This example demonstrates an exemplary method of enriching or selecting for a CD4+ T cell population and a CD8+ T cell population from an apheresis product sample obtained from a subject. The process is performed using a closed system with multiple chromatography columns for sequential positive selection of the CD4+ and CD8+ T cell populations from the same starting sample.

A. Enrichment for CD4+ T cell Population and CD8+ T Cell Population

[0305] In the exemplary process, depicted in Figure 1A, a series of immunochromatographic selection columns and removal columns are arranged in a closed apparatus **14** operably connected to each other and a peristaltic pump **8** through various tubing lines and valves **13** to control flow of liquid phase.

[0306] The first selection column **1** contains a chosen volume of an affinity chromatography matrix 3, such as an agarose resin, such as a resin as described in published U.S. Patent Appl. No. US2015/0024411 for the isolation of T cells, such as one with an exclusion limit designed to be greater than the size of a T cell, such as an agarose as obtained from Agarose Beads Technologies, Madrid, Spain, with a reduced exclusion size compared to Superflow™ Agarose, which has an exclusion size of 6 × 10^6 Daltons. The matrix in the first column is bound to multimers of Strep-Tactin$^®$ (e.g. containing a streptavidin mutant set forth in any of SEQ ID NO:12, 13, 15 or 16, IBA GmbH, Germany or as described in International Published PCT Appl. Nos. WO 2014/076277). The second selection column **2** contains a chosen volume of an affinity chromatography matrix **4,** such as an agarose resin, such as a resin as described above, which is also bound to multimers of Strep-Tactin$^®$.

[0307] A reservoir containing an anti-CD8 Fab fragment **18** is loaded to run through the pump **8**, tubing, and valves **13** such that the anti-CD8 Fab is applied to the first selection column **1,** whereby the anti-CD8 Fab becomes immobilized on the Strep-Tactin$^®$ on the affinity matrix **3** with a Twin Strep-Tag$^®$ (e.g. set forth in SEQ ID NO: 10; IBA GmbH) in the Fab Fragment, which is fused to the carboxy-terminus of its heavy chain (see e.g. published U.S. Patent Appl. No. US2015/0024411). In some embodiments, a washing buffer from a washing buffer reservoir **6**, such as phosphate buffered saline (PBS) containing 0.5% bovine serum albumin, human serum albumin, or recombinant human serum albumin, is loaded to run through the first selection column via operably coupled tubing. The flow-through is directed to the waste

container **10** via valves **13** operably connecting the first selection column and waste container. In some embodiments, the washing step is repeated a plurality of times.

[0308] A reservoir containing an anti-CD4 Fab fragment **19** is loaded to run through the pump **8**, tubing, and valves **13** such that the anti-CD4 Fab is applied to the second selection column **2**, whereby the anti-CD8 Fab becomes immobilized on the Strep-Tactin® on the affinity matrix **4** with a Twin Strep-Tag®. In some embodiments, a washing buffer from a washing buffer reservoir **6** is run through the second selection column via operably coupled tubing. The flow-through is directed to the waste container **10** via valves **13** operably connecting the second selection column and waste container. In some embodiments, the washing step is repeated a plurality of times.

[0309] The volume of affinity matrix reagent contained in the first selection column and the second selection column can be the same or different, and can be chosen based on the desired yield of the selection and/or desired ratio of CD4+ cells to CD8+ cells following selection. The volume so chosen is based on an assumption of an average yield of 1×10^8 cells being capable of selection per each 1 mL volume of filled column. In one exemplary process, the column volumes are the same, for example, using a 2 mL volume for the column for the anti-CD8 Fab fragment and a 2 mL column for the anti-CD4 Fab fragment. The column length and/or column diameter can be chosen to fit the desired volume, for example, to achieve a desired culture-initiation ratio of CD4+ cells to CD8+ cells. In some embodiments, to accommodate the volume of affinity matrix, a plurality of columns having immobilized thereto the same Fab reagent can be added directly in series and operably connected to each other via a tubing line.

