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(71) Applicant: **JUNO THERAPEUTICS, INC.** [US/US]; 400 Dexter Avenue N, Suite 1200, Seattle, Washington 98109 (US).

(72) Inventor: **WESNER, John Matthew**; 400 Dexter Avenue N, Suite 1200, Seattle, Washington 98109 (US).

(74) Agent: **AHN, Sejin** et al.; Morrison & Foerster LLP, 12531 High Bluff Drive, Suite 100, San Diego, California 92130-2040 (US).

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(54) Title: CLOSED-SYSTEM CRYOGENIC VESSELS

(57) Abstract: The present disclosure is directed to closed-system cryogenic vessels for biomedical material with needleless removal. The needleless removal can reduce damage to biomedical material inside the vessel, allow for greater recovery of biomedical material from the vessel, and reduce exposure risk to users of the closed-system cryogenic vessels during removal of the biomedical material from the vessel. In some aspects, the vessels can be used to store or package a composition of cells, such as a composition containing engineered cells, including in connection with adoptive cell therapy. Also provided are articles of manufacture, kits and methods.

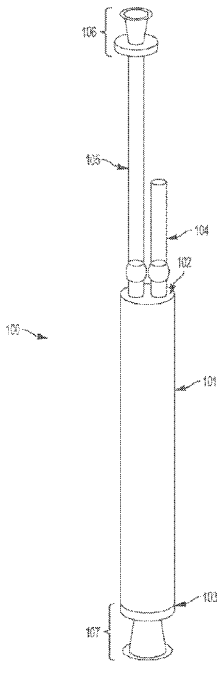

FIG. 1

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CLOSED-SYSTEM CRYOGENIC VESSELS

Cross-Reference to Related Applications

[0001] This application claims priority to U.S. provisional application 62/584,722, filed November 10, 2017, entitled "CLOSED-SYSTEM CRYOGENIC VESSELS," the contents of which are incorporated by reference in their entirety.

Field of the Disclosure

[0002] This disclosure relates in some aspects to vessels for biomedical material. More particularly, this disclosure relates in certain aspects to closed-system cryogenic vessels for biomedical material with needleless removal. In some aspects, the vessels can be used to store or package a composition of cells, such as a composition containing engineered cells, including in connection with adoptive cell therapy.

Background

[0003] Various biomedical materials such as cells and tissues can be inserted into a variety of vessels, such as for cryopreservation in order to extend the biomedical materials' viability for use in biomedical applications. When ready for use, the biomedical material can be removed from the vessel, such as by using a syringe needle. Improved vessels for storage of biomedical materials, including cells, are disclosed herein.

Summary

[0004] Provided are cryogenic vessels for biomedical material with needleless removal. In some embodiments, the cryogenic vessels are closed-system vessels. The biomedical material vessels disclosed herein can reduce risks during the removal of biomedical material. Specifically, needleless removal can reduce damage to biomedical material inside the vessel, allow for greater recovery of biomedical material from the vessel, and/or can reduce risk to users of the biomedical material vessels during removal of the biomedical material from the vessel. In addition, the retrieval ports of the biomedical material vessels disclosed herein can increase user efficiency when compared to retrieval ports that require a syringe needle.

[0005] In some embodiments, a biomedical material vessel includes a vial with a top and an open bottom; an inlet tube supported by the top of the vial, wherein the inlet tube is fluidly connected to an interior of the vial and includes an loading port; and a needleless retrieval port fluidly connected to the open bottom of the vial, wherein the needleless retrieval port provides direct access to biomedical material in the vial. In some embodiments, the loading port is a

needleless loading port. In some embodiments, the needleless loading port includes a luer lock connection fitting. In some embodiments, the biomedical material vessel includes a cap configured to engage with the luer lock connection fitting of the needleless loading port. In some embodiments, the needleless retrieval port includes a luer lock connection fitting. In some embodiments, the biomedical material vessel includes a cap configured to engage with the luer lock connection fitting of the needleless retrieval port. In some embodiments, the biomedical material vessel includes an air vent tube supported by the top of the vial and fluidly connected to an interior of the vial. In some embodiments, the air vent tube includes a filter. In some embodiments, the filter is a microbial barrier filter. In some embodiments, the top of the vial includes a tube adaptor fluidly connected between the inlet tube and the interior of the vial. In some embodiments, the top of the vial includes a tube adaptor fluidly connected between the air vent tube and the interior of the vial. In some embodiments, the openings of the two tube adaptors into the interior of the vial are separated by a wall. In some embodiments, the retrieval port is a self-closing needleless retrieval port. In some embodiments, the biomedical material vessel is made up of USP Class VI compliant material.

[0006] In some embodiments, a method of storing and retrieving biomedical material includes injecting biomedical material into a vial via a loading port of an inlet tube, wherein the inlet tube is supported by a top of the vial; and retrieving the biomedical material from the vial via a needleless retrieval port fluidly connected to an open bottom of the vial, wherein the needleless retrieval port provides direct access to the biomedical material in the vial. In some embodiments, the method includes cryogenically freezing the biomedical material in the vial and thawing the cryogenically frozen biomedical material in the vial. In some embodiments, the method includes sealing the inlet tube. In some embodiments, the method includes sealing an air vent tube supported by the top of the vial and fluidly connected to an interior of the vial. In some embodiments, the method includes cutting open the air vent tube such that air can be vented from the vial. In some embodiments, the loading port is a needleless loading port. In some embodiments, the needleless loading port includes a luer lock connection fitting. In some embodiments, the needleless retrieval port includes a luer lock connection fitting. In some embodiments, the air vent tube includes a filter and the air vent tube is sealed above a location of the filter in the air vent tube.

[0007] Additional advantages will be readily apparent to those skilled in the art from the following detailed description. The examples and descriptions herein are to be regarded as illustrative in nature and not restrictive.

Brief Description of the Drawings

[0008] Exemplary embodiments are described with reference to the accompanying figures, in which:

[0009] **FIG. 1** illustrates an example of a biomedical material vessel disclosed herein.

[0010] **FIG. 2** illustrates an example of a biomedical material vessel with caps disclosed herein.

[0011] **FIG. 3A** illustrates a cross section of a first self-closing retrieval port disclosed herein.

[0012] **FIG. 3B** illustrates a cross section of a first self-closing retrieval port when a syringe is attached to the retrieval port.

[0013] **FIG. 4A** illustrates a cross section of a second self-closing retrieval port disclosed herein.

[0014] **FIG. 4B** illustrates a cross section of a second self-closing retrieval port when a syringe is attached to the retrieval port.

[0015] In the Figures, like reference numbers correspond to like components unless otherwise stated. For example, vial 101 can be the same as vial 201. In addition, the figures are not drawn to scale.

Detailed Description

[0016] The biomedical material vessels disclosed herein can reduce one or more of the risks of vessels that require biomedical material retrieval via a needle, in some embodiments in which the needle is an external needle and/or a needle not connected to the vessel. In some cases, the needle can damage the biomedical material inside the vessel. For example, a needle can cause damage and/or stress to the biomedical material inside the vessel by shearing, breaking or accidentally contaminating the biomedical material. Second, a syringe needle may not be able to remove the entirety of the biomedical material from the vessel, for example, as the needle penetrates into the vessel. Moreover, needles, for example external needles, may present a danger to the handler of the needle, in some aspects due to inadvertent needle stick.

[0017] The biomedical material vessels disclosed herein can reduce or eliminate stress and/or damage to the biomedical material by providing a needleless retrieval port. The biomedical material vessels disclosed herein can, in some embodiments, allow for greater and/or complete recovery of the biomedical material by providing direct access to the biomedical material in the vessel. The biomedical vessels disclosed herein can reduce risk of inadvertent

needle stick to users during removal of the biomedical material and can increase efficiency of removal.

[0018] All publications, including patent documents, scientific articles and databases, referred to in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication were individually incorporated by reference. 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 that are herein incorporated by reference, the definition set forth herein prevails over the definition that is incorporated herein by reference.

[0019] The section heading used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

I. BIOMEDICAL MATERIAL VESSELS

[0020] The vessels described herein can be used for various biomedical materials such as human, animal, insect, and plant cells and/or tissues. In some embodiments, the vessels described herein are used for storage of such biomedical materials including cryogenic storage of the biomedical materials. **FIG. 1** illustrates an example of a biomedical material vessel disclosed herein. The biomedical material vessel can include a vial, such as vial 101. Any biomedical material inserted into the vessel can be stored in the vial for freezing, thawing, and/or subsequent removal. As such, the vial can be sized to receive a liquid biomedical material sample. In some embodiments, the vial has a storage volume of about 1 mL to 100 mL, such as at or at least or about at least or about 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL, 7 mL, 8 mL, 9 mL, 10 mL, 20 mL, 30 mL, 40 mL, 50 mL, 60 mL, 70 mL, 80 mL, 90 mL or 100 mL. In some embodiments, the vial has a storage volume of about 1-10 mL, about 2-10, about 1-5 mL, about 2-5 mL, about 10 mL, about 5 mL, or about 2 mL. In some embodiments, the vial is graduated. In addition, the vial can be sized to fit into a vessel storage device, such as a box or cane, such that multiple biomedical medical vessels can be transported, frozen, and/or thawed.

[0021] The vial can have top 102 and bottom 103. In some embodiments, the top can be an open top and/or the bottom can be an open bottom. When the top is open, the open top of the vial can be sealed by a cap. In some embodiments, the cap is hermetically sealed to the vial. In some embodiments, the cap is heat sealed to the vial. The cap and vial together can form a fluid-tight connection. In some embodiments, the cap can be built into the vial such that they are formed as a single piece.

[0022] The top of the vial can support air vent tube 104 and/or inlet tube 105. The air vent tube and/or the inlet tube can be fluidly connected to the interior of the vial 101. The top of the vial can include tube adaptors which can be fluidly connected (i.e., fluid-tight connection) between the air vent tube and the vial interior and between the inlet tube and the vial interior. In addition, the openings of the two tube adaptors into the vial interior can be separated by a wall. The wall can prevent fluid entering from the inlet tube through the tube adaptor from being drawn out the other adaptor towards the air vent tube.

[0023] In some embodiments, the air vent tube and/or the inlet tube can be flexible. As such, inlet tube can be manipulated to better insert a biomedical material sample into the vial. In addition, the flexible air vent tube and/or the flexible inlet tube can be maneuvered to avoid interfering with one another or other biomedical material vessels. The inlet tube can include loading port 106 for receiving a biomedical material sample. The loading port can be hermetically sealed to the inlet tube. In some embodiments, the loading port is heat sealed to the inlet tube. The inlet tube and loading port together can form a fluid-tight connection.

[0024] The loading port can be a variety of loading ports. For example, the loading port can be one for standard needles such as a needle septum. In the case of a needle septum, the needle septum can be configured to provide an air and liquid tight seal with the inlet tube. The needle septum can be pieced by a syringe needle so that a biomedical material sample can be injected into the vial. In some embodiments, the needle septum can be a self-sealing needle septum once the needle is removed.

[0025] In some embodiments, the loading port can be a needleless loading port. For example, the needleless loading port can be a Luer lock connection fitting as shown in **FIG. 1**. As such, a syringe with a Luer lock connection fitting can be screwed onto the Luer lock connection fitting of the loading port and a biomedical material sample can be inserted into the vial through the loading port and inlet tube 105. In some embodiments, the loading port Luer lock connection fitting can be a female Luer lock connection fitting. As such, a syringe with a male Luer lock connection fitting can be screwed onto the female Luer lock connection fitting of the loading port. Once the syringe is connected to the loading port, a biomedical material sample can be inserted into the vial through the loading port and the inlet tube.

[0026] When a biomedical material sample is inserted into the vial via the loading port and inlet tube, a segment of the sample may be desired. If a segment is desired, a user can pull back on the syringe plunger to create a clear space in the inlet tube above and below the liquid for the segment. The user can then push air into the inlet tube to place the liquid column segment at a

desired level. If a segment is desired, the user can seal (with a sealer designed for use with EVA tubing for example) the inlet tube above and below the liquid segment in the inlet tube. In addition, the user can also place an additional seal below the lower seal so that the segment can be easily folded over for storage. If no segment is desired, the user can place a single seal near the vial body.

[0027] In some embodiments, the air vent tube can include a filter within the air vent tube. In some embodiments, the filter can be gas permeable but impermeable to the biomedical material sample stored within the vial. The filter can also be a microbial barrier filter. The air vent tube can also be sealed similar to the inlet tube. In some embodiments, the air vent tube is sealed above the filter. Once the air vent tube and the inlet tube are sealed, the vessel can be a closed system. The closed system can protect the samples from exposure to harmful contaminants and sample leakage. Once a closed system is formed, excess tubing can be removed for easier storage. The vessels can then be placed into a box or on cane for storage at cryogenic temperatures.

[0028] When a segment is needed, the vessel can be removed from storage and the segment can be immediately cut off after removal. When the biomedical material sample in the vial is needed, the vessel can be removed from cryogenic storage and thawed. After thawing, the air vent tube can be cut open to open the vent passageway. The vessel can include retrieval port 107 fluidly connected to the bottom of the vial. The retrieval port can be used to remove the biomedical material sample from the vial after storage. The retrieval port can be hermetically sealed to the bottom of the vial. In some embodiments, the retrieval port is heat sealed to the bottom of the vial. In some embodiments, the retrieval port can close the open bottom of the vial. The bottom of the vial and the retrieval port together can form a fluid-tight connection. In some embodiments, the retrieval port can include a removable cover to protect the retrieval port.

[0029] In some embodiments, the retrieval port can be a needleless retrieval port. As such, the needleless retrieval port can provide direct access to the biomedical material sample within the vial. Accordingly, retrieval of the biomedical material sample does not require the puncturing of a needle septum. The needleless retrieval port can eliminate stress and/or damage (i.e., shearing or breaking) to biomedical material inside the vial during the retrieval process caused by a sharp needle. The needleless retrieval port can reduce the chances of accidental contamination of the biomedical material sample by introduction of a needle. In addition, because a needle does not need to penetrate into the vial, more of the biomedical material can be removed from the vial. Applicants have discovered that on average about 0.3 mL of biomedical

material sample is left in the vial when a needle syringe is used to remove the sample from a needle septum port. Accordingly, the needleless retrieval port can reduce the amount of left over biomedical material in the vial after removal to less than about 0.3 mL, about 0.25 mL, about 0.2 mL, about 0.15 mL, about 0.1 mL, about 0.05 mL, or about 0.025 mL. In some embodiments, the needleless retrieval port can remove the entirety of the biomedical material sample in the vial. Furthermore, a needleless retrieval port can remove the potential for exposure risk (i.e., needle prick) to the users who are doing the actual removal of the biomedical material sample from the vials.

[0030] In some embodiments, the retrieval port can be a Luer lock connection fitting as shown in **FIG. 1**. As such, a syringe with a Luer lock connection fitting can be screwed onto the Luer lock connection fitting of the retrieval port and the biomedical material sample can be removed through the retrieval port. In some embodiments, the retrieval port Luer lock connection fitting can be a female Luer lock connection fitting. As such, a syringe with a male Luer lock connection fitting can be screwed onto the female Luer lock connection fitting of the retrieval port. Once the syringe is connected to the retrieval port, the biomedical material sample can be removed from the vial through the retrieval port.

[0031] In some embodiments, the needleless retrieval port can be a self-closing retrieval port. For example, the self-closing retrieval port can be a self-closing female Luer. As such, when a syringe is attached to the self-closing female Luer, flue flows freely through the retrieval port. However, when the syringe is removed, the retrieval port can close tightly to eliminate liquid loss and prevent potential infection. Accordingly, having a self-closing retrieval port can enable a user to access the contents of the vessel without risk of the contents leaking when the syringe is removed.

[0032] **FIGS. 3A-3B** and **4A-4B** illustrate cross sections of self-closing retrieval ports with and without a syringe attached thereto. As shown in **FIG. 3A**, retrieval port 307 is closed. As such, compressible element 311 is not compressed, thereby closing the retrieval port. When a syringe 310 is attached to retrieval port 307, compressible element 311 is compressed, thereby opening the retrieval port, as shown in **FIG. 3B**. When syringe 310 is removed, compressible element 311 will decompress and close the retrieval port. With respect to **FIG. 4A**, retrieval port 407 is closed. As such, compressible element 411 covers fluid path window 412. When syringe 410 is attached to retrieval port 408, compressible element 411 is compressed such that compressible element 411 no longer covers fluid path window 412 as shown in **FIG. 4B**, thereby allowing fluid to pass through fluid path window 412. Once syringe 410 is removed,

compressible element 411 will decompress to cover fluid path window 412 and thereby close the retrieval port. Examples of self-closing retrieval ports include, but are not limited to, ICU Medical's needleless connectors; Vygon's Vadsite needleless connectors; Quest Medical's needle-free injection sites; and Becton Dickinson's SmartSite™ needle-free valves.

[0033] The retrieval port and/or the loading port can include caps 209 and 208, respectively, as shown in **FIG. 2**. The caps for the ports can protect the ports from damage. In addition, the cap on the retrieval port can act as a stand for which the vessel can rest upright. As such, the cap can provide a more balanced surface than the retrieval port itself for the vessel to rest upright. In some embodiments, the caps are configured to be caps for a luer lock connection fitting. For example, the caps can be configured to be caps for a female luer lock connection fitting. As such, the caps may be screw caps to screw onto the female luer lock connection fittings.

[0034] In some embodiments, all components of the biomedical material vessels disclosed herein are capable of withstanding cryogenic temperatures (i.e., as temperatures as low as -196°C) and subsequent thawing. In addition, all components of the biomedical vessels disclosed herein can be manufactured from USP Class VI compliant materials. In addition, the biomedical vessels disclosed herein can meet the requirements established in ISO 10993-17 and 18 for allowable limits of leachable substances. All components of the biomedical material vessels disclosed herein can be formed of a suitable plastic, such as polystyrene or polypropylene. In addition, the tubes disclosed herein can be capable of withstanding cryogenic temperatures without compromising the ability to heat seal. In some embodiments, the flexible tubing disclosed herein can be formed of TYGON® or a similar material.

[0035] In addition, the biomedical material vessels may be irradiated with ionizing radiation such as gamma radiation, electron beam, or high energy x-rays using a dose to ensure sterility of the biomedical material vessel. Furthermore, all of the various components of the vessel can be constructed from radiation-resistant materials, e.g., ethylene copolymers, silicones, styrene copolymers, polysulfones etc. In some embodiments, the vessels disclosed herein can be sterilized and ready-for-use without additional sterilization.

II. METHODS FOR PRODUCING AND PREPARING A CELL COMPOSITION

[0036] In some embodiments, the biomedical material vessels provided herein can be used for storing cells, such as in connection with processes including manufacturing, generating or producing a cell therapy. In some embodiments, the cell therapy includes cells, such as T cells, engineered with a recombinant receptor, such as a chimeric antigen receptor (CAR), e.g. CAR T

cells. In some embodiments, cells can be expressed from a closed system into one or more of the plurality of biomedical material vessels described herein. In some embodiments, cells can be formulated into the vials in an amount for dosage administration, such as for a single unit dosage administration or multiple dosage administration.

