A common drawback of adoptive cell therapy is that reaching sufficient cell numbers (approximately 10^9 cells) usually requires expanding cells *ex vivo* for several weeks and/or involves the use of multiple culture phases wherein the cells are typically frozen between phases. As a consequence, a large fraction of the cells may be lost to the effects of freeze-thawing; additionally prolonged culturing can cause T cells to become terminal effector cells that may die shortly after infusion to the patient before reaching a target cell, tissue and/or organ. Accordingly, there is a need in the art for shorter expansion protocols that avoid freeze-thawing cycles and result in younger and fitter lymphocyte populations, i.e. populations of antigen specific T cells that are not terminally differentiated and comprise a low fraction of terminal effectors.

2. SUMMARY

The present disclosure relates to an improved method for expanding lymphocytes, in particular antigen-specific lymphocytes, *ex vivo*. The methods of the disclosure bears the advantage that high cell numbers (e.g. at least approximately 10^7 cells) can be achieved from a patient sample in a controlled single culture vessel without the need to transfer the cell culture to a larger culture vessel during the process. Furthermore, the methods of the disclosure provide a more rapid expansion of the cells relative to available methods. As a consequence, younger cell populations, characterized by a small fraction of terminal effector cells and, preferably, high stemness can be obtained. Those characteristics allow younger cells to efficiently proliferate after re-infusion, reaching target cells, tissue(s) or organ(s) before differentiating into terminal effector cells. While the terminal effector cells are involved in the immediate attack of cancer cells, the younger cells are expected to provide a durable response.

The disclosure relates to the following items:

A1. A population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and less than 10% are triple positive for CD45RA, CD57 and KLRG1.

b) culturing lymphocytes in the presence of said one or more antigens, which lymphocytes are isolated from a tissue or blood sample from a subject;

wherein the lymphocytes are cultured in a conditioned culture medium.

B1a. In one aspect, the present disclosure refers to a method for expansion of a population of lymphocytes specific for one or more antigens in a controlled single culture vessel, the method comprising a step of a) culturing a tissue or blood sample from a subject in the presence of said one or more antigens, which sample is known or suspected to contain lymphocytes; or b) culturing lymphocytes in the presence of said one or more antigens, which lymphocytes are isolated from a tissue or blood sample from a subject; wherein the lymphocytes are cultured in a conditioned culture medium.

B2. The method according to item B1, wherein the conditioned culture medium is a culture medium in which at least one of the following parameters is monitored and adjusted if necessary: pH, dissolved oxygen (DO) concentration, glucose concentration, lactate concentration and/or temperature.

B3. The method according to item B2, wherein the conditioned culture medium is a culture medium in which pH, dissolved oxygen (DO) concentration, glucose concentration, lactate concentration and temperature are monitored and adjusted if necessary.

B4. The method according to any one of items B1 to B3, the method comprising a step of adjusting he culture volume to the expansion rate of the lymphocytes.

B5. The method according to item B4, wherein the culture volume increases at least by a factor of 2, 3, 4, 5 or 6 during expansion of the lymphocytes.

B6. The method according to any one of items B1 to B5, the method comprising a step of dynamic culturing the lymphocyte culture with the conditioned culture medium.

B7. The method according to any one of items B1 to B6, wherein the tissue sample is derived from a tumor, in particular wherein the tissue sample is a tumor sample.

B8. The method according to item B7, wherein the tumor comprises at least one neoantigen.

B26. The method according to any one of items B1 to B25, wherein the method comprises a step of activating the lymphocytes during culturing.

B27. The method according to item B26, wherein the activation step comprises the addition of a CD3 agonist to the culture medium.

B28. The method according to item B27, wherein the CD3 agonist is added to the culture medium 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 days after the beginning of the culturing.

B29. The method according to any one of items B21 to B28, wherein the culture medium is supplemented with human AB serum and/or IL-2.

B30. The method according to any one of items B1 to B29, wherein said culturing is continued until said T cell population reaches at least 10^7 cells.

B31. The method according to any one of items B1 to B30 wherein said culturing is performed at temperatures of greater than 0 °C during said single culture phase.

B32. The method according to any one of items B1 to B31, wherein said sample or said lymphocytes are maintained at temperatures greater than 0°C subsequent to isolation from said subject and prior to said culture.

B33. A population of lymphocytes obtainable by the method of any one of items B1 to B32.

B33a. In another aspect, the present disclosure refers to a population of lymphocytes obtainable by the method as defined herein.