[0310] To achieve selection of cells, an apheresis sample is applied to the first selection column 1, whereby CD8+ T cells, if present in the sample, remain bound to the resin 3 of the first column, as unselected cells (containing CD8- cells) pass through the column. The number of cells in the starting sample used in some examples is chosen to be above the number of cells to be selected based on the combined volume of the matrices, such as an amount greater than the capacity of the column for each selection (e.g. an amount having greater than 200 million CD8+ cells and greater than 200 million CD4+ cells, e.g., greater by at least about 10 % or 20 % or greater). The flow-through containing unselected cells (negative fraction) then passes from the first column to a second selection column 2 via operably coupled tubing. CD4+ T cells remain bound to the resin 4 in the second column. The flow through containing fluid and further unselected cells (negative cells) is directed to a waste container 10 via valves 13 operably connecting the second selection column and waste container.

[0311] In some embodiments, the first selection column can alternatively be an anti-CD4 affinity chromatography matrix and the second selection column can be an anti-CD8 affinity chromatography matrix.

[0312] From a washing buffer reservoir **6,** a washing buffer is run through the first column and

the second column via operably coupled tubing. The flow-through containing any cells that are washed from the first and second columns is directed to the waste container **10** via valves **13** operably connecting the second selection column and waste container. In some embodiments, the washing step is repeated a plurality of times.

[0313] From an elution buffer reservoir 7, a buffer containing an eluent, such as low concentrations of biotin or an analog thereof, for example 2.5 mM desthiobiotin, is loaded to run through the first column and the second column via operably coupled tubing. In some embodiments, the eluent contains cell culture media. The flow-through, containing enriched CD4+ and CD8+ cells and residual biotin or analog, is directed to a removal chamber **9** to remove biotin or an analog thereof. The removal chamber **9** is a column of Superflow™ Sepharose® beads having Strep-Tactin® immobilized thereto with a volume sufficient to remove biotin or a biotin analog from the sample, such as a column having a bed volume of 6 mL with a binding capacity of 300 nanomol biotin/mL. The flow-through containing enriched cells positively selected for CD4+ and CD8+ is directed to a culture vessel **12,** such as a bag, via valves operably connecting the removal chamber 9 and culture vessel. In some embodiments, the elution step is repeated.

[0314] In some embodiments, after performing the elution step, an activation buffer containing a T-cell activation reagent (e.g. anti-CD3, anti-CD28, IL-2, IL-15, IL-7, and/or IL-21) replaces the wash buffer and is directed through the removal chamber and flow through directed to the culture vessel. The positive fraction collected in the culture vessel is an isolated and combined CD4+ and CD8+ cell population.

B. Enrichment for a CD4+ T cell Population and a CD8+ T Cell Population, Enriched for Cells Expressing a Marker on Central Memory T (T$_{CM}$) Cells

[0315] In the exemplary process, depicted in Figure 1B, a series of immunochromatographic selection columns and removal columns are arranged in a closed apparatus **14** operably connected to each other and a peristaltic pump **8** through various tubing lines and valves **13** to control flow of liquid phase.

[0316] The first selection column **1** and second selection column **2** are the same as described above with respect to Example 2A. In addition, the process further includes a third selection column **15** that contains a chosen volume of an affinity matrix **17,** such as an agarose resin, such as a resin as described in published U.S. Patent Appl. No. US2015/0024411 for the isolation of T cells, such as one as described above in Example 2A.