[0037] In some embodiments, the biomaterial material vessels are used in connection with manufacturing, generating or producing a cell therapy, which can be carried out via a process that includes one or more further processing steps, such as steps for the isolation, separation, selection, activation or stimulation, transduction, cultivation, expansion, washing, suspension, dilution, concentration, and/or formulation of the cells. In some embodiments, the methods of generating or producing a cell therapy include isolating cells from a subject, preparing, processing, culturing under one or more stimulating conditions. In some embodiments, the method includes processing steps carried out in an order in which: cells, e.g. primary cells, are first isolated, such as selected or separated, from a biological sample; selected cells are incubated with viral vector particles for transduction, optionally subsequent to a step of stimulating the isolated cells in the presence of a stimulation reagent; culturing the transduced cells, such as to expand the cells; formulating the transduced cells in a composition and introducing the composition into a provided biomedical material vessel. In some embodiments, the generated engineered cells are re-introduced into the same subject, before or after cryopreservation. In some embodiments, the cells during one or more steps of the steps, including before and/or after isolation, selection, transduction and/or cultivation, the cells can be cryopreserved, and subsequently thawed.

[0038] In some embodiments, the one or more processing steps can include one or more of (a) washing a biological sample containing cells (e.g., a whole blood sample, a buffy coat sample, a peripheral blood mononuclear cells (PBMC) sample, an unfractionated T cell sample, a lymphocyte sample, a white blood cell sample, an apheresis product, or a leukapheresis product), (b) isolating, e.g. selecting, from the sample a desired subset or population of cells (e.g., CD4+ and/or CD8+ T cells), for example, by incubation of cells with a selection or immunoaffinity reagent for immunoaffinity-based separation; c) incubating the isolated, such as selected cells, with viral vector particles, (d) culturing, cultivating or expanding the cells such using methods as described and (e) formulating the transduced cells, such as in a pharmaceutically acceptable buffer, cryopreservative or other suitable medium. In some embodiments, the methods can further include (e) stimulating cells by exposing cells to stimulating conditions, which can be performed prior to, during and/or subsequent to the

incubation of cells with viral vector particles. In some embodiments, one or more further step of washing or suspending step, such as for dilution, concentration and/or buffer exchange of cells, can also be carried out prior to or subsequent to any of the above steps. In some aspects, the resulting engineered cell composition is introduced into one or more provided biomedical culture vessel.

[0039] In some embodiments, the provided methods are carried out such that one, more, or all steps in the preparation of cells for clinical use, e.g., in adoptive cell therapy, are carried out without exposing the cells to non-sterile conditions and without the need to use a sterile room or cabinet. In some embodiments of such a process, the cells are isolated, separated or selected, transduced, washed, optionally activated or stimulated and formulated, all within a closed system. In some aspects of such a process, the cells are expressed from a closed system and introduced into one or more of the biomaterial vessels. In some embodiments, the methods are carried out in an automated fashion. In some embodiments, one or more of the steps is carried out apart from the closed system or device.

[0040] In some embodiments, a closed system is used for carrying out one or more of the other processing steps of a method for manufacturing, generating or producing a cell therapy. In some embodiments, one or more or all of the processing steps, e.g., isolation, selection and/or enrichment, processing, incubation in connection with transduction and engineering, and formulation steps is carried out using a system, device, or apparatus in an integrated or self-contained system, and/or in an automated or programmable fashion. In some aspects, 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 aspects of the processing, isolation, engineering, and formulation steps. In one example, the system is a system as described in International Patent Application, Publication Number WO2009/072003, or US 20110003380 A1. In one example, the system is a system as described in International Publication Number WO2016/073602.

A. Isolation or Selection of Cells from Samples

[0041] In some embodiments, the processing steps include isolation of cells or compositions thereof from biological samples, such as those 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 aspects, 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 embodiments

are primary cells, e.g., primary human cells. In some embodiments, the cells comprise CD4+ and CD8+ T cells. In some embodiments, the cells comprise CD4+ or CD8+ T cells. The samples include tissue, fluid, and other samples taken directly from the subject. 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.

[0042] In some aspects, 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.

[0043] In some examples, cells from the circulating blood of a subject are obtained, e.g., by apheresis or leukapheresis. The samples, in some aspects, contain lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and/or platelets, and in some aspects contains cells other than red blood cells and platelets.

[0044] In some embodiments, 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 embodiments, the cells are washed with phosphate buffered saline (PBS). In some embodiments, the wash solution lacks calcium and/or magnesium and/or many or all divalent cations. In some aspects, 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 aspects, a washing step is accomplished by tangential flow filtration (TFF) according to the manufacturer's instructions. In some embodiments, the cells are resuspended in a variety of biocompatible buffers after washing, such as, for example, Ca^{++}/Mg^{++} free PBS. In certain embodiments, components of a blood cell sample are removed and the cells directly resuspended in culture media.

[0045] In some embodiments, the preparation methods include steps for freezing, e.g., cryopreserving, the cells, either before or after isolation, selection and/or enrichment and/or incubation for transduction and engineering, and/or after cultivation and/or harvesting of the

engineered cell. Exemplary methods for freezing, cryopreservation or cryogenic preservation of biological samples, such as T cells or T cell compositions, include those described in WO2018170188, which is incorporated by reference in its entirety. In some embodiments, the freeze and subsequent thaw step removes granulocytes and, to some extent, monocytes in the cell population. In some embodiments, 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 aspects may be used. In some embodiments, the cells are frozen, e.g., cryopreserved or cryopotected, in media and/or solution with a final concentration of or of about 12.5%, 12.0%, 11.5%, 11.0%, 10.5%, 10.0%, 9.5%, 9. 0%, 8.5%, 8.0%, 7.5%, 7.0%, 6.5%, 6.0%, 5.5%, or 5.0% DMSO, or between 1% and 15%, between 6% and 12%, between 5% and 10%, or between 6% and 8% DMSO. In particular embodiments, the cells are frozen, e.g., cryopreserved or cryoprotected, in media and/or solution with a final concentration of or of about 5.0%, 4.5%, 4.0%, 3.5%, 3.0%, 2.5%, 2.0%, 1.5%, 1.25%, 1.0%, 0.75%, 0.5%, or 0.25% HSA, or between 0.1% and -5%, between 0.25% and 4%, between 0.5% and 2%, or between 1% and 2% HSA. 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 generally then frozen to or to about −80° C. at a rate of or of about 1°C per minute and stored in the vapor phase of a liquid nitrogen storage tank.

[0046] In some embodiments, 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. In some embodiments, 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.

[0047] In some embodiments, at least a portion of the selection step includes incubation of cells with a selection reagent. The incubation with a selection reagent or reagents, e.g., as part of selection methods which may be performed using one or more selection reagents for selection of one or more different cell types based on the expression or presence in or on the cell of one or more specific molecules, such as surface markers, e.g., surface proteins, intracellular markers, or

nucleic acid. In some embodiments, any known method using a selection reagent or reagents for separation based on such markers may be used. In some embodiments, the selection reagent or reagents result in a separation that is affinity- or immunoaffinity-based separation. For example, the selection in some aspects includes incubation with a reagent or reagents for 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. In some embodiments, the selection and/or other aspects of the process is as described in International Patent Application Publication Number WO/2015/164675.

[0048] In some aspects of such processes, a volume of cells is mixed with an amount of a desired affinity-based selection reagent. The immunoaffinity-based selection can be carried out using any system or method that results in a favorable energetic interaction between the cells being separated and the molecule specifically binding to the marker on the cell, e.g., the antibody or other binding partner on the solid surface, e.g., particle. In some embodiments, methods are carried out using particles such as beads, e.g. magnetic beads, that are coated with a selection agent (e.g. antibody) specific to the marker of the cells. The particles (e.g. beads) can be incubated or mixed with cells in a container, such as a tube or bag, while shaking or mixing, with a constant cell density-to-particle (e.g., bead) ratio to aid in promoting energetically favored interactions. In other cases, the methods include selection of cells in which all or a portion of the selection is carried out in the internal cavity of a centrifugal chamber, for example, under centrifugal rotation. In some embodiments, incubation of cells with selection reagents, such as immunoaffinity-based selection reagents, is performed in a centrifugal chamber. In certain embodiments, the isolation or separation is carried out using a system, device, or apparatus described in International Patent Application, Publication Number WO2009/072003, or US 20110003380 A1. In one example, the system is a system as described in International Publication Number WO2016/073602.

[0049] In some embodiments, by conducting such selection steps or portions thereof (e.g., incubation with antibody-coated particles, e.g., magnetic beads) in the cavity of a centrifugal chamber, the user is able to control certain parameters, such as volume of various solutions, addition of solution during processing and timing thereof, which can provide advantages compared to other available methods. For example, the ability to decrease the liquid volume in

the cavity during the incubation can increase the concentration of the particles (e.g. bead reagent) used in the selection, and thus the chemical potential of the solution, without affecting the total number of cells in the cavity. This in turn can enhance the pairwise interactions between the cells being processed and the particles used for selection. In some embodiments, carrying out the incubation step in the chamber, e.g., when associated with the systems, circuitry, and control as described herein, permits the user to effect agitation of the solution at desired time(s) during the incubation, which also can improve the interaction.

[0050] In some embodiments, at least a portion of the selection step is performed in a centrifugal chamber, which includes incubation of cells with a selection reagent. In some aspects of such processes, a volume of cells is mixed with an amount of a desired affinity-based selection reagent that is far less than is normally employed when performing similar selections in a tube or container for selection of the same number of cells and/or volume of cells according to manufacturer's instructions. In some embodiments, an amount of selection reagent or reagents that is/are no more than 5%, no more than 10%, no more than 15%, no more than 20%, no more than 25%, no more than 50%, no more than 60%, no more than 70% or no more than 80% of the amount of the same selection reagent(s) employed for selection of cells in a tube or container-based incubation for the same number of cells and/or the same volume of cells according to manufacturer's instructions is employed.

[0051] In some embodiments, for selection, e.g., immunoaffinity-based selection of the cells, the cells are incubated in the cavity of the chamber in a composition that also contains the selection buffer with a selection reagent, such as a molecule that specifically binds to a surface marker on a cell that it desired to enrich and/or deplete, but not on other cells in the composition, such as an antibody, which optionally is coupled to a scaffold such as a polymer or surface, e.g., bead, e.g., magnetic bead, such as magnetic beads coupled to monoclonal antibodies specific for CD4 and CD8. In some embodiments, as described, the selection reagent is added to cells in the cavity of the chamber in an amount that is substantially less than (e.g. is no more than 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70% or 80% of the amount) as compared to the amount of the selection reagent that is typically used or would be necessary to achieve about the same or similar efficiency of selection of the same number of cells or the same volume of cells when selection is performed in a tube with shaking or rotation. In some embodiments, the incubation is performed with the addition of a selection buffer to the cells and selection reagent to achieve a target volume with incubation of the reagent of, for example, 10 mL to 200 mL, such as at least or about at least or about or 10 mL, 20 mL, 30 mL, 40 mL, 50 mL, 60 mL, 70 mL, 80 mL, 90

mL, 100 mL, 150 mL or 200 mL. In some embodiments, the selection buffer and selection reagent are pre-mixed before addition to the cells. In some embodiments, the selection buffer and selection reagent are separately added to the cells. In some embodiments, the selection incubation is carried out with periodic gentle mixing condition, which can aid in promoting energetically favored interactions and thereby permit the use of less overall selection reagent while achieving a high selection efficiency.

[0052] In some embodiments, the total duration of the incubation with the selection reagent is from 5 minutes to 6 hours or from about 5 minutes to about 6 hours, such as 30 minutes to 3 hours, for example, at least or about at least 30 minutes, 60 minutes, 120 minutes or 180 minutes.

[0053] In some embodiments, the incubation generally is carried out under mixing conditions, such as in the presence of spinning, generally at relatively low force or speed, such as speed lower than that used to pellet the cells, such as from 600 rpm to 1700 rpm or from about 600 rpm to about 1700 rpm (e.g. at or about or at least 600 rpm, 1000 rpm, or 1500 rpm or 1700 rpm), such as at an RCF at the sample or wall of the chamber or other container of from 80g to 100g or from about 80g to about 100g (e.g. at or about or at least 80 g, 85 g, 90 g, 95 g, or 100 g). In some embodiments, the spin is carried out using repeated intervals of a spin at such low speed followed by a rest period, such as a spin and/or rest for 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 seconds, such as a spin at approximately 1 or 2 seconds followed by a rest for approximately 5, 6, 7, or 8 seconds.

[0054] In some embodiments, such process is carried out within the entirely closed system to which the chamber is integral. In some embodiments, this process (and in some aspects also one or more additional step, such as a previous wash step washing a sample containing the cells, such as an apheresis sample) is carried out in an automated fashion, such that the cells, reagent, and other components are drawn into and pushed out of the chamber at appropriate times and centrifugation effected, so as to complete the wash and binding step in a single closed system using an automated program.

[0055] In some embodiments, after the incubation and/or mixing of the cells and selection reagent and/or reagents, the incubated cells are subjected to a separation to select for cells based on the presence or absence of the particular reagent or reagents. In some embodiments, the separation is performed in the same closed system in which the incubation of cells with the selection reagent was performed. In some embodiments, after incubation with the selection reagents, incubated cells, including cells in which the selection reagent has bound are transferred

into a system for immunoaffinity-based separation of the cells. In some embodiments, the system for immunoaffinity-based separation is or contains a magnetic separation column.

[0056] Such separation steps can be based on positive selection, in which the cells having bound the reagents, e.g. antibody or binding partner, are retained for further use, and/or negative selection, in which the cells having not bound to the reagent, e.g., antibody or binding partner, are retained. In some examples, both fractions are retained for further use. In some aspects, 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.

[0057] In some embodiments, the process steps further include negative and/or positive selection of the incubated and cells, such as using a system or apparatus that can perform an affinity-based selection. In some embodiments, 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 embodiments, 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. Multiple rounds of the same selection step, e.g., positive or negative selection step, can be performed. In certain embodiments, the positively or negatively selected fraction subjected to the process for selection, such as by repeating a positive or negative selection step. In some embodiments, selection is repeated twice, three times, four times, five times, six times, seven times, eight times, nine times or more than nine times. In certain embodiments, the same selection is performed up to five times. In certain embodiments, the same selection step is performed three times.

[0058] 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.

[0059] 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. In certain embodiments, one or more separation steps are repeated and/or performed more than once. In some embodiments, the positively or negatively selected fraction resulting from a separation step is subjected to the same separation step, such as by repeating the positive or negative selection step. In some embodiments, a single separation step is repeated and/or performed more than once, for example, to increase the yield of positively selected cells, to increase the purity of negatively selected cells, and/or to further remove the positively selected cells from the negatively selected fraction. In certain embodiments, one or more separation steps are performed and/or repeated two times, three times, four times, five times, six times, seven times, eight times, nine times, ten times, or more than ten times. In certain embodiments, the one or more selection steps are performed and/or repeated between one and ten times, between one and five times, or between three and five times. In certain embodiments, one or more selection steps are repeated three times.

[0060] For example, in some aspects, 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. In some embodiments, such cells are selected by incubation with one or more antibody or binding partner that specifically binds to such markers. In some embodiments, the antibody or binding partner can be conjugated, such as directly or indirectly, to a solid support or matrix to effect selection, such as a magnetic bead or paramagnetic bead. For example, CD3+, CD28+ T cells can be positively selected using anti-CD3/anti-CD28 conjugated magnetic beads (e.g., DYNABEADS® M-450 CD3/CD28 T Cell Expander, and/or ExpACT® beads).

[0061] In some embodiments, 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 aspects, a CD4+ or CD8+ selection step is used to separate CD4+ helper and CD8+ cytotoxic T 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.

[0062] In some embodiments, CD8+ T 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 embodiments, enrichment for central memory T (TCM) cells is carried out to increase efficacy, such as to improve long-term survival, expansion, and/or engraftment following administration, which in some aspects is particularly robust in such sub-populations. See Terakura et al., (2012) Blood.1:72–82; Wang et al. (2012) J Immunother. 35(9):689-701. In some embodiments, combining TCM-enriched CD8+ T cells and CD4+ T cells further enhances efficacy.

[0063] In embodiments, 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.

[0064] In some embodiments, the enrichment for central memory T (TCM) cells is based on positive or high surface expression of CD45RO, CD62L, CCR7, CD28, CD3, and/or CD 127; in some aspects, it is based on negative selection for cells expressing or highly expressing CD45RA and/or granzyme B. In some aspects, isolation of a CD8+ population enriched for TCM cells is carried out by depletion of cells expressing CD4, CD14, CD45RA, and positive selection or enrichment for cells expressing CD62L. In one aspect, enrichment for central memory T (TCM) 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.

[0065] Such selections in some aspects are carried out simultaneously and in other aspects are carried out sequentially, in either order. In some aspects, the same CD4 expression-based selection step used in preparing the CD8+ T cell population or subpopulation, also is used to generate the CD4+ T 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. In some embodiments, the selection for the CD4+ T cell population and the selection for the CD8+ T cell population are carried out simultaneously. In some embodiments, the CD4+ T cell population and the selection for the CD8+ T cell population are carried out sequentially, in either order. In some embodiments, methods for selecting cells can include those as described in published U.S. App. No. US20170037369. In some embodiments, the selected CD4+ T cell population and the selected CD8+ T cell population may be combined subsequent to the selecting. In some aspects,

the selected CD4+ T cell population and the selected CD8+ T cell population may be combined in a container or a bag, such as a bioreactor bag, or in the provided biomedical materials vessels. In some embodiments, the selected CD4+ T cell population and the selected CD8+ T cell population are separately processed, whereby the selected CD4+ T cell population is enriched in CD4+ T cells and incubated with a stimulatory reagent (e.g. anti-CD3/anti-CD28 magnetic beads), transduced with a viral vector encoding a recombinant protein (e.g. CAR) and cultivated under conditions to expand T cells and the selected CD8+ T cell population is enriched in CD8+ T cell and incubated with a stimulatory reagent (e.g. anti-CD3/anti-CD28 magnetic beads), transduced with a viral vector encoding a recombinant protein (e.g. CAR), the same recombinant protein as for engineering of the CD4+ T cells from the same donor, and cultivated under conditions to expand T cells, such as in accord with the provided methods.

[0066] In particular embodiments, a biological sample, e.g., a sample of PBMCs or other white blood cells, are subjected to selection of CD4+ T cells, where both the negative and positive fractions are retained. In certain embodiments, CD8+ T cells are selected from the negative fraction. In some embodiments, a biological sample is subjected to selection of CD8+ T cells, where both the negative and positive fractions are retained. In certain embodiments, CD4+ T cells are selected from the negative fraction.

[0067] In a particular example, a sample of PBMCs or other white blood cell sample is subjected to selection of CD4+ T 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.

[0068] CD4+ T helper cells may be sorted into naïve, central memory, and effector cells by identifying cell populations that have cell surface antigens. CD4+ lymphocytes can be obtained by standard methods. In some embodiments, naive CD4+ T lymphocytes are CD45RO-, CD45RA+, CD62L+, or CD4+ T cells. In some embodiments, central memory CD4+ T cells are CD62L+ and CD45RO+. In some embodiments, effector CD4+ T cells are CD62L- and CD45RO-.

[0069] In one example, to enrich for CD4+ T cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8. In some embodiments, the antibody or binding partner is bound to a solid support or matrix, such as a magnetic bead or paramagnetic bead, to allow for separation of cells for

positive and/or negative selection. For example, in some embodiments, 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).