B34. A population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and less than 10% are triple positive for CD45RA, CD57 and KLRG1.

B35. The population of lymphocytes according to item B34, wherein said T cells are specific for one or more antigens.

B35a. In yet another aspect, the present disclosure refers to a population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and less than 10% are triple positive for CD45RA, CD57 and KLRG1, in particular wherein said T cells are specific for one or more antigens.

B36. The population of lymphocytes according to any one of items B34 to B36, wherein less than 15% of said T cell portion secrete at least one protein from the group consisting of: TNF-α, IL-4 and IL-5.

B37. The population of lymphocytes according to any one of items B34 to B36, wherein at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the T cells in said T cell portion are $CD8^+$ T cells.

B38. The population of lymphocytes according to any one of items B34 to B37, wherein at least two T cells in said T cell portion are directed against different antigens.

B39. The population of lymphocytes according to item B38, wherein at least one antigen is a neoantigen.

B40. The population of lymphocytes according to any one of items B34 to B39, wherein said T cell portion comprises at least 10^7 T cells.

B41. A pharmaceutical composition comprising the population of lymphocytes according to any one of items B33 to B40.

B42. The pharmaceutical composition according to item B41, wherein the lymphocytes are suspended in a pharmaceutically acceptable buffer.

B43. The pharmaceutical composition according to item B42, wherein the pharmaceutically acceptable buffer comprises about 0.9% NaCl and, optionally, up to 15% DMSO.

B43a. In another aspect, the present disclosure refers to a pharmaceutical composition comprising the population of lymphocytes as described herein, in particular wherein the lymphocytes are suspended in pharmaceutically acceptable buffer, in particular wherein the pharmaceutically acceptable buffer comprises about 0.9% NaCl and, optionally, up to 15% DMSO.

B44. The population of lymphocytes according to any one of items B33 to B40 or the pharmaceutical composition according to any one of items B41 to B43 for use as a medicament.

B45. The population of lymphocytes according to any one of items B33 to B40 or the pharmaceutical composition according to any one of items B41 to B43 for use in cancer therapy.

B46. The population of lymphocytes or the pharmaceutical composition for use according to item B45, wherein the cancer therapy is adoptive cell therapy.

B46a. In another aspect, the present disclosure refers to the use of the population of lymphocytes as defined herein or the pharmaceutical composition as defined herein in the manufacture of a medicament for treating cancer, in particular wherein the treatment of cancer comprises a cancer therapy which is an adoptive cell therapy and/or wherein the cancer therapy is autologous cell therapy.

B47. The population of lymphocytes or the pharmaceutical composition for use according to item B45 or B46, wherein the cancer therapy is autologous cell therapy.

B48. A method for treating cancer, the method comprising the steps of:
a) providing a population of lymphocytes according to any one of items B33 to B40 or a pharmaceutical composition according to any one of items B41 to B43; and
b) infusing the population of lymphocytes or the pharmaceutical composition into a subject suffering from cancer.

B49. A method for treating cancer in a subject, the method comprising the steps of:
a) surgically removing a tumor from a subject or taking a biopsy from a subject's tumor;
b) identifying at least one tumor antigen in the tumor sample obtained in step (a);
c) expanding lymphocytes comprised in the tumor sample obtained in step (a) with the method according to any one of items B1 to B33, wherein the lymphocytes are expanded in the presence of at least one tumor antigen that has been identified in step (b) to be present in the tumor sample; d) infusing the expanded lymphocytes obtained in step (c) into the subject from which the tumor sample has been obtained.

B50. The method according to item B49, wherein the tumor antigen is a tumor-associated antigen or a tumor-specific antigen.

B51. The method according to any one of items B48 to B49, wherein the lymphocytes comprise tumor-infiltrating lymphocytes (TILs).

B52. The method according to item B51, wherein the TILs specifically recognize one or more tumor antigens.

B53. The method according to item B52, wherein at least one tumor antigen is a neoantigen.

Accordingly, in a particular example, the disclosure relates to a population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable and wherein at least 50% of said T cell portion are CD27 and/or CD28 positive.

That is, in certain examples, the disclosure relates to a population of lymphocytes comprising at least 90% CD3+ T cells. The term "CD3+ T cells", as used herein, refers to a type of cells that express the CD3 marker. "CD3", as used herein, refers to a cluster of differentiation 3, a protein complex composed of four distinct chains. In mammals, the complex contains a CD3γ chain, a CD3δ chain, and two CD3ε chains. These chains associate with a molecule known as the T cell receptor (TCR) and the ζ-chain to generate an activation signal in T lymphocytes. The TCR, ζ-chain, and CD3 molecules together comprise the TCR complex.