[0317] The anti-CD8 Fab and the anti-CD4 Fab are applied to the first column and second column, respectively, as described in Example 2A. A reservoir containing a further Fab fragment against a marker expressed on central memory T (T$_{CM}$) cells **20,** such as one of CD28, CD62L, CCR7, CD27 or CD127, is loaded to run through the pump **8,** tubing, and valves

13 such that the further Fab is applied to the third selection column **15,** whereby the further Fab becomes conjugated to the Strep-Tactin® on the affinity matrix **17** with a Twin Strep-Tag® (SEQ ID NO: 10; IBA GmbH) in the Fab Fragment, which is fused to the carboxy-terminus of its heavy chain, as described in Example 2A. In some embodiments, a washing buffer, as described in Example 2A, is loaded to run through the third selection column via operably coupled tubing. The flow-through is directed to the waste container **10** via valves **13** operably connecting the third selection column and waste container. In some embodiments, the washing step is repeated a plurality of times.

[0318] The volume of affinity matrix reagent contained in the first selection, second and/or third selection columns can be the same or different, and can be chosen based on the desired yield of the selection and/or desired ratio of CD4+ cells to CD8+ cells enriched for cells expressing a marker expressed on central memory T (T_{CM}) cells following selection. The volume so-chosen is based on the assumption of an average yield of 1×10^8 cells being capable of selection per each 1 mL volume of filled column. Also, for a particular chosen culture-initiation ratio, for example, a culture-initiation ratio of 1:1 of CD4+ cells to a population containing CD8+ cells enriched for a marker expressed on central memory T (T_{CM}) cells (e.g. a population of CD8+ cells enriched based on further selection for one of CD28, CD62L, CCR7, CD27 or CD127), the volume of the matrix for selecting the parent population of the enriched population, e.g. the CD8+ cells, is larger in comparison to the matrix used for selecting the other of the CD4+ or CD8+ T cell population, e.g. the CD4+ population. The amount or extent in which the volume of the matrix used to select for the parent population of the further enriched population, e.g. the matrix containing anti-CD8 Fab fragment, is greater than the volume of the other matrix or other matrices is chosen based on the fraction or percentage of the further enriched population of cells in the sample (e.g. CD8+/CD28+, CD8+/CD62L+, CD8+/CCR7+, CD8+/CD27+ or CD8+/CD127+) compared to the fraction or percentage of the parent population, e.g. CD8+ cells, present in the sample. This can be estimated based on averages among patients or healthy donors, or it can be measured for a given patient on which selection is being performed prior to determining the size of the columns to use.

[0319] In one exemplary process, the column volumes used in the process are, for example, a 2 mL column with anti-CD4 Fab fragments, a 6 mL column with anti-CD8 Fab fragments, and a 2 mL column with anti-CD62L Fab fragments. The column length and/or column diameter can be chosen to fit the desired volume, for example, to achieve a desired culture-initiation ratio of CD4+ cells to CD8+ cells enriched for cells expressing a marker expressed on central memory T (T_{CM}) cells, e.g.CD8+/CD28+, CD8+/CD62L+, CD8+/CCR7+, CD8+/CD27+ or CD8+/CD127+ cells. In some embodiments, to accommodate the volume of affinity matrix, a plurality of columns having immobilized thereto the same Fab reagent can be added directly in series and operably connected to each other via a tubing line.

[0320] To achieve selection of cells, an apheresis sample is applied to the first selection column 1, whereby CD8+ T cells, if present in the sample, remain bound to the resin of the first column as unselected cells (containing CD8- cells) pass through the column. The number of

cells in the starting sample used in some examples is chosen to be above the number of cells to be selected based on the combined volume of the matrices such as an amount greater than the capacity of the column for each selection (e.g. an amount greater than 200 million CD8+/CD62L+ cells and greater than 200 million CD4+ cells, e.g. greater by at least about 10% or 20 % or greater). The flow-through containing unselected cells (negative fraction) is directed to pass to the second column **2** via a valve **13** operably connecting the first and second column. CD4+ T cells remain bound to the resin in the second column. The flow-through containing fluid and further unselected cells (negative cells) is directed to a first waste container **10** via valves **13** operably connecting the second selection column and negative fraction container.