[0070] In some aspects, the incubated sample or composition of cells to be separated is incubated with a selection reagent containing small, magnetizable or magnetically responsive material, such as magnetically responsive particles or microparticles, such as paramagnetic beads (e.g., such as Dynalbeads or MACS® 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.

[0071] In some embodiments, the magnetic particle or bead comprises a magnetically responsive material bound to a specific binding member, such as an antibody or other binding partner. Many well-known magnetically responsive materials for use in magnetic separation methods are known, e.g., those described in Molday, U.S. Pat. No. 4,452,773, and in European Patent Specification EP 452342 B, which are hereby incorporated by reference. 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 also may be used.

[0072] 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.

[0073] In certain embodiments, the magnetically responsive particles are coated in primary antibodies or other binding partners, secondary antibodies, lectins, enzymes, or streptavidin. In certain embodiments, the magnetic particles are attached to cells via a coating of primary antibodies specific for one or more markers. In certain embodiments, 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 embodiments, streptavidin-coated magnetic particles are used in conjunction with biotinylated primary or secondary antibodies.

[0074] In some aspects, separation is achieved in a procedure in which 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 aspects, 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.

[0075] In some embodiments, the affinity-based selection is via magnetic-activated cell sorting (MACS) (Miltenyi Biotec, Auburn, CA). Magnetic Activated Cell Sorting (MACS), e.g., CliniMACS systems are capable of high-purity selection of cells having magnetized particles attached thereto. In certain embodiments, 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 embodiments, the non-target cells are labelled and depleted from the heterogeneous population of cells.

[0076] In some embodiments, the magnetically responsive particles are left attached to the cells that are to be subsequently incubated, cultured and/or engineered; in some aspects, the particles are left attached to the cells for administration to a patient. In some embodiments, 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 embodiments, the magnetizable particles are biodegradable.

B. Activation and Stimulation of Cells

[0077] In some embodiments, the one or more processing steps include a step of stimulating the isolated cells, such as selected cell populations. The incubation may be prior to or in connection with genetic engineering, such as genetic engineering resulting from embodiments of the transduction method described above. In some embodiments, the stimulation results in activation and/or proliferation of the cells, for example, prior to transduction.

[0078] In some embodiments, the processing steps include incubations of cells, such as selected cells, in which the incubation steps can include culture, cultivation, stimulation, activation, and/or propagation of cells. In some embodiments, the compositions or cells are incubated in the presence of stimulating conditions or a stimulatory agent. Such conditions include those designed to induce proliferation, 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 recombinant antigen receptor.

[0079] In some embodiments, the conditions for stimulation and/or activation 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.

[0080] In some embodiments, the stimulating conditions or agents include one or more agent, e.g., ligand, which is capable of stimulating or activating an intracellular signaling domain of a TCR complex. In some aspects, the agent turns on or initiates TCR/CD3 intracellular signaling cascade in a T cell. Such agents can include agents suitable to deliver a primary signal, e.g., to initiate activation of an ITAM-induced signal, such as antibodies, such as those specific for a TCR, e.g. anti-CD3. In some embodiments, the stimulating conditions include one or more agent, e.g. ligand, which is capable of stimulating a costimulatory receptor, e.g., anti-CD28 or anti-4-1BB. In some embodiments, such agents and/or ligands may be, bound to solid support such as a bead, and/or one or more cytokines. Among the stimulating agents are anti-CD3/anti-CD28 beads (e.g., DYNABEADS® M-450 CD3/CD28 T Cell Expander, and/or ExpACT® beads). 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). In some embodiments, the stimulating agents include IL-2, IL-7 and/or IL-15, for example, an IL-2 concentration of at least about 10 units/mL, at least about 50 units/mL, at least about 100 units/mL or at least about 200 units/mL.

[0081] 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.

[0082] In some aspects, incubation is carried out in accordance with techniques such as those described in US Patent No. 6,040,177 to Riddell et al., Klebanoff et al.(2012) J Immunother. 35(9): 651–660, Terakura et al. (2012) Blood.1:72–82, and/or Wang et al. (2012) J Immunother. 35(9):689-701.

[0083] In some embodiments, at least a portion of the incubation in the presence of one or more stimulating conditions or stimulatory agents is carried out in the internal cavity of a centrifugal chamber, for example, under centrifugal rotation, such as described in International

Publication Number WO2016/073602. In some embodiments, at least a portion of the incubation performed in a centrifugal chamber includes mixing with a reagent or reagents to induce stimulation and/or activation. In some embodiments, cells, such as selected cells, are mixed with a stimulating condition or stimulatory agent in the centrifugal chamber. In some aspects of such processes, a volume of cells is mixed with an amount of one or more stimulating conditions or agents that is far less than is normally employed when performing similar stimulations in a cell culture plate or other system.

[0084] In some embodiments, the stimulating agent is added to cells in the cavity of the chamber in an amount that is substantially less than (e.g. is no more than 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70% or 80% of the amount) as compared to the amount of the stimulating agent that is typically used or would be necessary to achieve about the same or similar efficiency of selection of the same number of cells or the same volume of cells when selection is performed without mixing in a centrifugal chamber, e.g. in a tube or bag with periodic shaking or rotation. In some embodiments, the incubation is performed with the addition of an incubation buffer to the cells and stimulating agent to achieve a target volume with incubation of the reagent of, for example, about 10 mL to about 200 mL, or about 20 mL to about 125 mL, such as at least or about at least or about 10 mL, 20 mL, 30 mL, 40 mL, 50 mL, 60 mL, 70 mL, 80 mL, 90 mL, 100 mL, 105 mL, 110 mL, 115 mL, 120 mL, 125 mL, 130 mL, 135 mL, 140 mL, 145 mL, 150 mL, 160 mL, 170 mL, 180 mL, 190 mL, or 200 mL. In some embodiments, the incubation buffer and stimulating agent are pre-mixed before addition to the cells. In some embodiments, the incubation buffer and stimulating agent are separately added to the cells. In some embodiments, the stimulating incubation is carried out with periodic gentle mixing condition, which can aid in promoting energetically favored interactions and thereby permit the use of less overall stimulating agent while achieving stimulating and activation of cells.

[0085] In some embodiments, the incubation generally is carried out under mixing conditions, such as in the presence of spinning, generally at relatively low force or speed, such as speed lower than that used to pellet the cells, such as from 600 rpm to 1700 rpm or from about 600 rpm to about 1700 rpm (e.g. at or about or at least 600 rpm, 1000 rpm, or 1500 rpm or 1700 rpm), such as at an RCF at the sample or wall of the chamber or other container of from 80g to 100g or from about 80g to about 100g (e.g. at or about or at least 80 g, 85 g, 90 g, 95 g, or 100 g). In some embodiments, the spin is carried out using repeated intervals of a spin at such low speed followed by a rest period, such as a spin and/or rest for 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10

seconds, such as a spin at approximately 1 or 2 seconds followed by a rest for approximately 5, 6, 7, or 8 seconds.

[0086] In some embodiments, the total duration of the incubation, e.g. with the stimulating agent, is between or between about 1 hour and 96 hours, 1 hour and 72 hours, 1 hour and 48 hours, 4 hours and 36 hours, 8 hours and 30 hours, 18 hours and 30 hours, or 12 hours and 24 hours, such as at least or about at least or about 6 hours, 12 hours, 18 hours, 24 hours, 36 hours or 72 hours. In some embodiments, the further incubation is for a time between or about between 1 hour and 48 hours, 4 hours and 36 hours, 8 hours and 30 hours or 12 hours and 24 hours, inclusive. .

C. Genetic Engineering

[0087] In some embodiments, the processing steps include introduction of a nucleic acid molecule encoding a recombinant protein. Among such recombinant proteins are recombinant receptors, such as any described in Section III. Introduction of the nucleic acid molecules encoding the recombinant protein, such as recombinant receptor, in the cell may be carried out using any of a number of known vectors. Such vectors include viral and non-viral systems, including lentiviral and gammaretroviral systems, as well as transposon-based systems such as PiggyBac or Sleeping Beauty-based gene transfer systems. Exemplary methods include those for transfer of nucleic acids encoding the receptors, including via viral, e.g., retroviral or lentiviral, transduction, transposons, and electroporation.

[0088] In certain embodiments, compositions of cells are engineered, e.g., transduced or transfected, prior to cultivating the cells, e.g., under conditions that promote proliferation and/or expansion. In particular embodiments, compositions of cells are engineered after the compositions have been stimulated, activated, and/or incubated under stimulating conditions. In particular embodiments, the compositions are stimulated compositions. In particular embodiments, the stimulated compositions have been previously cryopreserved and stored, and are thawed prior to engineering.

[0089] In some embodiments, gene transfer is accomplished by first stimulating the cell, such as by combining it with a stimulus that induces a response such as proliferation, survival, and/or activation, e.g., as measured by expression of a cytokine or activation marker, followed by transduction of the activated cells, and expansion in culture to numbers sufficient for clinical applications.

[0090] In some embodiments, 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), and human immunodeficiency virus (HIV).

[0091] In some embodiments, 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 embodiments, 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)).

[0092] Other approaches and vectors for transfer of the nucleic acids encoding the recombinant products are those described, e.g., in international patent application, Publication No.: WO2014055668, and U.S. Patent No. 7,446,190.

[0093] In some embodiments, the cells, e.g., T cells, may be transfected either during or after expansion e.g. with a T cell receptor (TCR) or a chimeric antigen receptor (CAR). This transfection for the introduction of the gene of the desired receptor can be carried out with any suitable retroviral vector, for example. The genetically modified cell population can then be liberated from the initial stimulus (the CD3/CD28 stimulus, for example) and subsequently be stimulated with a second type of stimulus e.g. via a de novo introduced receptor). This second type of stimulus may include an antigenic stimulus in form of a peptide/MHC molecule, the cognate (cross-linking) ligand of the genetically introduced receptor (e.g. natural ligand of a CAR) or any ligand (such as an antibody) that directly binds within the framework of the new receptor (e.g. by recognizing constant regions within the receptor). See, for example, Cheadle et al, "Chimeric antigen receptors for T-cell based therapy" Methods Mol Biol. 2012; 907:645-66 or Barrett et al., Chimeric Antigen Receptor Therapy for Cancer Annual Review of Medicine Vol. 65: 333-347 (2014).

[0094] In some cases, a vector may be used that does not require that the cells, e.g., T cells, are activated. In some such instances, the cells may be selected and/or transduced prior to activation. Thus, the cells may be engineered prior to, or subsequent to culturing of the cells, and in some cases at the same time as or during at least a portion of the culturing.

[0095] In some aspects, the cells further are engineered to promote expression of cytokines or other factors. Among additional nucleic acids, e.g., 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. See, e.g., Riddell et al., US Patent No. 6,040,177, at columns 14-17.

[0096] In some embodiments, the introducing is carried out by contacting one or more cells of a composition with a nucleic acid molecule encoding the recombinant protein, e.g. recombinant receptor. In some embodiments, the contacting can be effected with centrifugation, such as spinoculation (e.g. centrifugal inoculation). Such methods include any of those as described in International Publication Number WO2016/073602. Exemplary centrifugal chambers include those produced and sold by Biosafe SA, including those for use with the Sepax® and Sepax® 2 system, including an A-200/F and A-200 centrifugal chambers and various kits for use with such systems. Exemplary chambers, systems, and processing instrumentation and cabinets are described, for example, in US Patent No. 6,123,655, US Patent No. 6,733,433 and Published U.S. Patent Application, Publication No.: US 2008/0171951, and published international patent application, publication no. WO 00/38762, the contents of each of which are incorporated herein by reference in their entirety. Exemplary kits for use with such systems include, but are not limited to, single-use kits sold by BioSafe SA under product names CS-430.1, CS-490.1, CS-600.1 or CS-900.2.

[0097] In some embodiments, the system is included with and/or placed into association with other instrumentation, including instrumentation to operate, automate, control and/or monitor aspects of the transduction step and one or more various other processing steps performed in the system, e.g. one or more processing steps that can be carried out with or in connection with the centrifugal chamber system as described herein or in International Publication Number WO2016/073602. This instrumentation in some embodiments is contained within a cabinet. In some embodiments, the instrumentation includes a cabinet, which includes a housing containing control circuitry, a centrifuge, a cover, motors, pumps, sensors, displays, and

a user interface. An exemplary device is described in US Patent No. 6,123,655, US Patent No. 6,733,433 and US 2008/0171951.

[0098] In some embodiments, the system comprises a series of containers, e.g., bags, tubing, stopcocks, clamps, connectors, and a centrifuge chamber. In some embodiments, the containers, such as bags, include one or more containers, such as bags, containing the cells to be transduced and the viral vector particles, in the same container or separate containers, such as the same bag or separate bags. In some embodiments, the system further includes one or more containers, such as bags, containing medium, such as diluent and/or wash solution, which is pulled into the chamber and/or other components to dilute, resuspend, and/or wash components and/or compositions during the methods. The containers can be connected at one or more positions in the system, such as at a position corresponding to an input line, diluent line, wash line, waste line and/or output line.

[0099] In some embodiments, the chamber is associated with a centrifuge, which is capable of effecting rotation of the chamber, such as around its axis of rotation. Rotation may occur before, during, and/or after the incubation in connection with transduction of the cells and/or in one or more of the other processing steps. Thus, in some embodiments, one or more of the various processing steps is carried out under rotation, e.g., at a particular force. The chamber is typically capable of vertical or generally vertical rotation, such that the chamber sits vertically during centrifugation and the side wall and axis are vertical or generally vertical, with the end wall(s) horizontal or generally horizontal.

[0100] In some embodiments, the composition containing cells, viral particles and reagent can be rotated, generally at relatively low force or speed, such as speed lower than that used to pellet the cells, such as from 600 rpm to 1700 rpm or from about 600 rpm to about 1700 rpm (e.g. at or about or at least 600 rpm, 1000 rpm, or 1500 rpm or 1700 rpm). In some embodiments, the rotation is carried at a force, e.g., a relative centrifugal force, of from 100 g to 3200 g or from about 100 g to about 3200 g (e.g. at or about or at least at or about 100 g, 200 g, 300 g, 400 g, 500 g, 1000 g, 1500 g, 2000 g, 2500 g, 3000 g or 3200 g), as measured for example at an internal or external wall of the chamber or cavity. The term "relative centrifugal force" or RCF is generally understood to be the effective force imparted on an object or substance (such as a cell, sample, or pellet and/or a point in the chamber or other container being rotated), relative to the earth's gravitational force, at a particular point in space as compared to the axis of rotation. The value may be determined using well-known formulas, taking into

account the gravitational force, rotation speed and the radius of rotation (distance from the axis of rotation and the object, substance, or particle at which RCF is being measured).

[0101] In some embodiments, during at least a part of the genetic engineering, e.g. transduction, and/or subsequent to the genetic engineering the cells are transferred to a container such as a bag, e.g., a bioreactor bag assembly, for culture of the genetically engineered cells, such as for cultivation or expansion of the cells, as described above. In some embodiments, the container for cultivation or expansion of the cells is a bioreactor bag, such as a perfusion bag.

1. Vectors and Methods

[0102] In some embodiments, the processing steps include introduction of a nucleic acid molecule encoding a recombinant protein, into the cell, and may be carried out using any of a number of known vectors. In some embodiments, the vector contains the nucleic acid encoding the recombinant receptor. In particular embodiments, the vector is a viral vector a non-viral vector. In some cases, the vector is a viral vector, such as a retroviral vector, e.g., a lentiviral vector or a gammaretroviral vector.

[0103] In some cases, the nucleic acid sequence encoding the recombinant receptor, e.g., chimeric antigen receptor (CAR) contains a signal sequence that encodes a signal peptide. Non-limiting exemplary examples of signal peptides include, for example, the GMCSFR alpha chain signal peptide, the CD8 alpha signal peptide, or the CD33 signal peptide.

[0104] In some embodiments, the vectors include viral vectors, e.g., retroviral or lentiviral, non-viral vectors or transposons, e.g. *Sleeping Beauty* transposon system, vectors derived from simian virus 40 (SV40), adenoviruses, adeno-associated virus (AAV), lentiviral vectors or retroviral vectors, such as gamma-retroviral vectors, retroviral vector derived from the Moloney murine leukemia virus (MoMLV), 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).

[0105] In some embodiments, the viral vector or the non-viral DNA contains a nucleic acid that encodes a heterologous recombinant protein. In some embodiments, the heterologous recombinant molecule is or includes a recombinant receptor, *e.g.,* an antigen receptor, SB-transposons, *e.g.,* for gene silencing, capsid-enclosed transposons, homologous double stranded nucleic acid, *e.g.,* for genomic recombination or reporter genes (*e.g.,* fluorescent proteins, such as GFP) or luciferase).

2. Preparation of Viral Vector Particles for Transduction

[0106] In some embodiments, 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 embodiments, 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.

[0107] In some embodiments, the retroviral vector has a long terminal repeat sequence (LTR), e.g., a retroviral vector derived from the Moloney murine leukemia virus (MoMLV), myeloproliferative sarcoma virus (MPSV), murine embryonic stem cell virus (MESV), murine stem cell virus (MSCV), or spleen focus forming virus (SFFV). In some embodiments, 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 embodiment, 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.

[0108] 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.

[0109] In some embodiments, the viral vector particles contain a genome derived from a retroviral genome based vector, such as derived from a lentiviral genome based vector. In some aspects of the provided viral vectors, the heterologous nucleic acid encoding a recombinant receptor, such as a CAR, is contained and/or located between the 5′ LTR and 3′ LTR sequences of the vector genome.

[0110] In some embodiments, the viral vector genome is a lentivirus genome, such as an HIV-1 genome or an SIV genome. For example, lentiviral vectors have been generated by multiply attenuating virulence genes, for example, the genes env, vif, vpu and nef can be

deleted, making the vector safer for therapeutic purposes. Lentiviral vectors are known. See Naldini et al., (1996 and 1998); Zufferey et al., (1997); Dull et al., 1998, U.S. Pat. Nos. 6,013,516; and 5,994,136). In some embodiments, these viral vectors are plasmid-based or virus-based, and are configured to carry the essential sequences for incorporating foreign nucleic acid, for selection, and for transfer of the nucleic acid into a host cell. Known lentiviruses can be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; 10801 University Blvd., Manassas, Va. 20110-2209), or isolated from known sources using commonly available techniques.

[0111] Non-limiting examples of lentiviral vectors include those derived from a lentivirus, such as Human Immunodeficiency Virus 1 (HIV-1), HIV-2, an Simian Immunodeficiency Virus (SIV), Human T-lymphotropic virus 1 (HTLV-1), HTLV-2 or equine infection anemia virus (E1AV). For example, lentiviral vectors have been generated by multiply attenuating the HIV virulence genes, for example, the genes env, vif, vpr, vpu and nef are deleted, making the vector safer for therapeutic purposes. Lentiviral vectors are known in the art, see Naldini et al., (1996 and 1998); Zufferey et al., (1997); Dull et al., 1998, U.S. Pat. Nos. 6,013,516; and 5,994,136). In some embodiments, these viral vectors are plasmid-based or virus-based, and are configured to carry the essential sequences for incorporating foreign nucleic acid, for selection, and for transfer of the nucleic acid into a host cell. Known lentiviruses can be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; 10801 University Blvd., Manassas, Va. 20110-2209), or isolated from known sources using commonly available techniques.