In certain examples, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of the lymphocytes in the population of lymphocytes are CD3+ T cells.

The skilled person is aware of methods to determine the percentage of CD3+ T cells in a population of cells. For example, the percentage of CD3+ T cells in a population of cells may be determined by flow cytometry, using antibodies directed against CD3 and/or other suitable T cell-specific surface markers.

In certain examples, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of the lymphocytes in the population of lymphocytes are CD3+ and CD45+ T cells, as determined by flow cytometry.

In certain examples the population of lymphocytes may comprise up to 10% of impurities.

In certain examples, the population of lymphocytes is obtained by contacting a patient sample comprising lymphocytes or isolated lymphocytes with B cells, in particular antigen-presenting B cells. Thus, in certain examples, the population of lymphocytes may comprise a population of B cells. In certain examples, the population of lymphocytes comprises less than 5%, less than 4%, less than 3%, less than 2% or less than 1% B cells.

Alternatively, the population of lymphocytes may comprise between 0.1% and 5% B cells, between 0.1% and 4% B cells, between 0.1% and 3% B cells, between 0.1% and 2% B cells, or between 0.1% and 1% B cells.

Even if lymphocytes are initially cultured in the presence of B cells, it is to be understood that the final population of lymphocytes may be free of B cells. That is because B cells are usually not able to survive in T cell specific media for prolonged periods. Thus, in certain examples, the population of lymphocytes according is substantially free of B cells. That is, the number of B cells in the population may be below the limit of quantification by flow cytometry.

The skilled person is aware of methods to determine the percentage of B cells in a population of cells. For example, B cells may be identified by flow cytometry using antibodies against B cell specific surface markers, such as CD19 or CD20.

The term "B cell", as used herein, refers to a type of lymphocyte that plays a major role in the humoral immune response, as opposed to the cell-mediated immune response, which is governed by T cells. B cells are characterized by the presence of a B cell receptor (BCR) on their outer surface which allows the B cell to bind to its specific antigen. The principal functions

of a B cell are (i) to produce antibodies against the specific antigens which it recognizes, (ii) to perform the role of antigen-presenting cells (APCs) and (iii) to eventually develop into memory B cells after activation by interacting with its cognate antigen. B cells are an essential component of the adaptive immune system. The term "B cell" includes long-lived plasma cells and memory B cells. The term "long-lived plasma B cell", as used herein, refers to a sub-type of B cells that reside primarily in the bone marrow and continuously secrete antibodies. The term "memory B cell", as used herein, refers to a sub-type of B cells that are formed following a primary infection and activation by interacting with its cognate antigen, reside primarily in peripheral lymphoid tissues and, upon re-encounter with the priming antigen, differentiate into antibody-secreting cells (ASC) thus amplifying the antibody response. In certain examples, the B cell is a memory B cell.

Other impurities may be cells that were comprised in the sample from which the lymphocytes and/or the B cells originate. For example, in certain examples, the lymphocytes originate from tumor samples. In such examples, the preparation of lymphocytes may comprise a residual fraction of tumor cells. The abundance of tumor cells in the final population of lymphocytes can be determined by flow cytometry, for example by determining the abundance of CD45-negative cells in the population of lymphocytes. Alternatively or in addition, residual tumor cells in the population of lymphocytes may be detected by qPCR as known in the art.

In certain examples, the population of lymphocytes comprises less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3% or less than 2% of B cells (CD19+ or CD20+) and/or tumor cells (CD45-).

Alternatively, the population of lymphocytes may comprise between 0.1% and 10%, between 0.1% and 9%, between 0.1% and 8, between 0.1% and 7%, between 0.1% and 6%, between 0.1% and 5%, between 0.1% and 4% B cells, between 0.1% and 3%, between 0.1% and 2%, or between 0.1% and 1% of B cells (CD19+ or CD20+) and/or tumor cells (CD45-).