[0321] From a washing buffer reservoir **6,** a washing buffer, such as described in Example 2A, is loaded to run through the first column and the second column via a valve and tubes operably connecting the columns. The flow-through containing any cells that are washed from the first and second columns is directed to the waste container **10** via valves **13** operably connecting the second selection column and waste container. In some embodiments, the washing step is repeated a plurality of times.

[0322] From an elution buffer reservoir 7, a buffer containing an eluent, such as biotin or an analog thereof, for example 2.5 mM desthiobiotin, is loaded to run through the second column **2** via valves **13** and tubing operably connecting the elution buffer reservoir and second column. In some embodiments, the elution buffer contains cell culture media. The flow-through, containing enriched CD4+ cells and residual biotin, is directed to a removal chamber **9** to remove biotin, such as described in Example 2A, which is operably connected to the second column via a valve **13** and tubing. The flow-through containing enriched cells positively selected for CD4+ is directed to a culture vessel **12,** such as a bag, via valves **13** operably connecting the removal chamber 9 and culture vessel. In some embodiments, the elution step is repeated. In some embodiments, after performing the elution step, an activation buffer containing a T-cell activation reagent (e.g. anti-CD3, anti-CD28, IL-2, IL-15, IL-7, and/or IL-21) replaces the wash buffer and is directed via valves and tubing from the washing buffer reservoir to the second column, removal chamber and into the culture vessel. The positive fraction collected in the culture vessel is an isolated CD4+ cell population.

[0323] From an elution buffer reservoir **7,** a buffer containing an eluent, such as biotin, is loaded to run through the first column **1** via a valve **13** and tubing operably connecting the elution buffer reservoir and first column. In some embodiments, the elution buffer contains cell culture media. The flow-through, containing enriched CD8+ cells and residual biotin or an analog thereof, is directed to a removal chamber **9** to remove biotin or a biotin analog, such as described in Example 2A, which is operably connected to the first column via a valve **13** and tubing. The flow-through containing enriched cells positively selected for CD8+ is directed to pass to a third column **15** via a valve **13** operably connecting the removal chamber **9** and third column. A CD62L+ subset of the CD8+ cells remain bound to the resin in the third column. The flow through containing fluid and further unselected cells (negative cells) is directed to a second waste container **11** via valves **13** operably connecting the third selection column and

second waste container.

[0324] From a washing buffer reservoir 6, a washing buffer, such as described in Example 2A, is loaded to run through the third column 15 via valves 13 and tubing operably connecting the wash buffer reservoir and third column. The flow through containing any cells that are washed from the third column is directed to the second waste container 11 via valves 13 operably connecting the third selection column and second waste container. In some embodiments, the washing step is repeated a plurality of times.

[0325] From an elution buffer reservoir 7, a buffer containing an eluent, such as biotin or an analog thereof, for example 2.5 mM desthiobiotin, is loaded to run through the third column 15 via a valve 13 and tubing operably connecting the elution buffer reservoir and third column. In some embodiments, the elution buffer contains cell culture media. The flow-through, containing CD8+ cells enriched for central memory T (T_{CM}) cells expressing one of CD28, CD62L, CCR7, CD27 or CD127,and residual biotin or analog thereof, is directed to a removal chamber 9 to remove biotin or a biotin analog, such as described in Example 2A, which is operably connected to the third column via a valve 13 and tubing. The flow-through containing enriched T cell memory cells positively selected for CD8 and a marker expressed on central memory T (T_{CM}) cells, such as one of CD28, CD62L, CCR7, CD27 or CD127, is directed to the culture vessel 12, such as a bag, via valves 13 operably connecting the removal chamber 9 and culture vessel. In some embodiments, the culture vessel contains a T-cell activation reagent, a cell culture media, or both. In some embodiments, the elution step is repeated. In some embodiments, after performing the elution step, an activation buffer containing a T-cell activation reagent (e.g. anti-CD3, anti-CD28, IL-2, IL-15, IL-7, and/or IL-21) replaces the wash buffer and is directed via valves and tubing from the washing buffer reservoir to the first and/or third column, removal chamber and into the culture vessel. The positive fraction containing CD8+ cells and cells positive for a marker on central memory T (T_{CM}) cells, such as one of CD28, CD62L, CCR7, CD27 or CD127, is collected in the culture vessel with the CD4+ positive fraction previously collected. In some embodiments, the steps of the process can be performed in a different order, such as by first enriching for cells containing CD8+ cells and cells positive for a marker on central memory T (T_{CM}) cells, such as one of CD28, CD62L, CCR7, CD27 or CD127 before enriching CD4+ cells.