[0112] The viral vector genome is typically constructed in a plasmid form that can be transfected into a packaging or producer cell line. In any of such examples, the nucleic acid encoding a recombinant protein, such as a recombinant receptor, is inserted or located in a region of the viral vector, such as generally in a non-essential region of the viral genome. In some embodiments, the nucleic acid is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication defective.

[0113] Any of a variety of known methods can be used to produce retroviral particles whose genome contains an RNA copy of the viral vector genome. In some embodiments, at least two components are involved in making a virus-based gene delivery system: first, packaging plasmids, encompassing the structural proteins as well as the enzymes necessary to generate a viral vector particle, and second, the viral vector itself, i.e., the genetic material to be transferred. Biosafety safeguards can be introduced in the design of one or both of these components.

[0114] In some embodiments, the packaging plasmid can contain all retroviral, such as HIV-1, proteins other than envelope proteins (Naldini et al., 1998). In other embodiments, viral vectors can lack additional viral genes, such as those that are associated with virulence, e.g. vpr, vif, vpu and nef, and/or Tat, a primary transactivator of HIV. In some embodiments, lentiviral vectors, such as HIV-based lentiviral vectors, comprise only three genes of the parental virus: gag, pol and rev, which reduces or eliminates the possibility of reconstitution of a wild-type virus through recombination.

[0115] In some embodiments, the viral vector genome is introduced into a packaging cell line that contains all the components necessary to package viral genomic RNA, transcribed from the viral vector genome, into viral particles. Alternatively, the viral vector genome may comprise one or more genes encoding viral components in addition to the one or more sequences, e.g., recombinant nucleic acids, of interest. In some aspects, in order to prevent replication of the genome in the target cell, however, endogenous viral genes required for replication are removed and provided separately in the packaging cell line.

[0116] In some embodiments, a packaging cell line is transfected with one or more plasmid vectors containing the components necessary to generate the particles. In some embodiments, a packaging cell line is transfected with a plasmid containing the viral vector genome, including the LTRs, the cis-acting packaging sequence and the sequence of interest, i.e. a nucleic acid encoding an antigen receptor, such as a CAR; and one or more helper plasmids encoding the virus enzymatic and/or structural components, such as Gag, pol and/or rev. In some embodiments, multiple vectors are utilized to separate the various genetic components that generate the retroviral vector particles. In some such embodiments, providing separate vectors to the packaging cell reduces the chance of recombination events that might otherwise generate replication competent viruses. In some embodiments, a single plasmid vector having all of the retroviral components can be used.

[0117] In some embodiments, the retroviral vector particle, such as lentiviral vector particle, is pseudotyped to increase the transduction efficiency of host cells. For example, a retroviral vector particle, such as a lentiviral vector particle, in some embodiments is pseudotyped with a VSV-G glycoprotein, which provides a broad cell host range extending the cell types that can be transduced. In some embodiments, a packaging cell line is transfected with a plasmid or polynucleotide encoding a non-native envelope glycoprotein, such as to include xenotropic, polytropic or amphotropic envelopes, such as Sindbis virus envelope, GALV or VSV-G.

[0118] In some embodiments, the packaging cell line provides the components, including viral regulatory and structural proteins, that are required in trans for the packaging of the viral genomic RNA into lentiviral vector particles. In some embodiments, the packaging cell line may be any cell line that is capable of expressing lentiviral proteins and producing functional lentiviral vector particles. In some aspects, suitable packaging cell lines include 293 (ATCC CCL X), 293T, HeLA (ATCC CCL 2), D17 (ATCC CCL 183), MDCK (ATCC CCL 34), BHK (ATCC CCL-10) and Cf2Th (ATCC CRL 1430) cells.

[0119] In some embodiments, the packaging cell line stably expresses the viral protein(s). For example, in some aspects, a packaging cell line containing the gag, pol, rev and/or other structural genes but without the LTR and packaging components can be constructed. In some embodiments, a packaging cell line can be transiently transfected with nucleic acid molecules encoding one or more viral proteins along with the viral vector genome containing a nucleic acid molecule encoding a heterologous protein, and/or a nucleic acid encoding an envelope glycoprotein.

[0120] In some embodiments, the viral vectors and the packaging and/or helper plasmids are introduced via transfection or infection into the packaging cell line. The packaging cell line produces viral vector particles that contain the viral vector genome. Methods for transfection or infection are well known. Non-limiting examples include calcium phosphate, DEAE-dextran and lipofection methods, electroporation and microinjection.

[0121] When a recombinant plasmid and the retroviral LTR and packaging sequences are introduced into a special cell line (e.g., by calcium phosphate precipitation for example), the packaging sequences may permit the RNA transcript of the recombinant plasmid to be packaged into viral particles, which then may be secreted into the culture media. The media containing the recombinant retroviruses in some embodiments is then collected, optionally concentrated, and used for gene transfer. For example, in some aspects, after cotransfection of the packaging plasmids and the transfer vector to the packaging cell line, the viral vector particles are recovered from the culture media and titered by standard methods used by those of skill in the art.

[0122] In some embodiments, a retroviral vector, such as a lentiviral vector, can be produced in a packaging cell line, such as an exemplary HEK 293T cell line, by introduction of plasmids to allow generation of lentiviral particles. In some embodiments, a packaging cell is transfected and/or contains a polynucleotide encoding gag and pol, and a polynucleotide encoding a recombinant receptor, such as an antigen receptor, for example, a CAR. In some embodiments,

the packaging cell line is optionally and/or additionally transfected with and/or contains a polynucleotide encoding a rev protein. In some embodiments, the packaging cell line is optionally and/or additionally transfected with and/or contains a polynucleotide encoding a non-native envelope glycoprotein, such as VSV-G. In some such embodiments, approximately two days after transfection of cells, e.g. HEK 293T cells, the cell supernatant contains recombinant lentiviral vectors, which can be recovered and titered.

[0123] Recovered and/or produced retroviral vector particles can be used to transduce target cells using the methods as described. Once in the target cells, the viral RNA is reverse-transcribed, imported into the nucleus and stably integrated into the host genome. One or two days after the integration of the viral RNA, the expression of the recombinant protein, e.g. antigen receptor, such as CAR, can be detected.

D. Cultivating and/or Expansion of Cells

[0124] In some embodiments, biomedical materials that can be stored or transferred in the provided biomedical materials vessels, or in the provided articles of manufacture, include cells that have been engineered using methods that include include one or more steps for cultivating engineered cells, e.g., cultivating cells under conditions that promote proliferation and/or expansion. In some embodiments, engineered cells are cultivated under conditions that promote proliferation and/or expansion subsequent to a step of genetically engineering, e.g., introducing a recombinant polypeptide to the cells by transduction or transfection. In particular embodiments, the cells are cultivated after the cells have been incubated under stimulating conditions and transduced or transfected with a recombinant polynucleotide, e.g., a polynucleotide encoding a recombinant receptor. In some embodiments, the cultivation produces one or more cultivated compositions of enriched T cells. In some embodiments, such conditions may be designed to induce proliferation, expansion, activation, and/or survival of cells in the population. In particular embodiments, the stimulating 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 promote growth, division, and/or expansion of the cells.

[0125] In some embodiments, the engineered cells are cultured in a container that can be filled, e.g. via the feed port, with cell media and/or cells for culturing of the added cells. The cells can be from any cell source for which culture of the cells is desired, for example, for expansion and/or proliferation of the cells.

[0126] In some aspects, the culture media is an adapted culture medium that supports that growth, cultivation, expansion or proliferation of the cells, such as T cells. In some aspects, the medium can be a liquid containing a mixture of salts, amino acids, vitamins, sugars or any combination thereof. In some embodiments, the culture media further contains one or more stimulating conditions or agents, such as to stimulate the cultivation, expansion or proliferation of cells during the incubation. In some embodiments, the stimulating condition is or includes one or more cytokines, such as selected from IL-2, IL-7 or IL-15. In some embodiments, the cytokine is a recombinant cytokine. In particular embodiments, the one or more cytokines are human recombinant cytokines. In certain embodiments, the one or more cytokines bind to and/or are capable of binding to receptors that are expressed by and/or are endogenous to T cells. In particular embodiments, the one or more cytokines is or includes a member of the 4-alpha-helix bundle family of cytokines. In some embodiments, members of the 4-alpha-helix bundle family of cytokines include, but are not limited to, interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-7 (IL-7), interleukin-9 (IL-9), interleukin 12 (IL-12), interleukin 15 (IL-15), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF). In some embodiments, the one or more cytokines is or includes IL-15. In particular embodiments, the one or more cytokines is or includes IL-7. In particular embodiments, the one or more cytokines is or includes recombinant IL-2.

[0127] In some embodiments, the concentration of the one or more cytokine in the culture media during the culturing or incubation, independently, is from or from about 1 IU/mL to 1500 IU/mL, such as from or from about 1 IU/mL to 100 IU/mL, 2 IU/mL to 50 IU/mL, 5 IU/mL to 10 IU/mL, 10 IU/mL to 500 IU/mL, 50 IU/mL to 250 IU/mL or 100 IU/mL to 200 IU/mL, 50 IU/mL to 1500 IU/mL, 100 IU/mL to 1000 IU/mL or 200 IU/mL to 600 IU/mL. In some embodiments, the concentration of the one or more cytokine, independently, is at least or at least about 1 IU/mL, 5 IU/mL, 10 IU/mL, 50 IU/mL, 100 IU/mL, 200 IU/mL, 500 IU/mL, 1000 IU/mL or 1500 IU/mL.

[0128] In some aspects, the cells are incubated for at least a portion of time after transfer of the engineered cells and culture media. In some embodiments, the stimulating conditions generally include a temperature suitable for the growth of primary immune cells, such as 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 embodiments, the composition of enriched T cells is incubated at a temperature of 25 to 38°C, such as 30 to 37°C, for example at or about 37 ºC ± 2 ºC. In some embodiments, the incubation is carried out for a time period

until the culture, e.g. cultivation or expansion, results in a desired or threshold density, concentration, number or dose of cells. In some embodiments, the incubation is carried out for a time period until the culture, e.g. cultivation or expansion, results in a desired or threshold density, concentration, number or dose of viable cells. In some embodiments, the incubation is greater than or greater than about or is for about or 24 hours, 48 hours, 72 hours, 96 hours, 5 days, 6 days, 7 days, 8 days, 9 days or more.

[0129] In some embodiments, the cells are incubated under conditions to maintain a target amount of carbon dioxide in the cell culture. In some aspects, this ensures optimal cultivation, expansion and proliferation of the cells during the growth. In some aspects, the amount of carbon dioxide (CO_2) is between 10% and 0% (v/v) of said gas, such as between 8% and 2% (v/v) of said gas, for example an amount of or about 5% (v/v) CO_2.

[0130] In particular embodiments, the cultivation is performed in a closed system. In certain embodiments, the cultivation is performed in a closed system under sterile conditions. In particular embodiments, the cultivation is performed in the same closed system as one or more steps of the provided systems. In some embodiments the composition of enriched T cells is removed from a closed system and placed in and/or connected to a bioreactor for the cultivation. Examples of suitable bioreactors for the cultivation include, but are not limited to, GE Xuri W25, GE Xuri W5, Sartorius BioSTAT RM 20 | 50, Finesse SmartRocker Bioreactor Systems, and Pall XRS Bioreactor Systems. In some embodiments, the bioreactor is used to perfuse and/or mix the cells during at least a portion of the cultivation step.

[0131] In some embodiments, cells cultivated while enclosed, connected, and/or under control of a bioreactor undergo expansion during the cultivation more rapidly than cells that are cultivated without a bioreactor, e.g., cells that are cultivated under static conditions such as without mixing, rocking, motion, and/or perfusion. In some embodiments, cells cultivated while enclosed, connected, and/or under control of a bioreactor reach or achieve a threshold expansion, cell count, and/or density within 14 days, 10 days, 9 days, 8 days, 7 days, 6 days, 5 days, 4 days, 3 days, 2 days, 60 hours, 48 hours, 36 hours, 24 hours, or 12 hours. In some embodiments, cells cultivated while enclosed, connected, and/or under control of a bioreactor reach or achieve a threshold expansion, cell count, and/or density at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 100%, at least 150%, at least 1-fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold than cells cultivated in an exemplary and/or alternative process where cells are not cultivated while enclosed, connected, and/or under control of a bioreactor.

[0132] In some embodiments, the mixing is or includes rocking and/or motioning. In some embodiments, cells are incubated using containers, e.g., bags, which are used in connection with a bioreactor. In some cases, the bioreactor can be subject to motioning or rocking, which, in some aspects, can increase oxygen transfer. Motioning the bioreactor may include, but is not limited to rotating along a horizontal axis, rotating along a vertical axis, a rocking motion along a tilted or inclined horizontal axis of the bioreactor or any combination thereof. In some embodiments, at least a portion of the incubation is carried out with rocking. The rocking speed and rocking angle may be adjusted to achieve a desired agitation. In some embodiments the rock angle is or is about 20°, 19°, 18°, 17°, 16°, 15°, 14°, 13°, 12°, 11°, 10°, 9°, 8°, 7°, 6°, 5°, 4°, 3°, 2° or 1°. In certain embodiments, the rock angle is between 6-16°. In other embodiments, the rock angle is between 7-16°. In other embodiments, the rock angle is between 8-12°. In some embodiments, the rock rate is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 1 12, 13, 14 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 rpm. In some embodiments, the rock rate is between 4 and 12 rpm, such as between 4 and 6 rpm, inclusive. At least a portion of the cell culture expansion is performed with a rocking motion, such as at an angle of between 5° and 10°, such as 6°, at a constant rocking speed, such as a speed of between 5 and 15 RPM, such as 6 RMP or 10 RPM.

[0133] In some embodiments, a composition comprising cells, such as engineered T cells, e.g. engineered CD4+ T cells or engineered CD8+ T cells, is cultivated in the presence of a surfactant. In particular embodiments, cultivating the cells of the composition reduces the amount of shear stress that may occur during the cultivation, e.g., due to mixing, rocking, motion, and/or perfusion. In particular embodiments, the composition of cells, such as engineered T cells, e.g. engineered CD4+ T cells or engineered CD8+ T cells, is cultivated with the surfactant and at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 99.9% of the T cells survive, e.g., are viable and/or do not undergo necrosis, programed cell death, or apoptosis, during or at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or more than 7 days after the cultivation is complete. In particular embodiments, the composition of cells, such as engineered T cells, e.g. engineered CD4+ T cells or engineered CD8+ T cells, is cultivated in the presence of a surfactant and less than 50%, less than 40%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, less than 5%, less than 1%, less than 0.1% or less than 0.01% of the cells undergo cell death, e.g., programmed cell death, apoptosis, and/or necrosis, such as due to shearing or shearing-induced stress.

[0134] In particular embodiments, a composition of cells, such as engineered T cells, e.g. engineered CD4+ T cells or engineered CD8+ T cells, is cultivated in the presence of between 0.1 µl/ml and 10.0 µl/ml, between 0.2 µl/ml and 2.5 µl/ml, between 0.5 µl/ml and 5 µl/ml, between 1 µl/ml and 3 µl/ml, or between 2 µl/ml and 4 µl/ml of the surfactant. In some embodiments, the composition of cells, such as engineered T cells, e.g. engineered CD4+ T cells or engineered CD8+ T cells, is cultivated in the presence of, of about, or at least 0.1 µl/ml, 0.2 µl/ml, 0.4 µl/ml, 0.6 µl/ml, 0.8 µl/ml, 1 µl/ml, 1.5 µl/ml, 2.0 µl/ml, 2.5 µl/ml, 5.0 µl/ml, 10 µl/ml, 25 µl/ml, or 50 µl/ml of the surfactant. In certain embodiments, the composition of cells is cultivated in the presence of or of about 2 µl/ml of the surfactant.

[0135] In some embodiments, a surfactant is or includes an agent that reduces the surface tension of liquids and/or solids. For example, a surfactant includes a fatty alcohol (e.g., steryl alcohol), a polyoxyethylene glycol octylphenol ether (e.g., Triton X-100), or a polyoxyethylene glycol sorbitan alkyl ester (e.g., polysorbate 20, 40, 60). In certain embodiments the surfactant is selected from the group consisting of Polysorbate 80 (PS80), polysorbate 20 (PS20), poloxamer 188 (P188). In an exemplary embodiment, the concentration of the surfactant in chemically defined feed media is about 0.0025% to about 0.25% (v/v) of PS80; about 0.0025% to about 0.25% (v/v) of PS20; or about 0.1% to about 5.0% (w/v) of P188.

[0136] In some embodiments, the surfactant is or includes an anionic surfactant, a cationic surfactant, a zwitterionic surfactant, or a nonionic surfactant added thereto. Suitable anionic surfactants include but are not limited to alkyl sulfonates, alkyl phosphates, alkyl phosphonates, potassium laurate, triethanolamine stearate, sodium lauryl sulfate, sodium dodecylsulfate, alkyl polyoxyethylene sulfates, sodium alginate, dioctyl sodium sulfosuccinate, phosphatidyl glycerol, phosphatidyl inosine, phosphatidylinositol, diphosphatidylglycerol, phosphatidylserine, phosphatidic acid and their salts, sodium carboxymethylcellulose, cholic acid and other bile acids (e.g., cholic acid, deoxycholic acid, glycocholic acid, taurocholic acid, glycodeoxycholic acid) and salts thereof (e.g., sodium deoxycholate).

[0137] In some embodiments, suitable nonionic surfactants include: glyceryl esters, polyoxyethylene fatty alcohol ethers, polyoxyethylene sorbitan fatty acid esters (polysorbates), polyoxyethylene fatty acid esters, sorbitan esters, glycerol monostearate, polyethylene glycols, polypropylene glycols, cetyl alcohol, cetostearyl alcohol, stearyl alcohol, aryl alkyl polyether alcohols, polyoxyethylene-polyoxypropylene copolymers (poloxamers), poloxamines, methylcellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, noncrystalline cellulose, polysaccharides including starch and starch derivatives such

as hydroxyethylstarch (HES), polyvinyl alcohol, and polyvinylpyrrolidone. In certain embodiments, the nonionic surfactant is a polyoxyethylene and polyoxypropylene copolymer and preferably a block copolymer of propylene glycol and ethylene glycol. Such polymers are sold under the tradename POLOXAMER, also sometimes referred to as PLURONIC® F68 or Kolliphor® P188. Among polyoxyethylene fatty acid esters is included those having short alkyl chains. One example of such a surfactant is SOLUTOL® HS 15, polyethylene-660-hydroxystearate.

[0138] In some embodiments, suitable cationic surfactants may include, but are not limited to, natural phospholipids, synthetic phospholipids, quaternary ammonium compounds, benzalkonium chloride, cetyltrimethyl ammonium bromide, chitosans, lauryl dimethyl benzyl ammonium chloride, acyl carnitine hydrochlorides, dimethyl dioctadecyl ammomium bromide (DDAB), dioleyoltrimethyl ammonium propane (DOTAP), dimyristoyl trimethyl ammonium propane (DMTAP), dimethyl amino ethane carbamoyl cholesterol (DC-Chol), 1,2-diacylglycero-3-(O-alkyl) phosphocholine, O-alkylphosphatidylcholine, alkyl pyridinium halides, or long-chain alkyl amines such as, for example, n-octylamine and oleylamine.