Example 3: Generation of a CD4+ and CD8+ T cell Composition by Sequential Purification in a Closed System For Use in Genetic Engineering and Adoptive Cell Therapy

[0326] This example demonstrates a procedure for selecting and generating a composition of cells containing CD4+ and CD8+ T cells, such as CD4+ and CD8+ T cells present in a culture-initiation ratio, for incubation/activation and transduction in methods associated with genetic engineering of cells for use in connection with adoptive cell therapy.

[0327] A composition of cells generated by selection of CD4+ and CD8+ cells, performed as described in either Example 2A (CD4+ and CD8+) or Example 2B (CD4+ and CD8+ enriched for CD62L+), is incubated under stimulating conditions, such as using anti-CD3/anti-CD28 in the presence IL-2 (100 IU/mL), for example, for 72 hours at 37 °C. Stimulated cells then are genetically engineered by introducing into the cells recombinant genes for expression of recombinant antigen receptors, such as chimeric antigen receptors (CARs) or recombinant TCRs, for example, by viral transduction. In some embodiments, following the introduction, cells are further incubated, generally at 37 degrees C, for example, to allow for cell expansion.

[0328] The method results in an output composition with engineered CD4$^+$ and CD8$^+$ T cells. In some embodiments, based on the chosen volume of the selection columns, the ratio of CD4+ to CD8+ cells in the composition that is incubated under the stimulating conditions prior to engineering (culture-initiation ratio) results in a particular desired output ratio of CD4+ to CD8+ cells or of engineered CD4+ cells to engineered CD8+ cells, or such a ratio that is within a certain range of tolerated error of such a desired output ratio, following the incubation, stimulation and/or engineering steps. In some embodiments, such a desired output ratio or ratio within the range of tolerated error is achieved a certain tolerated percentage of the time.

Example 4: Selection of Cells using Sub-Optimal Yield Concentrations of Fab-Coated Surfaces

[0329] Human apheresis-derived PBPC samples, at a range of different cell numbers, were incubated in a single composition with magnetic microbeads conjugated to anti-CD4 Fabs and magnetic microbeads conjugated to anti-CD8 Fabs, with gentle mixing for about 30 minutes. This incubation was followed by elution of non-selected cells, and recovery using magnetic field carried out as described above. Incubation using between 0.05 and 1.5 mL of each of microbead reagent per million cells produced low yields of CD4+ or CD8+ cells, respectively (cells recovered for the respective selection compared with positive cells in the incubation), which in this study were generally in the range of 15-70 %). On average, greater yields were observed at higher concentrations of reagents per cell using such suboptimal yield concentrations.

[0330] Accordingly, in one example, a selection is carried out, in which PBMCs are incubated with suboptimal concentrations per cell number of beads coupled to a CD4-binding and beads coupled to a CD8-binding agent, and cells recovered using a magnetic field. Based on the observed relationship between concentration of the reagent per cell and yield for the beads used, one or the other of the CD4-binding or CD8-binding reagent is included at a higher concentration (e.g., higher number of Cd4 or CD8 binding molecules included per cell), thereby producing an output ratio of CD8+: CD4+ cells following selection, which is greater than or less than 1, such as 1.5:1 or 2:1 or 3:1 or 1:1.5, 1:2, or 1:3. The output ratio may be selected based on the desired ratio of CD8 vs. CD4 cells in a composition containing engineered cells to be produced following one or more additional steps, such as activation, transduction, and/or

expansion.

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FREMGANGSMÅDER TIL ISOLERING, DYRKNING OG GENMANIPULERING AF
IMMUNCELLEPOPULATIONER TIL ADOPTIV TERAPI

PATENTKRAV

1.   Sammensætning, der omfatter en dosis genmanipulerede CD4+- og CD8+-T-celler, til anvendelse i en fremgangsmåde til behandling af en autoimmun eller inflammatorisk sygdom hos et individ, hvor:

(i)   CD4+- og CD8+-T-cellerne forekommer i et forhold på mellem 5:1 og 1:5;

(ii)   dosen af CD4+- og CD8+-T-celler udgør mellem 1 x 10^6 og 1 x 10^{11} celler;

(iii)   CD4+- og CD8+-T-cellerne er genmanipuleret med en kimær antigenreceptor (CAR), hvor CAR'en:

(a)   genkender et antigen, der udtrykkes af celler fra den autoimmune eller inflammatoriske sygdom, hvor antigenet er CD19;

(b)   omfatter et ekstracellulært antigengenkendelsesdomæne og et intracellulært signaleringsdomæne, der omfatter en ITAM-holdig sekvens og et intracellulært signaleringsdomæne af et co-stimulerende T-celle-molekyle; og

(iv)   CD4+- og CD8+-T-cellerne stammer fra en prøve, der er opnået fra individet med den autoimmune eller inflammatoriske sygdom.

2.   Sammensætning til anvendelse ifølge krav 1, hvor CD4+- og CD8+-T-cellerne forekommer i et forhold på mellem 1:3 og 3:1.

3.   Sammensætning til anvendelse ifølge krav 1, hvor CD4+- og CD8+-T-cellerne forekommer i et forhold på mellem 2:1 og 1:5.

4.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-3, hvor dosen af CD4+- og CD8+-T-celler udgør mellem 1 x 10^6 og 1 x 10^9 celler.

5.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-4, hvor dosen af CD4+- og CD8+-T-celler udgør mellem 1 x 10^6 og 25 x 10^6 celler.

6.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-5, hvor dosen af CD4+- og CD8+-T-celler udgør 5 x 10^6 celler.

7.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-5, hvor dosen af CD4+- og CD8+-T-celler udgør 10 x 10^6 celler.

8.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-5, hvor dosen af CD4+- og CD8+-T-celler udgør 20 x 10^6 celler.

9.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-8, hvor prøven er en blodprøve eller en blodafledt prøve, eventuelt hvor prøven er en prøve af hvide blodlegemer, en afereseprøve, en leukafereseprøve, en prøve af mononukleære celler fra perifert blod og fuldblod.

10.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-9, hvor prøven er afledt af et aferese- eller et leukafereseprodukt.