[0139] Zwitterionic surfactants are electrically neutral but possess local positive and negative charges within the same molecule. Suitable zwitterionic surfactants include but are not limited to zwitterionic phospholipids. Suitable phospholipids include phosphatidylcholine, phosphatidylethanolamine, diacyl-glycero-phosphoethanolamine (such as dimyristoyl-glycero-phosphoethanolamine (DMPE), dipalmitoyl-glycero-phosphoethanolamine (DPPE), distearoyl-glycero-phosphoethanolamine (DSPE), and dioleolyl-glycero-phosphoethanolamine (DOPE)). Mixtures of phospholipids that include anionic and zwitterionic phospholipids may be employed in this invention. Such mixtures include but are not limited to lysophospholipids, egg or soybean phospholipid or any combination thereof. The phospholipid, whether anionic, zwitterionic or a mixture of phospholipids, may be salted or desalted, hydrogenated or partially hydrogenated or natural semi-synthetic or synthetic.

[0140] In certain embodiments, the surfactant is poloxamer, e.g., poloxamer 188. In some embodiments, a composition of cells is cultivated in the presence of between 0.1 µl/ml and 10.0 µl/ml, between 0.2 µl/ml and 2.5 µl/ml, between 0.5 µl/ml and 5 µl/ml, between 1 µl/ml and 3 µl/ml, or between 2 µl/ml and 4 µl/ml of poloxamer. In some embodiments, the composition of cells is cultivated in the presence of, of about, or at least 0.1 µl/ml, 0.2 µl/ml, 0.4 µl/ml, 0.6 µl/ml, 0.8 µl/ml, 1 µl/ml, 1.5 µl/ml, 2.0 µl/ml, 2.5 µl/ml, 5.0 µl/ml, 10 µl/ml, 25

µl/ml, or 50 µl/ml of the surfactant. In certain embodiments, the composition of cells is cultivated in the presence of or of about 2 µl/ml of poloxamer.

[0141] In some aspects, the CD4+ and CD8+ cells are each separately expanded or expanded together until they each reach a threshold amount or cell density. In particular embodiments, the cultivation ends, such as by harvesting cells, when cells achieve a threshold amount, concentration, and/or expansion. In particular embodiments, the cultivation ends when the cell achieve or achieve about or at least a 1.5-fold expansion, a 2-fold expansion, a 2.5-fold expansion, a 3-fold expansion, a 3.5-fold expansion, a 4-fold expansion, a 4.5-fold expansion, a 5-fold expansion, a 6-fold expansion, a 7-fold expansion, a 8-fold expansion, a 9-fold expansion, a 10-fold expansion, or greater than a 10-fold expansion, e.g., with respect and/or in relation to the amount of density of the cells at the start or initiation of the cultivation. In some embodiments, the threshold expansion is a 4-fold expansion, e.g., with respect and/or in relation to the amount of density of the cells at the start or initiation of the cultivation. In some embodiments, the cultivation ends, such as by harvesting cells, when the cells achieve a threshold total amount of cells, e.g., threshold cell count. In some embodiments, the cultivation ends when the cells achieve a threshold total nucleated cell (TNC) count. In some embodiments, the cultivation ends when the cells achieve a threshold viable amount of cells, e.g., threshold viable cell count. In some embodiments, the threshold cell count is or is about or is at least of 50 $x10^6$ cells, 100 $x10^6$ cells, 200 $x10^6$ cells, 300 $x10^6$ cells, 400 $x10^6$ cells, 600 $x10^6$ cells, 800 $x10^6$ cells, 1000 $x10^6$ cells, 1200 $x10^6$ cells, 1400 $x10^6$ cells, 1600 $x10^6$ cells, 1800 $x10^6$ cells, 2000 $x10^6$ cells, 2500 $x10^6$ cells, 3000 $x10^6$ cells, 4000 $x10^6$ cells, 5000 $x10^6$ cells, 10,000 $x10^6$ cells, 12,000 $x10^6$ cells, 15,000 $x10^6$ cells or 20,000 $x10^6$ cells, or any of the foregoing threshold of viable cells.

[0142] In particular embodiments, the cultivation ends when the cells achieve a threshold cell count. In some embodiments, the cultivation ends at, at about, or within 6 hours, 12 hours, 24 hours, 36 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 or more days, after the threshold cell count is achieved. In particular embodiments, the cultivation is ended at or about 1 day after the threshold cell count is achieved. In certain embodiments, the threshold density is, is about, or is at least 0.1 $x10^6$ cells/ml, 0.5 $x10^6$ cells/ml, 1 $x10^6$ cells/ml, 1.2 $x10^6$ cells/ml, 1.5 $x10^6$ cells/ml, 1.6 $x10^6$ cells/ml, 1.8 $x10^6$ cells/ml, 2.0 $x10^6$ cells/ml, 2.5 $x10^6$ cells/ml, 3.0 $x10^6$ cells/ml, 3.5 $x10^6$ cells/ml, 4.0 $x10^6$ cells/ml, 4.5 $x10^6$ cells/ml, 5.0 $x10^6$ cells/ml, 6 $x10^6$ cells/ml, 8 $x10^6$ cells/ml, or 10 $x10^6$ cells/ml, or any of the foregoing threshold of viable cells. In particular embodiments, the cultivation ends when the cells achieve a threshold density. In

some embodiments, the cultivation ends at, at about, or within 6 hours, 12 hours, 24 hours, 36 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 or more days, after the threshold density is achieved. In particular embodiments, the cultivation is ended at or about 1 day after the threshold density is achieved.

[0143] In some embodiments, at least a portion of the incubation is carried out under static conditions. In some embodiments, at least a portion of the incubation is carried out with perfusion, such as to perfuse out spent media and perfuse in fresh media during the culture. In some embodiments, the method includes a step of perfusing fresh culture medium into the cell culture, such as through a feed port. In some embodiments, the culture media added during perfusion contains the one or more stimulating agents, e.g. one or more recombinant cytokine, such as IL-2, IL-7 and/or IL-15. In some embodiments, the culture media added during perfusion is the same culture media used during a static incubation.

[0144] In some embodiments, subsequent to the incubation, the container, e.g., bag, is re-connected to a system for carrying out the one or more other processing steps of for manufacturing, generating or producing the cell therapy, such as is re-connected to the system containing the centrifugal chamber. In some aspects, cultured cells are transferred from the bag to the internal cavity of the chamber for formulation of the cultured cells.

E. Compositions and Formulations

[0145] In some embodiments, the dose of cells comprising cells engineered with a recombinant antigen receptor, e.g. CAR or TCR, is provided as a composition or formulation, such as a pharmaceutical composition or formulation. Such compositions can be used in accord with adoptive cell therapy methods, including methods for the prevention or treatment of diseases, conditions, and disorders, or in detection, diagnostic, and prognostic methods. In some embodiments, such compositions or formulations can be stored, contained or transferred in the provided biomedical materials vessels, and/or as a component of the provided articles of manufacture.

[0146] In some cases, the cells are processed in one or more steps (e.g. carried out in the centrifugal chamber and/or closed system) for manufacturing, generating or producing a cell therapy and/or engineered cells may include formulation of cells, such as formulation of genetically engineered cells resulting from the provided transduction processing steps prior to or after the culturing, e.g. cultivation and expansion, and/or one or more other processing steps as described. In some cases, the cells can be formulated in an amount for dosage administration, such as for a single unit dosage administration or multiple dosage administration. In some

embodiments, the provided methods associated with formulation of cells include processing transduced cells, such as cells transduced and/or expanded using the processing steps described above, in a closed system. In some embodiments, the formulated cells can be transferred or introduced into the biomedical material vessels, e.g., vials, provided herein.

[0147] In certain embodiments, one or more compositions of cells, such as engineered and cultivated T cells, are formulated. In particular embodiments, one or more compositions of cells, such as engineered and cultivated T cells, are formulated after the one or more compositions have been engineered and/or cultivated.

[0148] In some embodiments, T cells, such as CD4+ and/or CD8+ T cells, generated by one or more of the processing steps are formulated. In some aspects, a plurality of compositions are separately manufactured, produced or generated, each containing a different population and/or sub-types of cells from the subject, such as for administration separately or independently, optionally within a certain period of time. For example, separate formulations of engineered cells containing different populations or sub-types of cells can include CD8+ and CD4+ T cells, respectively, and/or CD8+- and CD4+-enriched populations, respectively, e.g., CD4+ and/or CD8+ T cells each individually including cells genetically engineered to express the recombinant receptor. In some embodiments, at least one composition is formulated with CD4+ T cells genetically engineered to express the recombinant receptor. In some embodiments, at least one composition is formulated with CD8+ T cells genetically engineered to express the recombinant receptor. In some embodiments, the administration of the dose comprises administration of a first composition comprising a dose of CD8+ T cells or a dose of CD4+ T cells and administration of a second composition comprising the other of the dose of CD4+ T cells and the CD8+ T cells. In some embodiments, a first composition comprising a dose of CD8+ T cells or a dose of CD4+ T cells is administered prior to the second composition comprising the other of the dose of CD4+ T cells and the CD8+ T cells. In some embodiments, the administration of the dose comprises administration of a composition comprising both of a dose of CD8+ T cells and a dose of CD4+ T cells.

[0149] In certain embodiments, the one or more compositions of cells, such as engineered and cultivated T cells, are or include two separate compositions, e.g., separate engineered and/or cultivated compositions, of cells. In particular embodiments, two separate compositions of cells, e.g., two separate compositions of CD4+ T cells and CD8+ T cells selected, isolated, and/or enriched from the same biological sample, separately engineered and separately cultivated, are separately formulated. In certain embodiments, the two separate compositions

include a composition of CD4+ T cells, such as a composition of engineered and/or cultivated CD4+ T cells. In particular embodiments, the two separate compositions include a composition of CD8+ T cells, such as a composition of engineered and/or cultivated CD8+ T cells. In some embodiments, two separate compositions of CD4+ T cells and CD8+ T cells, such as separate compositions of engineered and cultivated CD4+ T cells and engineered and cultivated CD8+ T cells, are separately formulated. In some embodiments, a single composition of cells is formulated. In certain embodiments, the single composition is a composition of CD4+ T cells, such as a composition of engineered and/or cultivated CD4+ T cells. In some embodiments, the single composition is a composition of CD4+ and CD8+ T cells that have been combined from separate compositions prior to the formulation.

[0150] In some embodiments, separate compositions of CD4+ and CD8+ T cells, such as separate compositions of engineered and cultivated CD4+ and CD8+ T cells are combined into a single composition and are formulated. In certain embodiments, separate formulated compositions of CD4+ and CD8+ T cells are combined into a single composition after the formulation has been performed and/or completed. In particular embodiments, separate compositions of CD4+ and CD8+ T cells, such as separate compositions of engineered and cultivated CD4+ and CD8+ T cells, are separately formulated as separate compositions.

[0151] In some embodiments, cells can be formulated into a container, such as a vial, such as any vial in the biomedical materials vessels provided herein. In some embodiments, the cells are formulated between 0 days and 10 days, between 0 and 5 days, between 2 days and 7 days, between 0.5 days, and 4 days, or between 1 day and 3 days after the cells after the threshold cell count, density, and/or expansion has been achieved during the cultivation. In certain embodiments, the cells are formulated at or at or about or within 12 hours, 18 hours, 24 hours, 1 day, 2 days, or 3 days after the threshold cell count, density, and/or expansion has been achieved during the cultivation. In some embodiments, the cells are formulated within or within about 1 day after the threshold cell count, density, and/or expansion has been achieved during the cultivation.

[0152] In some embodiments, the cells are formulated in a pharmaceutically acceptable buffer, which may, in some aspects, include a pharmaceutically acceptable carrier or excipient. In some embodiments, the processing includes exchange of a medium into a medium or formulation buffer that is pharmaceutically acceptable or desired for administration to a subject. In some embodiments, the processing steps can involve washing the transduced and/or expanded cells to replace the cells in a pharmaceutically acceptable buffer that can include one or more

optional pharmaceutically acceptable carriers or excipients. Exemplary of such pharmaceutical forms, including pharmaceutically acceptable carriers or excipients, can be any described below in conjunction with forms acceptable for administering the cells and compositions to a subject. The pharmaceutical composition in some embodiments contains the cells in amounts effective to treat or prevent the disease or condition, such as a therapeutically effective or prophylactically effective amount.

[0153] A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

[0154] In some aspects, the choice of carrier is determined in part by the particular cell and/or by the method of administration. 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 aspects, 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. Carriers are described, e.g., by Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980). Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG).

[0155] Buffering agents in some aspects are included in the compositions. Suitable buffering agents include, for example, citric acid, sodium citrate, phosphoric acid, potassium phosphate, and various other acids and salts. In some aspects, 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).

[0156] The formulations can include aqueous solutions. The formulation or composition may also contain more than one active ingredient useful for the particular indication, disease, or condition being treated with the cells, preferably those with activities complementary to the cells, where the respective activities do not adversely affect one another. Such active ingredients are suitably present in combination in amounts that are effective for the purpose intended. Thus, in some embodiments, the pharmaceutical composition further includes 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, and/or vincristine.

[0157] Compositions in some embodiments are provided as sterile liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions, dispersions, or viscous compositions, which may in some aspects be buffered to a selected pH. Liquid compositions can comprise carriers, which can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol) and suitable mixtures thereof. Sterile injectable solutions can be prepared by incorporating the cells in a solvent, such as in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, dextrose, or the like. The compositions can contain auxiliary substances such as wetting, dispersing, or emulsifying agents (e.g., methylcellulose), pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, and/or colors, depending upon the route of administration and the preparation desired. Standard texts may in some aspects be consulted to prepare suitable preparations.

[0158] Various additives which enhance the stability and sterility of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, and sorbic acid. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0159] In some embodiments, the formulation buffer contains a cryopreservative. In some embodiments, the cell are formulated with a cyropreservative solution that contains 1.0% to 30% DMSO solution, such as a 5% to 20% DMSO solution or a 5% to 10% DMSO solution. In some embodiments, the cryopreservation solution is or contains, for example, PBS containing 20% DMSO and 8% human serum albumin (HSA), or other suitable cell freezing media. In some embodiments, the cryopreservative solution is or contains, for example, at least or about 7.5% DMSO. In some embodiments, the processing steps can involve washing the transduced and/or expanded cells to replace the cells in a cryopreservative solution. In some embodiments, the cells are frozen, e.g., cryopreserved or cryoprotected, in media and/or solution with a final concentration of or of about 12.5%, 12.0%, 11.5%, 11.0%, 10.5%, 10.0%, 9.5%, 9. 0%, 8.5%, 8.0%, 7.5%, 7.0%, 6.5%, 6.0%, 5.5%, or 5.0% DMSO, or between 1% and 15%, between 6% and 12%, between 5% and 10%, or between 6% and 8% DMSO. In particular embodiments, the cells are frozen, e.g., cryopreserved or cryoprotected, in media and/or solution with a final concentration of or of about 5.0%, 4.5%, 4.0%, 3.5%, 3.0%, 2.5%, 2.0%, 1.5%, 1.25%, 1.0%, 0.75%, 0.5%, or 0.25% HSA, or between 0.1% and 5%, between 0.25% and 4%, between 0.5% and 2%, or between 1% and 2% HSA.

[0160] In particular embodiments, the composition of enriched T cells, e.g., T cells that have been stimulated, engineered, and/or cultivated, are formulated, cryopreserved, and then stored for an amount of time, for example, in the provided biomedical materials vessel. In certain embodiments, the formulated, cryopreserved cells are stored until the cells are released for infusion. In particular embodiments, the formulated cryopreserved cells are stored for between 1 day and 6 months, between 1 month and 3 months, between 1 day and 14 days, between 1 day and 7 days, between 3 days and 6 days, between 6 months and 12 months, or longer than 12 months. In some embodiments, the cells are cryopreserved and stored for, for about, or for less than 1 days, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days. In certain embodiments, the cells are thawed and administered to a subject after the storage. In certain embodiments, the cells are stored for or for about 5 days.

[0161] In some embodiments, the formulation is carried out using one or more processing step including washing, diluting or concentrating the cells, such as the cultured or expanded cells. In some embodiments, the processing can include dilution or concentration of the cells to a desired concentration or number, such as unit dose form compositions including the number of cells for administration in a given dose or fraction thereof. In some embodiments, the processing steps can include a volume-reduction to thereby increase the concentration of cells as

desired. In some embodiments, the processing steps can include a volume-addition to thereby decrease the concentration of cells as desired. In some embodiments, the processing includes adding a volume of a formulation buffer to transduced and/or expanded cells. In some embodiments, the volume of formulation buffer is from 10 mL to 1000 mL or from about 10 mL to about 1000 mL, such as at least or about at least or about 50 mL, 100 mL, 200 mL, 300 mL, 400 mL, 500 mL, 600 mL, 700 mL, 800 mL, 900 mL or 1000 mL.

[0162] In some embodiments, the cells are cultured, such as stimulated engineered and/or cultivated in a container, e.g., bag or a centrifugal chamber. In some aspects, the container is a first container and the cultured cells are expressed or transferred from the first container, e.g. bag or centrifugal chamber, to a second container, such as biomedical material vessels, that is operably linked to the first container. In some embodiments, the biomedical material vessels are configured for integration and or operable connection and/or is integrated or operably connected, to the first container, e.g. bag or centrifugal chamber, used for one or more of the previous processing steps. In some embodiments, the biomedical material vessel is connected to the first container, e.g. bag or centrifugal chamber, at an output line or output position. In some cases, the first container, e.g. bag or centrifugal chamber, is connected to the vial of the biomedical material vessel at the inlet tube.

[0163] In some embodiments, such processing steps for formulating a cell composition is carried out in a closed system. Exemplary of such processing steps can be performed using a centrifugal chamber in conjunction with one or more systems or kits associated with a cell processing system, such as a centrifugal chamber produced and sold by Biosafe SA, including those for use with the Sepax® or Sepax 2® cell processing systems. An exemplary system and process is described in International Publication Number WO2016/073602. In some embodiments, the method includes effecting expression or transfer from the internal cavity of the centrifugal chamber a formulated composition, which is the resulting composition of cells formulated in a formulation buffer, such as pharmaceutically acceptable buffer, in any of the above embodiments as described. In some embodiments, the expression or transfer of the formulated composition is to a container, such as vials of the biomedical material vessels described herein, that is operably linked as part of a closed system with the centrifugal chamber. In some embodiments, the biomedical material vessels are configured for integration and or operable connection and/or is integrated or operably connected, to a closed system or device that carries out one or more processing steps. In some embodiments, the biomedical material vessel is connected to a system at an output line or output position. In some cases, the closed system is

connected to the vial of the biomedical material vessel at the inlet tube. Exemplary closed systems for use with the biomedical material vessels described herein include the Sepax® and Sepax® 2 system.

[0164] In some embodiments, the composition can be transferred from the first container, such as a centrifugal chamber or cell processing system, to the provided biomedical material vessels via a multi-port output kit containing a multi-way tubing manifold associated at each end of a tubing line with a port to which one or a plurality of containers, e.g. biomedical material vessels, can be connected for expression of the formulated composition. In some aspects, a desired number or plurality of such vials, can be sterilely connected to one or more, generally two or more, such as at least 3, 4, 5, 6, 7, 8 or more of the ports of the multi-port output. For example, in some embodiments, one or more containers, e.g., biomedical material vessels, can be attached to the ports, or to fewer than all of the ports. Thus, in some embodiments, the system can effect expression of the output composition into a plurality of vials of the biomedical material vessels.