11.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-10, hvor den autoimmune eller inflammatoriske sygdom er valgt fra gruppen bestående af artritis, Type I-diabetes, systemisk lupus
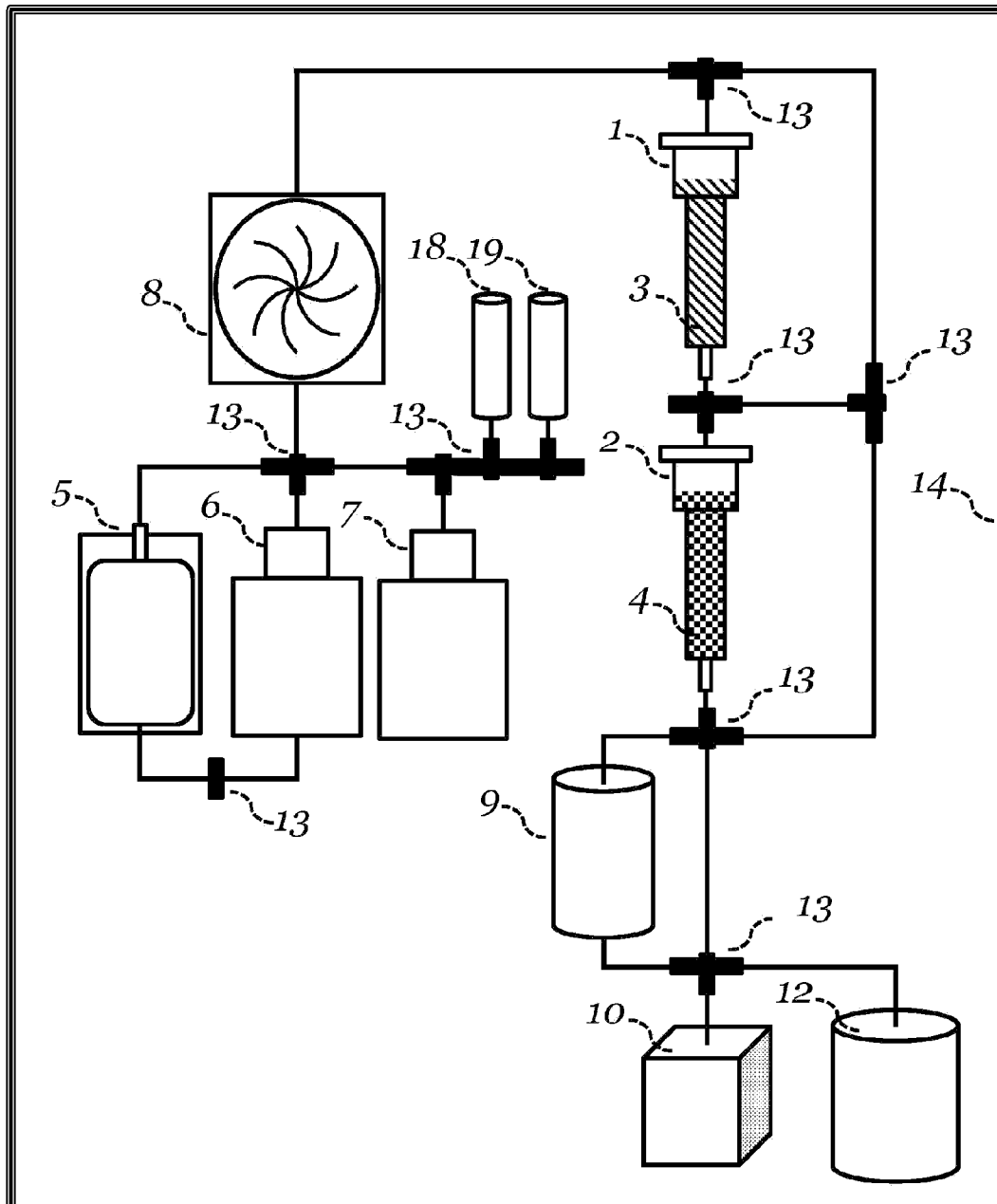
- 2 -

erythematosus (SLE), inflammatorisk tarmsygdom, psoriasis, sklerodermi, autoimmun thyroidea-sygdom, Graves' sygdom, Crohns sygdom, multipel sklerose, astma og en sygdom eller lidelse, der er forbundet med transplantation.

12.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-11, hvor den autoimmune eller inflammatoriske sygdom er SLE.

13.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-12, hvor CAR'en omfatter et enkeltkædet antistoffragment (scFV), der binder CD19.

14.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-13, hvor det intracellulære cellulære signaleringsdomæne er afledt af CD3zeta.

15.   Sammensætning til anvendelse ifølge et hvilket som helst af kravene 1-14, hvor CAR'en indbefatter et signaleringsdomæne og/eller en transmembran del af en co-stimulerende receptor, eventuelt valgt blandt CD28, 4-1BB, OX40, DAP10 og ICOS.
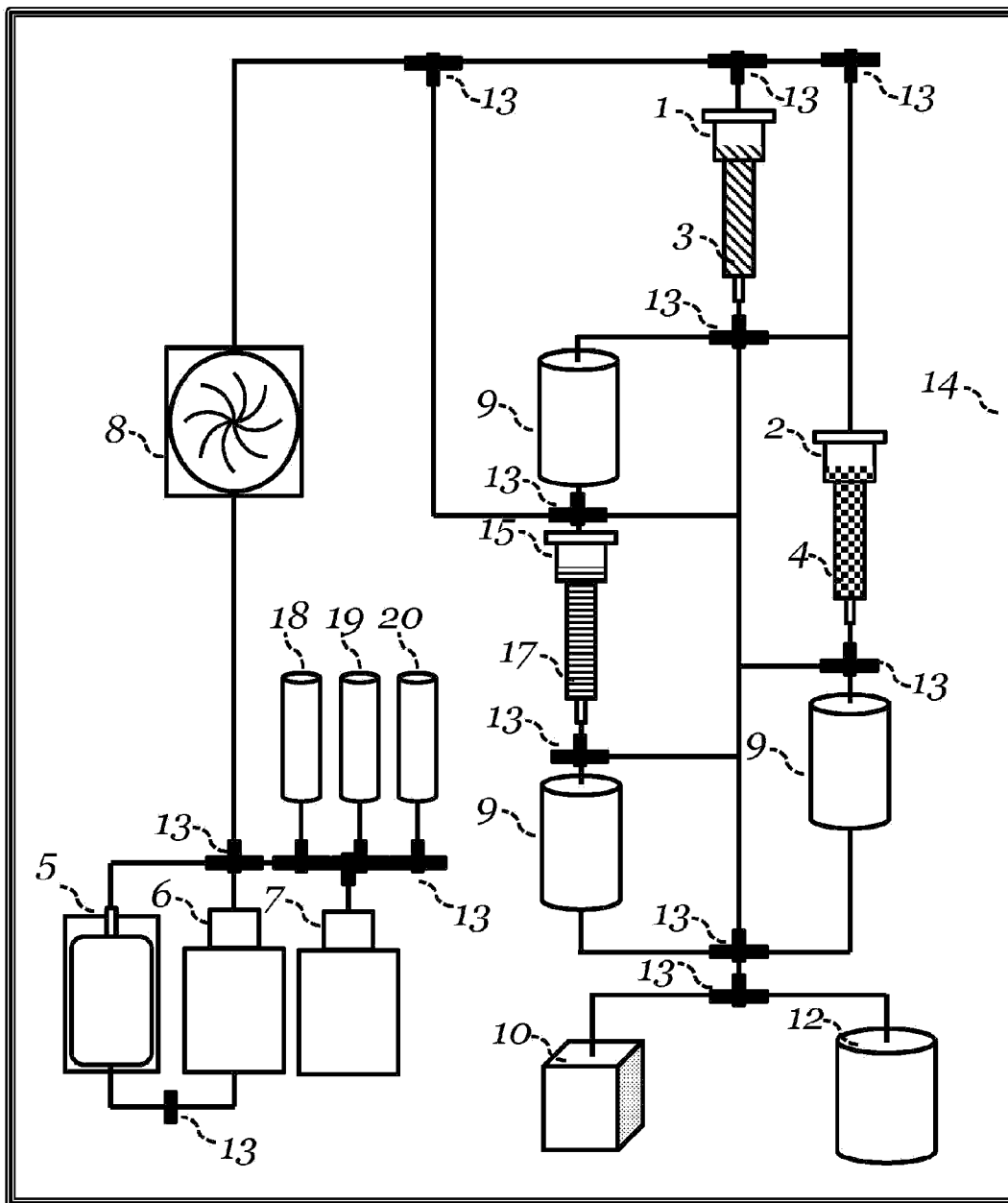
DRAWINGS

Drawing

FIGURE 1A

Tubing ————

FIGURE 1B

Tubing ———

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.