[0165] In some aspects, cells can be expressed or transferred to the one or more of the plurality of output containers, e.g., vials of the biomedical material vessels, in an amount for dosage administration, such as for a single unit dosage administration or multiple dosage administration. For example, in some embodiments, the vials of the biomedical material vessels, may each contain the number of cells for administration in a given dose or fraction thereof. Thus, each vial, in some aspects, may contain a single unit dose for administration or may contain a fraction of a desired dose such that more than one of the plurality of vials, such as two of the vials, or 3 of the vials, together constitute a dose for administration.

[0166] Thus, the vials in the biomedical materials vessels described herein, generally contain the cells to be administered, e.g., one or more unit doses thereof. The unit dose may be an amount or number of the cells to be administered to the subject or twice the number (or more) of the cells to be administered. It may be the lowest dose or lowest possible dose of the cells that would be administered to the subject.

[0167] In some embodiments, each of the vials individually comprises a unit dose of the cells. Thus in some embodiments, each of the containers comprises the same or approximately or substantially the same number of cells. In some embodiments, each unit dose contains at least or about at least 1×10^6, 2×10^6, 5×10^6, 1×10^7, 5×10^7, 1×10^8, 2.5×10^8, or 5×10^8engineered cells, total cells, T cells, or PBMCs. In some embodiments, each unit dose contains at least at or about 2.5×10^7, at or about 5.0×10^7, at or about 1.5×10^8, at or about 3.0

x 10^8, at or about 4.5 x 10^8, at or about 8.0 x 10^8 or at or about 1.2 x 10^9engineered cells, total cells, T cells, or PBMCs. In some embodiments, each unit dose contains no more than at or about 2.5 x 10^7, at or about 5.0 x 10^7, at or about 1.5 x 10^8, at or about 3.0 x 10^8, at or about 4.5 x 10^8, at or about 8.0 x 10^8 or at or about 1.2 x 10^9engineered cells, total cells, T cells, or PBMCs. In some aspects, exemplary dose of cells that can be contained in the vials include any doses described herein, e.g., in Section IV. In some aspects, exemplary dose of cells that can be administered to a subject include any doses described herein, e.g., in Section IV.

[0168] In some embodiments, the volume of the formulated cell composition in each container is 10 mL to 100 mL, such as at least or about at least or about 20 mL, 30 mL, 40 mL, 50 mL, 60 mL, 70 mL, 80 mL, 90 mL or 100 mL. In some embodiments, the cells in the vials can be cryopreserved. In some embodiments, the vials can be stored in liquid nitrogen until further use.

[0169] In some embodiments, such cells produced by the method, or a composition comprising such cells, are administered to a subject for treating a disease or condition.

III. RECOMBINANT PROTEINS

[0170] In some embodiments, the biomedical material vessels provided herein can be used for preserving, storing and/or transferring biomedical materials such as compositions containing cells, such as in connection with processes including manufacturing, generating or producing a cell therapy, that are engineered to express a recombinant protein, such as a recombinant receptor. In some embodiments, the compositions or cells that can be stored in the provided biomedical material vessels include cells, e.g., T cells, engineered with a recombinant receptor, such as a chimeric antigen receptor (CAR), e.g. CAR T cells. In some embodiments, the composition or cells can be formulated into the vials in an amount for dosage administration, such as for a single unit dosage administration or multiple dosage administration, for therapy, such as adoptive cell therapy.

[0171] In some embodiments, the compositions or cells that can be kept, preserved, transferred or stored in the provided biomedical material vessels include those cells that have been engineered and/or cultivated as described herein, e.g., in Section II. In some embodiments, the methods for culturing, such as for expansion or cultivation of cells, is carried out on cells genetically engineered, e.g. transduced, with a recombinant protein. In some embodiments, the recombinant protein is or includes a recombinant receptor, e.g. an antigen receptor. The antigen receptor may include a functional non-TCR antigen receptors, including chimeric antigen

receptors (CARs), and other antigen-binding receptors such as transgenic T cell receptors (TCRs). The receptors may also include other receptors, such as other chimeric receptors, such as receptors that bind to particular ligands and having transmembrane and/or intracellular signaling domains similar to those present in a CAR.

[0172] Exemplary antigen receptors, including CARs, and methods for engineering and introducing such receptors into cells, include those described, for example, in international patent application publication numbers WO200014257, WO2013126726, WO2012/129514, WO2014031687, WO2013/166321, WO2013/071154, WO2013/123061, U.S. patent application publication numbers US2002131960, US2013287748, US20130149337, U.S. Patent Nos.: 6,451,995, 7,446,190, 8,252,592, 8,339,645, 8,398,282, 7,446,179, 6,410,319, 7,070,995, 7,265,209, 7,354,762, 7,446,191, 8,324,353, and 8,479,118, and European patent application number EP2537416, 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. In some aspects, the antigen receptors include a CAR as described in U.S. Patent No.: 7,446,190, and those described in International Patent Application Publication No.: WO/2014055668 A1. Examples of the CARs include CARs as disclosed in any of the aforementioned publications, such as WO2014031687, US 8,339,645, US 7,446,179, US 2013/0149337, U.S. Patent No.: 7,446,190, US Patent No.: 8,389,282, Kochenderfer et al., 2013, Nature Reviews Clinical Oncology, 10, 267-276 (2013); Wang et al. (2012) J. Immunother. 35(9): 689-701; and Brentjens et al., Sci Transl Med. 2013 5(177). See also WO2014031687, US 8,339,645, US 7,446,179, US 2013/0149337, U.S. Patent No.: 7,446,190, and US Patent No.: 8,389,282.

[0173] In some embodiments, the nucleic acid(s) encoded the recombinant protein further encodes one or more marker, e.g., for purposes of confirming transduction or engineering of the cell to express the receptor and/or selection and/or targeting of cells expressing molecule(s) encoded by the polynucleotide. In some aspects, such a marker may be encoded by a different nucleic acid or polynucleotide, which also may be introduced during the genetic engineering process, typically via the same method, e.g., transduction by any of the methods provided herein, e.g., via the same vector or type of vector.

[0174] In some aspects, the marker, e.g., transduction marker, is a protein and/or is a cell surface molecule. Exemplary markers are truncated variants of a naturally-occurring, e.g., endogenous markers, such as naturally-occurring cell surface molecules. In some aspects, the variants have reduced immunogenicity, reduced trafficking function, and/or reduced signaling

function compared to the natural or endogenous cell surface molecule. In some embodiments, the marker is a truncated version of a cell surface receptor, such as truncated EGFR (tEGFR). In some aspects, the marker includes all or part (e.g., truncated form) of CD34, an NGFR, or epidermal growth factor receptor (e.g., tEGFR). In some embodiments, the nucleic acid encoding the marker is operably linked to a polynucleotide encoding for a linker sequence, such as a cleavable linker sequence. See, e.g., WO2014/031687. In some embodiments, a single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three genes (*e.g.* encoding the molecule involved in modulating a metabolic pathway and encoding the recombinant receptor) separated from one another by sequences encoding a self-cleavage peptide (*e.g.*, 2A sequences) or a protease recognition site (*e.g.*, furin). The ORF thus encodes a single polypeptide, which, either during (in the case of 2A) or after translation, is processed into the individual proteins. In some cases, the peptide, such as T2A, can cause the ribosome to skip (ribosome skipping) synthesis of a peptide bond at the C-terminus of a 2A element, leading to separation between the end of the 2A sequence and the next peptide downstream (*see*, for example, de Felipe. *Genetic Vaccines and Ther.* 2:13 (2004) and deFelipe *et al. Traffic* 5:616-626 (2004)). Many 2A elements are known. Examples of 2A sequences that can be used in the methods and nucleic acids disclosed herein, without limitation, 2A sequences from the foot-and-mouth disease virus (F2A), equine rhinitis A virus (E2A), Thosea asigna virus (T2A), and porcine teschovirus-1 (P2A) as described in U.S. Patent Publication No. 20070116690.

[0175] In some embodiments, the marker is a molecule, e.g., cell surface protein, not naturally found on T cells or not naturally found on the surface of T cells, or a portion thereof.

[0176] In some embodiments, the molecule is a non-self molecule, e.g., non-self protein, i.e., one that is not recognized as "self" by the immune system of the host into which the cells will be adoptively transferred.

[0177] In some embodiments, the marker serves no therapeutic function and/or produces no effect other than to be used as a marker for genetic engineering, e.g., for selecting cells successfully engineered. In other embodiments, the marker may be a therapeutic molecule or molecule otherwise exerting some desired effect, such as a ligand for a cell to be encountered in vivo, such as a costimulatory or immune checkpoint molecule to enhance and/or dampen responses of the cells upon adoptive transfer and encounter with ligand.

A. Chimeric Antigen Receptors

[0178] In some embodiments, a CAR is generally a genetically engineered receptor with an extracellular ligand binding domain, such as an extracellular portion containing an antibody or fragment thereof, linked to one or more intracellular signaling components. In some embodiments, the chimeric antigen receptor includes a transmembrane domain and/or intracellular domain linking the extracellular domain and the intracellular signaling domain. 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.

[0179] In some embodiments, CARs are constructed with a 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 and/or any of the antigens described. Thus, the CAR typically includes one or more antigen-binding fragment, domain, or portion of an antibody, or one or more antibody variable domains, and/or antibody molecules. In some embodiments, the CAR includes an antigen-binding portion or portions of an antibody molecule, such as a variable heavy chain (VH) or antigen-binding portion thereof, or a single-chain antibody fragment (scFv) derived from the variable heavy (VH) and variable light (VL) chains of a monoclonal antibody (mAb).

[0180] In some embodiments, the CAR contains an antibody or an antigen-binding fragment (e.g. scFv) that specifically recognizes an antigen, such as an intact antigen, expressed on the surface of a cell.

[0181] In some embodiments, the antigen is or includes αvβ6 integrin (avb6 integrin), B cell maturation antigen (BCMA), B7-H3, B7-H6, carbonic anhydrase 9 (CA9, also known as CAIX or G250), a cancer-testis antigen, cancer/testis antigen 1B (CTAG, also known as NY-ESO-1 and LAGE-2), carcinoembryonic antigen (CEA), a cyclin, cyclin A2, C-C Motif Chemokine Ligand 1 (CCL-1), CD19, CD20, CD22, CD23, CD24, CD30, CD33, CD38, CD44, CD44v6, CD44v7/8, CD123, CD133, CD138, CD171, chondroitin sulfate proteoglycan 4 (CSPG4), epidermal growth factor protein (EGFR), type III epidermal growth factor receptor mutation (EGFR vIII), epithelial glycoprotein 2 (EPG-2), epithelial glycoprotein 40 (EPG-40), ephrinB2, ephrin receptor A2 (EPHa2), estrogen receptor, Fc receptor like 5 (FCRL5; also known as Fc receptor homolog 5 or FCRH5), fetal acetylcholine receptor (fetal AchR), a folate binding protein (FBP), folate receptor alpha, ganglioside GD2, O-acetylated GD2 (OGD2), ganglioside GD3, glycoprotein 100 (gp100), glypican-3 (GPC3), G Protein Coupled Receptor 5D (GPRC5D), Her2/neu (receptor tyrosine kinase erb-B2), Her3 (erb-B3), Her4 (erb-B4), erbB

dimers, Human high molecular weight-melanoma-associated antigen (HMW-MAA), hepatitis B surface antigen, Human leukocyte antigen A1 (HLA-A1), Human leukocyte antigen A2 (HLA-A2), IL-22 receptor alpha(IL-22Rα), IL-13 receptor alpha 2 (IL-13Rα2), kinase insert domain receptor (kdr), kappa light chain, L1 cell adhesion molecule (L1-CAM), CE7 epitope of L1-CAM, Leucine Rich Repeat Containing 8 Family Member A (LRRC8A), Lewis Y, Melanoma-associated antigen (MAGE)-A1, MAGE-A3, MAGE-A6, MAGE-A10, mesothelin (MSLN), c-Met, murine cytomegalovirus (CMV), mucin 1 (MUC1), MUC16, natural killer group 2 member D (NKG2D) ligands, melan A (MART-1), neural cell adhesion molecule (NCAM), oncofetal antigen, Preferentially expressed antigen of melanoma (PRAME), progesterone receptor, a prostate specific antigen, prostate stem cell antigen (PSCA), prostate specific membrane antigen (PSMA), Receptor Tyrosine Kinase Like Orphan Receptor 1 (ROR1), survivin, Trophoblast glycoprotein (TPBG also known as 5T4), tumor-associated glycoprotein 72 (TAG72), Tyrosinase related protein 1 (TRP1, also known as TYRP1 or gp75), Tyrosinase related protein 2 (TRP2, also known as dopachrome tautomerase, dopachrome delta-isomerase or DCT), vascular endothelial growth factor receptor (VEGFR), vascular endothelial growth factor receptor 2 (VEGFR2), Wilms Tumor 1 (WT-1), a pathogen-specific or pathogen-expressed antigen, or an antigen associated with a universal tag, and/or biotinylated molecules, and/or molecules expressed by HIV, HCV, HBV or other pathogens. Antigens targeted by the receptors in some embodiments include antigens associated with a B cell malignancy, such as any of a number of known B cell marker. In some embodiments, the antigen is or includes CD20, CD19, CD22, ROR1, CD45, CD21, CD5, CD33, Igkappa, Iglambda, CD79a, CD79b or CD30.

[0182] In some embodiments, the antigen is or includes a pathogen-specific or pathogen-expressed antigen. In some embodiments, the antigen is a viral antigen (such as a viral antigen from HIV, HCV, HBV, etc.), bacterial antigens, and/or parasitic antigens.

[0183] In some embodiments, the CAR contains a TCR-like antibody, such as an antibody or an antigen-binding fragment (e.g. scFv) that specifically recognizes an intracellular antigen, such as a tumor-associated antigen, presented on the cell surface as a MHC-peptide complex. In some embodiments, an antibody or antigen-binding portion thereof that recognizes an MHC-peptide complex can be expressed on cells as part of a recombinant receptor, such as an antigen receptor. Among the antigen receptors are functional non-TCR antigen receptors, such as chimeric antigen receptors (CARs). Generally, a CAR containing an antibody or antigen-binding

fragment that exhibits TCR-like specificity directed against peptide-MHC complexes also may be referred to as a TCR-like CAR.

[0184] In some embodiments, the extracellular portion of the CAR, such as an antibody portion thereof, further includes a spacer, such as a spacer region between the antigen-recognition component, e.g. scFv, and a transmembrane domain. The spacer may be or include at least a portion of an immunoglobulin constant region or variant or modified version thereof, such as a hinge region, e.g., an IgG4 hinge region, and/or a C_H1/C_L and/or Fc region. In some embodiments, the constant region or portion is of a human IgG, such as IgG4 or IgG1. The spacer can be of a length that provides for increased responsiveness of the cell following antigen binding, as compared to in the absence of the spacer. In some examples, the spacer is at or about 12 amino acids in length or is no more than 12 amino acids in length. Exemplary spacers include those having at least about 10 to 229 amino acids, about 10 to 200 amino acids, about 10 to 175 amino acids, about 10 to 150 amino acids, about 10 to 125 amino acids, about 10 to 100 amino acids, about 10 to 75 amino acids, about 10 to 50 amino acids, about 10 to 40 amino acids, about 10 to 30 amino acids, about 10 to 20 amino acids, or about 10 to 15 amino acids, and including any integer between the endpoints of any of the listed ranges. In some embodiments, a spacer region has about 12 amino acids or less, about 119 amino acids or less, or about 229 amino acids or less. Exemplary spacers include IgG4 hinge alone, IgG4 hinge linked to C_H2 and C_H3 domains, or IgG4 hinge linked to the C_H3 domain. Exemplary spacers include, but are not limited to, those described in Hudecek et al. (2013) Clin. Cancer Res., 19:3153 or international patent application publication number WO2014/031687.

[0185] The extracellular ligand binding, such as antigen recognition domain, generally is linked to one or more intracellular signaling components, such as signaling components that mimic activation through an antigen receptor complex, such as a TCR complex, in the case of a CAR, and/or signal via another cell surface receptor. In some embodiments, a transmembrane domain links the extracellular ligand binding and intracellular signaling domains. In some embodiments, the CAR includes a transmembrane domain fused to the extracellular domain. In one embodiment, a transmembrane domain that naturally is associated with one of the domains in the receptor, e.g., 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.

[0186] The transmembrane domain in some embodiments is derived either from a natural or from a synthetic source. Where the source is natural, the domain in some aspects 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, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 or CD154. The transmembrane domain in some embodiments is synthetic. In some aspects, the synthetic transmembrane domain comprises predominantly hydrophobic residues such as leucine and valine. In some aspects, a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. In some embodiments, the linkage is by linkers, spacers, and/or transmembrane domain(s).

[0187] In some embodiments, 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.

[0188] The recombinant receptor, e.g., the CAR, generally includes at least one intracellular signaling component or components. In some embodiments, the receptor includes an intracellular component of a TCR complex, such as a TCR CD3 chain that mediates T-cell activation and cytotoxicity, e.g., CD3 zeta chain. Thus, in some aspects, the antigen-binding portion is linked to one or more cell signaling modules. In some embodiments, cell signaling modules include CD3 transmembrane domain, CD3 intracellular signaling domains, and/or other CD transmembrane domains. In some embodiments, the receptor, e.g., 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 aspects, the CAR or other chimeric receptor includes a chimeric molecule between CD3-zeta (CD3-ζ) or Fc receptor γ and CD8, CD4, CD25 or CD16.

[0189] In some embodiments, upon ligation of the CAR or other chimeric receptor, the cytoplasmic domain or intracellular signaling domain of the receptor activates at least one of the normal effector functions or responses of the immune cell, e.g., T cell engineered to express the CAR. 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 embodiments, a truncated portion of an intracellular signaling domain of an antigen receptor component or costimulatory molecule is used in place of an intact immunostimulatory chain, for example, if it transduces the effector function signal. In some embodiments, the intracellular

signaling domain or domains include the cytoplasmic sequences of the T cell receptor (TCR), and in some aspects also those of co-receptors that in the natural context act in concert with such receptors 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.

[0190] 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 embodiments, to promote full activation, a component for generating secondary or co-stimulatory signal is also included in the CAR. In other embodiments, the CAR does not include a component for generating a costimulatory signal. In some aspects, an additional CAR is expressed in the same cell and provides the component for generating the secondary or costimulatory signal.

[0191] T cell activation is in some aspects described as being mediated by at least 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 aspects, the CAR includes one or both of such signaling components.

[0192] In some aspects, the CAR includes a primary cytoplasmic signaling sequence that regulates primary activation of the TCR complex. 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, CD8, CD22, CD79a, CD79b, and CD66d. In some embodiments, cytoplasmic signaling molecule(s) in the CAR contain(s) a cytoplasmic signaling domain, portion thereof, or sequence derived from CD3 zeta.

[0193] In some embodiments, the CAR includes a signaling domain and/or transmembrane portion of a costimulatory receptor, such as CD28, 4-1BB, OX40, CD27, DAP10, and ICOS. In some aspects, the same CAR includes both the activating and costimulatory components.

[0194] In some embodiments, the activating domain is included within one CAR, whereas the costimulatory component is provided by another CAR recognizing another antigen. In some embodiments, the CARs include activating or stimulatory CARs, and costimulatory CARs, both expressed on the same cell (see WO2014/055668). In some aspects, the CAR is the stimulatory or activating CAR; in other aspects, it is the costimulatory CAR. In some embodiments, the

cells further include inhibitory CARs (iCARs, see Fedorov et al., Sci. Transl. Medicine, 5(215) (December, 2013), such as a CAR recognizing a different antigen, whereby an activating signal delivered through a CAR recognizing a first antigen is diminished or inhibited by binding of the inhibitory CAR to its ligand, e.g., to reduce off-target effects.

[0195] In some embodiments, 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 embodiments, the intracellular signaling domain of the CD8+ cytotoxic T cells is different than the intracellular signaling domain of the CD4+ helper T cells.

[0196] In certain embodiments, the intracellular signaling region comprises a CD28 transmembrane and signaling domain linked to a CD3 (e.g., CD3-zeta) intracellular domain. In some embodiments, the intracellular signaling region comprises a chimeric CD28 and CD137 (4-1BB, TNFRSF9) co-stimulatory domains, linked to a CD3 zeta intracellular domain.

[0197] In some embodiments, the CAR encompasses one or more, e.g., two or more, costimulatory domains and an activation domain, e.g., primary activation domain, in the cytoplasmic portion. Exemplary CARs include intracellular components of CD3-zeta, CD28, and 4-1BB.

[0198] In some cases, CARs are referred to as first, second, and/or third generation CARs. In some aspects, a first generation CAR is one that solely provides a CD3-chain induced signal upon antigen binding; in some aspects, a second-generation CARs is one that provides such a signal and costimulatory signal, such as one including an intracellular signaling domain from a costimulatory receptor such as CD28 or CD137; in some aspects, a third generation CAR in some aspects is one that includes multiple costimulatory domains of different costimulatory receptors.

[0199] In some embodiments, the chimeric antigen receptor includes an extracellular ligand-binding portion, such as an antigen-binding portion, such as an antibody or fragment thereof and in intracellular domain. In some embodiments, the antibody or fragment includes an scFv or a single-domain V_H antibody and the intracellular domain contains an ITAM. In some aspects, the intracellular signaling domain includes a signaling domain of a zeta chain of a CD3-zeta (CD3ζ) chain. In some embodiments, the chimeric antigen receptor includes a transmembrane domain linking the extracellular domain and the intracellular signaling domain. In some aspects, the transmembrane domain contains a transmembrane portion of CD28. The extracellular domain and transmembrane can be linked directly or indirectly. In some embodiments, the extracellular domain and transmembrane are linked by a spacer, such as any described herein. In some

embodiments, the chimeric antigen receptor contains an intracellular domain of a T cell costimulatory molecule, such as between the transmembrane domain and intracellular signaling domain. In some aspects, the T cell costimulatory molecule is CD28 or 4-1BB.

[0200] In some embodiments, the CAR contains an antibody, e.g., an antibody fragment, a transmembrane domain that is or contains a transmembrane portion of CD28 or a functional variant thereof, and an intracellular signaling domain containing a signaling portion of CD28 or functional variant thereof and a signaling portion of CD3 zeta or functional variant thereof. In some embodiments, the CAR contains an antibody, e.g., antibody fragment, a transmembrane domain that is or contains a transmembrane portion of CD28 or a functional variant thereof, and an intracellular signaling domain containing a signaling portion of a 4-1BB or functional variant thereof and a signaling portion of CD3 zeta or functional variant thereof. In some such embodiments, the receptor further includes a spacer containing a portion of an Ig molecule, such as a human Ig molecule, such as an Ig hinge, e.g. an IgG4 hinge, such as a hinge-only spacer.

[0201] In some embodiments, the transmembrane domain of the receptor, e.g., the CAR is a transmembrane domain of human CD28 or variant thereof, e.g., a 27-amino acid transmembrane domain of a human CD28 (Accession No.: P10747.1). In some embodiments, the intracellular domain comprises an intracellular costimulatory signaling domain of human CD28 or functional variant thereof, such as a 41 amino acid domain thereof and/or such a domain with an LL to GG substitution at positions 186-187 of a native CD28 protein. In some embodiments, the intracellular domain comprises an intracellular costimulatory signaling domain of 4-1BB or functional variant thereof, such as a 42-amino acid cytoplasmic domain of a human 4-1BB (Accession No. Q07011.1). In some embodiments, the intracellular signaling domain comprises a human CD3 zeta stimulatory signaling domain or functional variant thereof, such as an 112 AA cytoplasmic domain of isoform 3 of human CD3ζ (Accession No.: P20963.2) or a CD3 zeta signaling domain as described in U.S. Patent No.: 7,446,190. In some aspects, the spacer contains only a hinge region of an IgG, such as only a hinge of IgG4 or IgG1. In other embodiments, the spacer is an Ig hinge, e.g., and IgG4 hinge, linked to a C_H2 and/or C_H3 domains. In some embodiments, the spacer is an Ig hinge, e.g., an IgG4 hinge, linked to C_H2 and C_H3 domains. In some embodiments, the spacer is an Ig hinge, e.g., an IgG4 hinge, linked to a C_H3 domain only. In some embodiments, the spacer is or comprises a glycine-serine rich sequence or other flexible linker such as known flexible linkers.

[0202] For example, in some embodiments, the CAR includes: an extracellular ligand-binding portion, such as an antigen-binding portion, such as an antibody or fragment thereof,

including sdAbs and scFvs, that specifically binds an antigen, e.g. an antigen described herein; a spacer such as any of the Ig-hinge containing spacers; a transmembrane domain that is a portion of CD28 or a variant thereof; an intracellular signaling domain containing a signaling portion of CD28 or functional variant thereof; and a signaling portion of CD3 zeta signaling domain or functional variant thereof. In some embodiments, the CAR includes: an extracellular ligand-binding portion, such as an antigen-binding portion, such as an antibody or fragment thereof, including sdAbs and scFvs, that specifically binds an antigen, e.g. an antigen described herein; a spacer such as any of the Ig-hinge containing spacers; a transmembrane domain that is a portion of CD28 or a variant thereof; an intracellular signaling domain containing a signaling portion of 4-1BB or functional variant thereof; and a signaling portion of CD3 zeta signaling domain or functional variant thereof. In some embodiments, such CAR constructs further includes a T2A ribosomal skip element and/or a truncated EGFR (e.g., tEGFR) sequence, *e.g.*, downstream of the CAR.

B. T Cell Receptors (TCRs)

[0203] In some embodiments, the recombinant protein is or includes a recombinant T cell receptor (TCR). In some embodiments, 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.

[0204] In some embodiments, the TCR is one that has been cloned from naturally occurring T cells. In some embodiments, a high-affinity T cell clone for a target antigen (e.g., a cancer antigen) is identified and isolated from a patient. In some embodiments, 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 embodiments, 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.

[0205] In some embodiments, after the T-cell clone is obtained, the TCR alpha and beta chains are isolated and cloned into a gene expression vector. In some embodiments, the TCR alpha and beta genes are linked via a picornavirus 2A ribosomal skip peptide so that both chains are coexpressed. In some embodiments, the nucleic acid encoding a TCR further includes a marker to confirm transduction or engineering of the cell to express the receptor.

IV. ARTICLES OF MANUFACTURE AND KITS

[0206] Provided herein are articles of manufacture that include a composition containing cells, such as engineered cells, contained in one or more biomedical material vessels, such as for storage or packaging in the vial. In some embodiments, the composition of cells contains a pharmaceutically acceptable excipient. In some embodiments, the composition of cells contains a cryoprotectant, such as DMSO. The articles of manufacture may include a label providing information about the engineered cells and/or instructions for their use or administration. The articles of manufacture may include a label or package insert on or associated with the provided biomedical material vessels. The label or package insert may provide instructions for use of the cells, such as engineered cells and/or the biomedical material vessels. The label or package insert may indicate that the composition is used for treating a disease or condition.

[0207] Also provided are kits containing the articles of manufacture and/or one or more additional components for use in connection with administering the dose of engineered cells or a cell therapy to a subject, such as one or more other doses of engineered cells, reagents for diagnosing a subject having a disease or condition or one or more additional pharmaceutical compositions, including a composition containing another therapeutic agent for treating the disease or condition or for administration in combination with the engineered cells, and optionally instructions for use, for example, instructions for administering the engineered cell composition to a subject having a disease or condition, for example, in connection with adoptive cell therapy methods and/or instructions for using or operating the provided biomedical materials vessels. The one or more other components can be packaged in a container such as a further biomedical material vessel as provided or another vial, syringe, bottle, IV solution bag, etc. It may further include other materials such as other buffers, diluents, filters, needles, and/or syringes.

[0208] In some embodiments, the articles of manufacture and/or kits include instructions for administering the cells to a subject for treating a disease or condition. In some embodiments, the instructions specify particular instructions for administration of the cells for cell therapy, e.g., doses, timing, selection and/or identification of subjects for administration and conditions for administration. In some embodiments, the instructions can be included as a label or package insert accompanying the compositions for administration.

[0209] In some embodiments, the instructions specify the dose of cells to be administered. For example, in some embodiments, the dose specified in the instructions include a total recombinant receptor (e.g., CAR)-expressing cells between about 1×10^6 and 3×10^8, e.g., in the

range of about 1 x 10^7 to 2 x 10^8 such cells, such as 1 x 10^7, 5 x 10^7, 1 x 10^8 or 1.5 x 10^8 total such cells, or the range between any two of the foregoing values. In some embodiments, the patient is administered multiple doses, and each of the doses or the total dose can be within any of the foregoing values.

[0210] In some embodiments, the article of manufacture comprise a plurality of CD4+ T cells expressing a recombinant receptor and/or a plurality of CD8+ T cells expressing a recombinant receptor. In some embodiments, the article of manufacture comprise a plurality of CD4+ T cells expressing a recombinant receptor, and further comprises, in the same vessel, a plurality of CD8+ T cells expressing a recombinant receptor. In some embodiments, a cryoprotectant is included with the cells. In some embodiments, the article of manufacture comprise a plurality of CD4+ T cells expressing a recombinant receptor and a plurality of CD8+ T cells expressing a recombinant receptor, contained in separate vessels.

[0211] In some embodiments, the container such as the vial of the biomedical material vessel comprises greater than or greater than about 10 x 10^6 T cells or recombinant receptor-expressing T cells, greater than or greater than about 15 x 10^6 T cells or recombinant receptor-expressing T cells, greater than or greater than about 25 x 10^6 T cells or recombinant receptor-expressing T cell. In some aspects, the vial comprises between about 10 million cells per mL and about 70 million cells per mL, between about 10 million cells per mL and about 50 million cells per mL, between about 10 million cells per mL and about 25 million cells per mL, between about 10 million cells per mL and about 15 million cells per mL, 15 million cells per mL and about 70 million cells per mL, between about 15 million cells per mL and about 50 million cells per mL, between about 15 million cells per mL and about 25 million cells per mL, between about 25 million cells per mL and about 70 million cells per mL, between about 25 million cells per mL and about 50 million cells per mL, and between about 50 million cells per mL and about 70 million cells per mL.

[0212] In some embodiments, the kit comprises a plurality of biomedical material vessels each containing a vial containing a plurality of cells, e.g. engineered cells, or unit dose of cells specified for administration.

[0213] In some embodiments, the plurality of vials or unit dose of cells or the cells in the article or kit, collectively, comprises a dose of cells comprising between at or about 1 x 10^6 and at or about 2 x 10^9 total recombinant receptor-expressing cells, total T cells, or total peripheral blood mononuclear cells (PBMCs), between at or about 2.5 x 10^7 such cells and at or about 1.2 x 10^9 such cells, between at or about 5.0 x 10^7 such cells and at or about 4.5 x 10^8 such cells, or

between at or about 1.5 x 10^8 such cells and at or about 3.0 x 10^8 such cells., each inclusive. In some embodiments, the plurality of vials or unit dose of cells, collectively, comprises a dose of cells comprising at or about 2.5 x 10^7, at or about 5.0 x 10^7, at or about 1.5 x 10^8, at or about 3.0 x 10^8, at or about 4.5 x 10^8, at or about 8.0 x 10^8 or at or about 1.2 x 10^9 total recombinant receptor-expressing cells, total T cells, or total PBMCs. In some embodiments, the plurality of vials or unit dose of cells, collectively, comprises a dose of cells comprising from or from about 1 x 10^5 to 5 x 10^8 total recombinant receptor-expressing T cells or total T cells, 1 x 10^5 to 5 x 10^8 total recombinant receptor-expressing T cells or total T cells, from or from about 5 x 10^5 to 1 x 10^7 total recombinant receptor-expressing T cells or total T cells, or from or from about 1 x 10^6 to 1 x 10^7 total recombinant receptor-expressing T cells or total T cells; or total PBMCs at each range, each inclusive.

[0214] In some aspects, the article or kit comprises one or more unit dose of the CD4+ and CD8+ cells or of the CD4+receptor+ cells and CD8+receptor+ cells, wherein the unit dose comprises between at or about 1 x 10^7 and at or about 2 x 10^8 recombinant receptor-expressing T cells, between at or about 5 x 10^7 and at or about 1.5 x 10^8 recombinant receptor-expressing T cells, at or about 5 x 10^7 recombinant receptor-expressing T cells, at or about 1 x 10^8 recombinant receptor-expressing T cells, or at or about 1.5 x 10^8 recombinant receptor-expressing T cells, optionally wherein the information or instructions in the article or kit specifies administration of one or of a plurality of unit doses and/or a volume corresponding to such one or plurality of unit doses. In some cases, the article or kit comprises one or more unit doses of the CD8+ cells, wherein the dose comprises between at or about 5 x 10^6 and at or about 1 x 10^8 recombinant receptor-expressing CD8+ T cells, the dose comprises between at or about 1 x 10^7 and at or about 0.75 x 10^8 recombinant receptor-expressing CD8+ T cells, the dose comprises at or about 2.5 x 10^7 recombinant receptor-expressing CD8+ T cells, or the dose comprises at or about 5 x 10^7 recombinant receptor-expressing CD8+ T cells, or the dose comprises at or about 0.75 x 10^8 recombinant receptor-expressing CD8+ T cells, optionally wherein the information in the article or kit specifies administration of one or of a plurality of unit doses and/or a volume corresponding to such one or plurality of unit doses.

[0215] In some embodiments, the plurality of vials or unit dose of cells or the cells in the article or kit, collectively, comprise a dose of cells comprising no more than at or about 2.5 x 10^7, no more than at or about 5.0 x 10^7, no more than at or about 1.5 x 10^8, no more than at or about 3.0 x 10^8, no more than at or about 4.5 x 10^8, no more than at or about 8.0 x 10^8 or no more than at or about 1.2 x 10^9 total recombinant receptor-expressing cells, total T cells, or total

PBMCs. In some embodiments, the cells in the article or kit, collectively, comprise a dose of cells comprising no more than 1 x 10^8 total recombinant receptor-expressing T cells or total T cells, no more than 1 x 10^7 total recombinant receptor-expressing T cells or total T cells, no more than 0.5 x 10^7 total recombinant receptor-expressing T cells or total T cells, no more than 1 x 10^6 total recombinant receptor-expressing T cells or total T cells, no more than 0.5 x 10^6 total recombinant receptor-expressing T cells or total T cells or total PBMCs at each number.

[0216] In some embodiments, the T cells of the dose include CD4+ T cells, CD8+ T cells or CD4+ and CD8+ T cells.

[0217] In some embodiments, the instructions can specify dosage regimen and timing of the administration. For example, in some embodiments, the instructions can specify administering to the subject multiple doses, e.g., two or more doses, of the cells. In some embodiments, the instructions specify the timing of the multiple doses, e.g., the second dose being administered approximately 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21 days after the first dose; and/or the dosage amount in each dose.

[0218] In some embodiments, the article of manufacture or kit comprises a plurality of CD4+ T cells expressing a recombinant receptor, and instructions for administering, to a subject having a disease or condition, all or a portion of the plurality of CD4+ T cells and further administering CD8+ T cells expressing a recombinant receptor. In some embodiments, the instructions specify administering the CD4+ T cells prior to administering the CD8+ cells. In some cases, the instructions specify administering the CD8+ T cells prior to administering the CD4+ cells. In some embodiments, the article of manufacture or kit comprises a plurality of CD8+ T cells expressing a recombinant receptor, and instructions for administering, to a subject having a disease or condition, all or a portion of the plurality of CD8+ T cells and CD4+ T cells expressing a recombinant receptor. In some embodiments, the instructions specify dosage regimen and timing of the administration of the cells.

[0219] In some aspects, the instructions specify administering all or a portion of the CD4+ T cells and the all or a portion of the CD8+ T cells 0 to 12 hours apart, 0 to 6 hours apart or 0 to 2 hours apart. In some cases, the instructions specify administering the CD4+ T cells and the CD8+ T cells no more than 2 hours, no more than 1 hour, no more than 30 minutes, no more than 15 minutes, no more than 10 minutes or no more than 5 minutes apart.

[0220] In some embodiments, the instructions specify the dose or number of cells or cell type(s) and/or a ratio of cell types, e.g., individual populations or sub-types, such as the CD4+ to CD8+ ratio. In some embodiments, the populations or sub-types of cells, such as $CD8^+$ and

$CD4^+$ T cells. For example, in some embodiments, the instructions specify that the cells are administered at or within a tolerated range of an output ratio of multiple cell populations or sub-types, such as CD4+ and CD8+ cells or sub-types, of 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 aspects, 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.

[0221] In certain embodiments, the instructions specify 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 and/or that amount of cells per kilogram of body weight, 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 and/or per kilogram of body weight. Dosages may vary depending on attributes particular to the disease or disorder and/or patient and/or other treatments.

[0222] In some embodiments, for example, where the subject is a human, the dose includes fewer than about 2×10^9 total recombinant receptor (e.g., CAR)-expressing cells, T cells, or peripheral blood mononuclear cells (PBMCs), e.g., in the range of about 1×10^6 to 2×10^9 such cells, such as 5×10^6, 1×10^7, 2.5×10^7, 5×10^7, 1×10^8, 1.5×10^8, 3×10^8, 4.5×10^8, 8×10^8 or 1.2×10^9 total such cells, or the range between any two of the foregoing values. In some embodiments, for example, where the subject is a human, the dose includes fewer than about 5 x

10^8 total recombinant receptor (e.g., CAR)-expressing cells, T cells, or peripheral blood mononuclear cells (PBMCs), e.g., in the range of about 1 x 10^6 to 5 x 10^8 such cells, such as 2 x 10^6, 5 x 10^6, 1 x 10^7, 5 x 10^7, 1 x 10^8, or 5 x 10^8 or total such cells, or the range between any two of the foregoing values.

[0223] In some embodiments, the instructions specify the administration of a dose comprising a number of cell between at or about 1 x 10^6 and at or about 2 x 10^9 total recombinant receptor-expressing cells, total T cells, or total peripheral blood mononuclear cells (PBMCs), between at or about 2.5 x 10^7 such cells and at or about 1.2 x 10^9 such cells, between at or about 5.0 x 10^7 such cells and at or about 4.5 x 10^8 such cells, or between at or about 1.5 x 10^8 such cells and at or about 3.0 x 10^8 such cells., each inclusive. In some embodiments, the instructions specify the administration of a dose comprising at or about 2.5 x 10^7, at or about 5.0 x 10^7, at or about 1.5 x 10^8, at or about 3.0 x 10^8, at or about 4.5 x 10^8, at or about 8.0 x 10^8 or at or about 1.2 x 10^9 total recombinant receptor-expressing cells, total T cells, or total PBMCs. In some embodiments, the instructions specify the administration of a dose comprising a number of cell from or from about 1 x 10^5 to 5 x 10^8 total recombinant receptor-expressing cells, total T cells, or total peripheral blood mononuclear cells (PBMCs), from or from about 5 x 10^5 to 1 x 10^7 total recombinant receptor-expressing cells, total T cells, or total peripheral blood mononuclear cells (PBMCs) or from or from about 1 x 10^6 to 1 x 10^7 total recombinant receptor-expressing cells, total T cells, or total peripheral blood mononuclear cells (PBMCs), each inclusive.

[0224] In some embodiments, the cell therapy comprises administration of a dose of cells comprising a number of cells at least or about at least 1 x 10^5 total recombinant receptor-expressing cells, total T cells, or total peripheral blood mononuclear cells (PBMCs), such at least or at least 1 x 10^6, at least or about at least 1 x 10^7, at least or about at least 1 x 10^8 of such cells. In some embodiments, the number is with reference to the total number of CD3+ or CD8+, in some cases also recombinant receptor-expressing (e.g. CAR+) cells. In some embodiments, the cell therapy comprises administration of a dose comprising a number of cell from or from about 1 x 10^5 to 5 x 10^8 CD3+ or CD8+ total T cells or CD3+ or CD8+ recombinant receptor-expressing cells, from or from about 5 x 10^5 to 1 x 10^7 CD3+ or CD8+ total T cells or CD3+ or CD8+ recombinant receptor-expressing cells, or from or from about 1 x 10^6 to 1 x 10^7 CD3+ or CD8+ total T cells or CD3+ or CD8+recombinant receptor-expressing cells, each inclusive. In some embodiments, the cell therapy comprises administration of a dose comprising a number of cell from or from about 1 x 10^5 to 5 x 10^8 total CD3+/CAR+ or CD8+/CAR+ cells, from or from

about 5 x 10^5 to 1 x 10^7 total CD3+/CAR+ or CD8+/CAR+ cells, or from or from about 1 x 10^6 to 1 x 10^7 total CD3+/CAR+ or CD8+/CAR+ cells, each inclusive.

[0225] In some embodiments, the T cells of the dose include CD4+ T cells, CD8+ T cells or CD4+ and CD8+ T cells.

[0226] In some embodiments, for example, where the subject is human, the instructions specify the CD8+ T cells of the dose, including in a dose including CD4+ and CD8+ T cells, includes between about 1 x 10^6 and 5 x 10^8 total recombinant receptor (e.g., CAR)-expressing CD8+cells, e.g., in the range of about 5 x 10^6 to 1 x 10^8 such cells, such cells 1 x 10^7, 2.5 x 10^7, 5 x 10^7, 7.5 x 10^7, 1 x 10^8, or 5 x 10^8 total such cells, or the range between any two of the foregoing values. In some embodiments, the patient is administered multiple doses, and each of the doses or the total dose can be within any of the foregoing values. In some embodiments, the dose of cells comprises the administration of from or from about 1 x 10^7 to 0.75 x 10^8 total recombinant receptor-expressing CD8+ T cells, 1 x 10^7 to 2.5 x 10^7 total recombinant receptor-expressing CD8+ T cells, from or from about 1 x 10^7 to 0.75 x 10^8 total recombinant receptor-expressing CD8+ T cells, each inclusive. In some embodiments, the dose of cells comprises the administration of or about 1 x 10^7, 2.5 x 10^7, 5 x 10^7 7.5 x 10^7, 1 x 10^8, or 5 x 10^8 total recombinant receptor-expressing CD8+ T cells.

[0227] In some embodiments, the instructions specify that the dose of cells, e.g., recombinant receptor-expressing T cells, is administered to the subject as a single dose or is administered only one time within a period of two weeks, one month, three months, six months, 1 year or more.

[0228] In some embodiments, the label or package insert may provide instructions for using or operating the provided biomedical material vessels, such as according to any of the methods storing and retrieving biomedical material provided herein. In some embodiments, the label or package insert may provide instructions for loading, inserting or filling the vials in the biomedical material vessels, e.g., with biomedical material sample, such as any cells or cell compositions described herein. In some embodiments, the label or package insert may provide instructions for manipulating, storing, freezing and/or thawing the biomedical material vessels. In some embodiments, the label or package insert may provide instructions for retrieving or unloading the biomedical material sample, such as any cells or cell compositions described herein, from the biomedical material vessels.

Definitions

[0229] 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.

[0230] Reference to "about" a value or parameter herein includes (and describes) variations that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X". In addition, reference to phrases "less than", "greater than", "at most", "at least", "less than or equal to", "greater than or equal to", or other similar phrases followed by a string of values or parameters is meant to apply the phrase to each value or parameter in the string of values or parameters. For example, a statement that the sample volume left over after removal from the vial can be less than about 0.3 mL, about 0.25 mL, or about 0.2 mL is meant to mean that the sample volume left over after removal from the vial can be less than about 0.3 mL, less than about 0.25 mL, or less than about 0.2 mL.

[0231] As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. It is also to be understood that the term "and/or" as used herein refers to and encompasses any and all possible combinations of one or more of the associated listed items. It is further to be understood that the terms "includes, "including," "comprises," and/or "comprising," when used herein, specify the presence of stated features, integers, steps, operations, elements, components, and/or units but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, units, and/or groups thereof.

[0232] The term "vector," as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors." Among the vectors are viral vectors, such as retroviral, e.g., gammaretroviral and lentiviral vectors.

[0233] The terms "host cell," "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced,

including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

[0234] 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 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.

[0235] 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 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.

[0236] 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.

[0237] As used herein, a "subject" is a mammal, such as a human or other animal, and typically is human.

[0238] This application discloses several numerical ranges in the text and figures. The numerical ranges disclosed inherently support any range or value within the disclosed numerical ranges, including the endpoints, even though a precise range limitation is not stated verbatim in

the specification because this disclosure can be practiced throughout the disclosed numerical ranges.

[0239] The above description is presented to enable a person skilled in the art to make and use the disclosure, and is provided in the context of a particular application and its requirements. Various modifications to the preferred embodiments will be readily apparent to those skilled in the art, and the generic principles defined herein may be applied to other embodiments and applications without departing from the spirit and scope of the disclosure. Thus, this disclosure is not intended to be limited to the embodiments shown, but is to be accorded the widest scope consistent with the principles and features disclosed herein.

Claims

1. A biomedical material vessel comprising:

a vial comprising a top and an open bottom;

an inlet tube supported by the top of the vial, wherein the inlet tube is fluidly connected to an interior of the vial and comprises a loading port; and

a needleless retrieval port fluidly connected to the open bottom of the vial, wherein the needleless retrieval port provides direct access to biomedical material in the vial.

2. The biomedical material vessel of claim 1, wherein the loading port is a needleless loading port.

3. The biomedical material vessel of claim 2, wherein the needleless loading port comprises a luer lock connection fitting.

4. The biomedical material vessel of claim 3, further comprising a first cap configured to engage with the luer lock connection fitting of the needleless loading port.

5. The biomedical material vessel of any of claims 1-4, wherein the needleless retrieval port comprises a luer lock connection fitting.

6. The biomedical material vessel of claim 5, further comprising a second cap configured to engage with the luer lock connection fitting of the needleless retrieval port.

7. The biomedical material vessel of any of claims 1-6, further comprising an air vent tube supported by the top of the vial and fluidly connected to an interior of the vial.

8. The biomedical material vessel of claim 7, wherein the air vent tube comprises a filter.

9. The biomedical material vessel of claim 8, wherein the filter is a microbial barrier filter.

10. The biomedical material vessel of any of claims 1-9, wherein the top of the vial comprises a first tube adaptor fluidly connected between the inlet tube and the interior of the vial.

11. The biomedical material vessel of any of claims 7-10, wherein the top of the vial comprises a second tube adaptor fluidly connected between the air vent tube and the interior of the vial.

12. The biomedical material vessel of claim 11, wherein openings of the first tube adaptor and the second tube adaptor into the interior of the vial are separated by a wall.

13. The biomedical vessel of claims 1-12, wherein the retrieval port is a self-closing needleless retrieval port.

14. The biomedical material vessel of any of claims 1-13, wherein the biomedical material vessel is made up of USP Class VI compliant material.

15. A method of storing and retrieving biomedical material, the method comprising:

injecting biomedical material into a vial via a loading port of an inlet tube, wherein the inlet tube is supported by a top of the vial; and

retrieving the biomedical material from the vial via a needleless retrieval port fluidly connected to an open bottom of the vial, wherein the needleless retrieval port provides direct access to the biomedical material in the vial.

16. The method of claim 15, further comprising cryogenically freezing the biomedical material in the vial and thawing the cryogenically frozen biomedical material in the vial.

17. The method of claim 15 or claim 16, further comprising sealing the inlet tube.

18. The method of any of claims 15-17, further comprising sealing an air vent tube supported by the top of the vial and fluidly connected to an interior of the vial.

19. The method of claim 18, further comprising cutting open the air vent tube such that air can be vented from the vial.

20. The method of any of claims 15-19, wherein the loading port is a needleless loading port.

21. The method of claim 20, wherein the needleless loading port comprises a luer lock connection fitting.

22. The method of any of claims 15-21, wherein the needleless retrieval port comprises a luer lock connection fitting.

23. The method of any of claim 18-22, wherein the air vent tube comprises a filter and the air vent tube is sealed above a location of the filter in the air vent tube.

24. The method of claims 15-23, wherein the retrieval port is a self-closing needleless retrieval port.

25. An article of manufacture comprising the biomedical material vessel of any of claims 1-14 and a composition comprising a cell therapy.

26. The article of manufacture of claim 25, wherein the cell therapy comprises genetically engineered cells expressing a recombinant receptor.

27. The article of manufacture of claim 26, wherein the recombinant receptor is a T cell receptor (TCR) or a chimeric antigen receptor (CAR).

28. The article of manufacture of claim 26 or claim 27, wherein the genetically engineered cells comprises T cells.

29. The article of manufacture of claim 28, wherein the T cells are CD4+ and/or CD8+.

30. The article of manufacture of any of claims 26-29, wherein the genetically engineered cells comprises cells that are isolated from a subject, optionally a human subject.

31. The article of manufacture of claim 30, wherein the genetically engineered cells comprises cells that are autologous to the subject.

32. The article of manufacture of claim 30, wherein the genetically engineered cells comprises cells that are allogeneic to the subject.

33. The article of manufacture of any of claims 25-32, wherein the composition further comprises a cryoprotectant.

34. The article of manufacture of any of claims 25-32, further comprising a label comprising information about the cell therapy or instructions for administering the cell therapy.

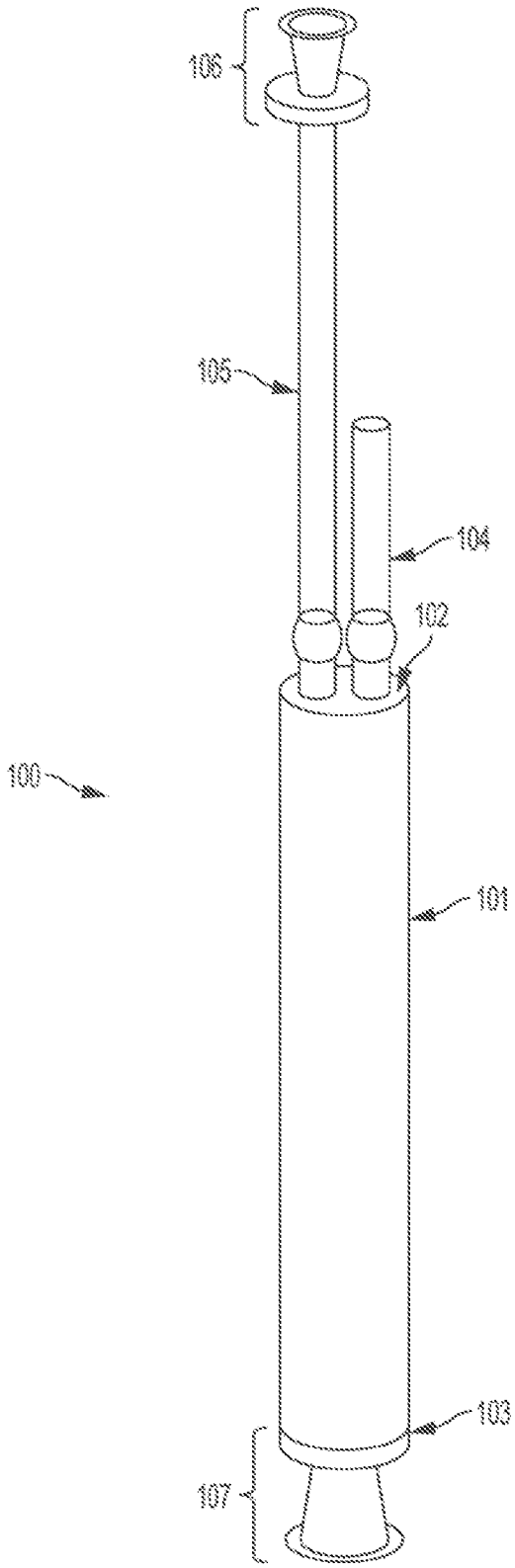

FIG. 1

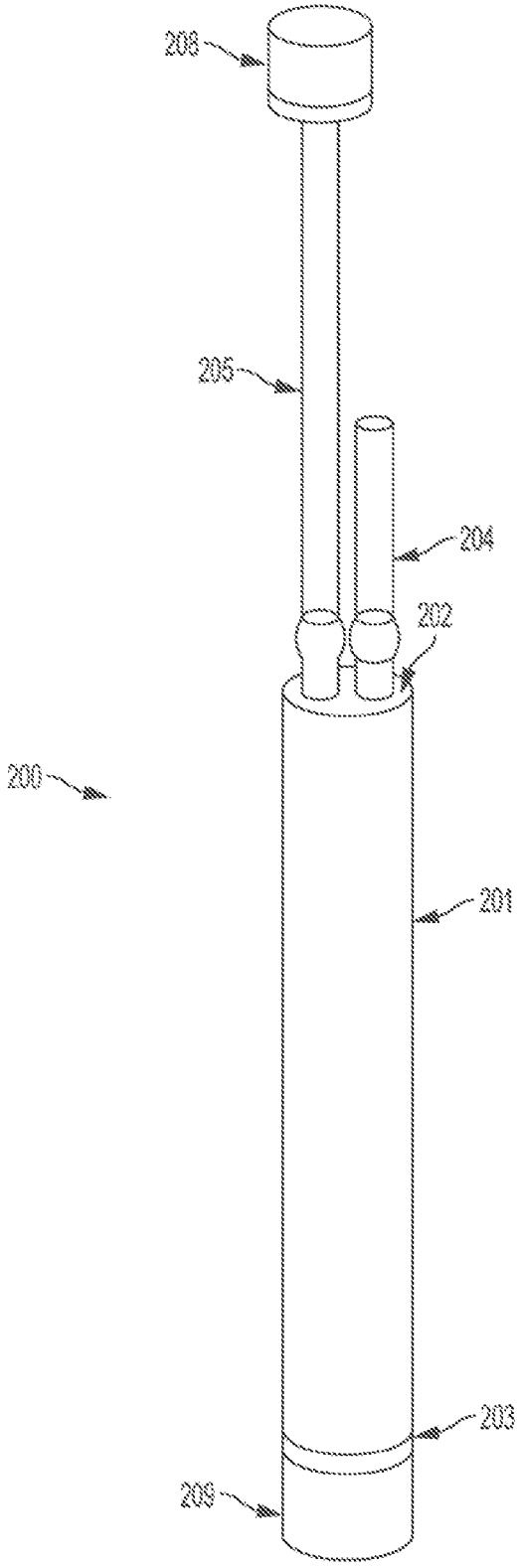

FIG. 2

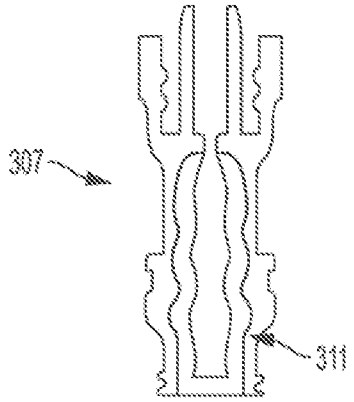

FIG. 3A

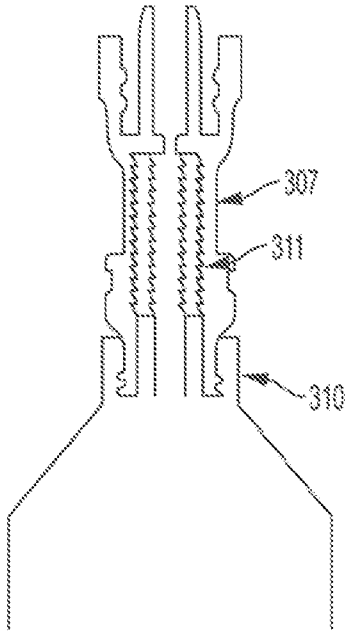

FIG. 3B

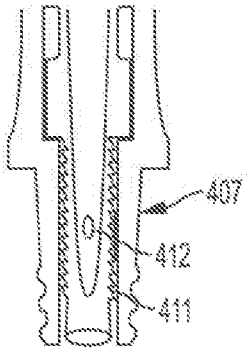

FIG. 4A

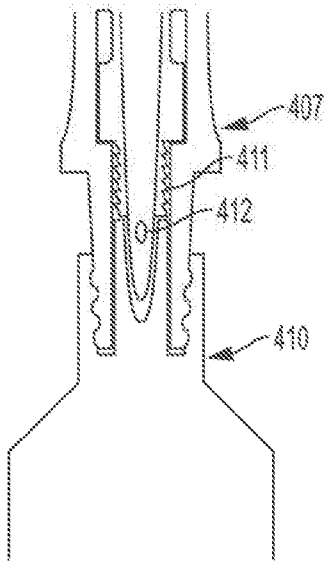

FIG. 4B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/060185

A. CLASSIFICATION OF SUBJECT MATTER
INV. B01L3/00
ADD. C12N15/09 A01N1/02 B01L7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C40B C12N A01N B01L A61J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2017/099832 A1 (WOODS ERIK JOHN [US]) 13 April 2017 (2017-04-13) paragraphs [0003], [0007], [0015], [0072] - [0079], [0085], [0089]; figures 1-9	1-34
Y	US 5 451 374 A (MOLINA ROGER V [US]) 19 September 1995 (1995-09-19) column 8 line 54- column 9 line 32; claim 1; figures 9-10	1-34
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 February 2019	12/02/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Goodman, Marco

1

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 7 175 615 B2 (ICU MEDICAL INC [US]) 13 February 2007 (2007-02-13) column 3 - column 4; figure 3	1-14
A	US 2002/185186 A1 (JULIAR RENA [US] ET AL) 12 December 2002 (2002-12-12) paragraphs [0003], [0004], [0055], [0056]; figure 9	1-14

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