The population of lymphocytes according to the disclosure may further comprise NK cells (CD3-, CD56+) and/or NKT cells (CD3+, CD56+). In certain examples, the population of lymphocytes may thus comprise between 0.1% and 10%, between 0.1% and 9%, between 0.1% and 8, between 0.1% and 7%, between 0.1% and 6%, between 0.1% and 5%, between 0.1% and 4% B cells, between 0.1% and 3%, between 0.1% and 2%, or between 0.1% and 1% of B cells (CD19+ or CD20+) and/or tumor cells (CD45-) and/or NK cells(CD3-, CD56+) and/or NKT cells (CD3+, CD56+).

Within the present disclosure, it is preferred that at least 70% of the CD3+ T cells in the population of cells are viable cells. Various methods to determine the viability of a T cell are known in the art and are commercially available. Without limitation, the viability of T cells in the population of lymphocytes may be determined in a proliferation assay or by live/dead staining.

In certain examples, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% of the CD3+ T cells in the population of lymphocytes are viable CD3+ T cells.

In certain examples, the viability of the CD3+ T cells in the population of lymphocytes can be determined by flow cytometry using the cell surface marker Annexin V (AnnV) and the nucleic acid dye 7-Amino-Actinomycin D (7AAD) or propidium iodide (PI). Viable cells are double negative for AnnV and 7AAD/PI. Early apoptotic cells are positive for AnnV and negative for 7AAD/PI. Late apoptotic cells are positive for AnnV and positive for 7AAD/PI. Dead cells are negative for AnnV and positive for 7AAD/PI.

In certain examples, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% of the CD3+ T cells in the population of lymphocytes are double negative for AnnV and 7AAD/PI.

Annexin V (or annexin V) is a cellular protein in the annexin group. In flow cytometry, annexin V is commonly used to detect apoptotic cells by its ability to bind to phosphatidylserine, a marker of apoptosis when it is on the outer leaflet of the plasma membrane.

7-Aminoactinomycin D (7-AAD) is a fluorescent chemical compound with a strong affinity for DNA. It is used as a fluorescent marker for DNA in fluorescence microscopy and flow cytometry. It is taken up by cells when the cell membrane integrity is lost and intercalates in double-stranded DNA, with a high affinity for GC-rich regions, making it useful for chromosome banding studies.

Propidium iodide (or PI) is a fluorescent intercalating agent that can be used to stain cells and nucleic acids. PI binds to DNA by intercalating between the bases with little or no sequence preference. Propidium iodide is used as a DNA stain in flow cytometry to evaluate cell viability or DNA content in cell cycle analysis, or in microscopy to visualize the nucleus and other DNA-containing organelles. Propidium Iodide is not membrane-permeable, making it useful to differentiate necrotic, apoptotic and healthy cells based on membrane integrity.

Viability may further be determined by using a cell counter such as, without limitation, a NucleoCounter NC-202. That is, in certain examples, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% of the CD3+ T cells in the population of lymphocytes are viable as determined with a cell counter, in particular with a NucleoCounter NC-202.

Viability may further be determined by trypan blue cell counting as known in the art. That is, in certain examples, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% of the CD3+ T cells in the population of lymphocytes are viable as determined by trypan blue cell counting.

It is to be understood that viability will differ depending on the method with which it is determined, in particular due to variations in the expression of cell markers such as AnnV. It is thus sufficient if viability of at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% of the CD3+ T cells in the population of lymphocytes can be achieved with at least one suitable method known in the art, preferably one of the methods disclosed herein.

Further, it is preferred that at least 50% of the CD3+ T cells in the population of lymphocytes are CD27 and/or CD28 positive cells. In certain examples, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% of the T cell portion is CD27 and/or CD28 positive. It is preferred that at least 75% of the T cell portion is CD27 and/or CD28 positive.

Alternatively, at least 20% of the CD3+ T cells in the population of lymphocytes are CD27 and/or CD28 positive cells. In certain examples, at least 25%, at least 30%, at least 35%, at least 40%, at least 45% or at least 50% of the T cell portion is CD27 and/or CD28 positive.

CD27 is a member of the tumor necrosis factor receptor superfamily. This receptor is required for generation and long-term maintenance of T cell immunity. It binds to ligand CD70 and plays a key role in regulating B cell activation and immunoglobulin synthesis. CD27 is predominantly expressed in naïve, central memory (CM) and effector memory (EM) T cells but not in terminal effector (TE) T cells.

CD28 is one of the proteins expressed on T cells that provide co-stimulatory signals required for T cell activation and survival. T cell stimulation through CD28 in addition to the T cell receptor (TCR) can provide a potent signal for the production of various interleukins. Similarly to CD27, CD28 is predominantly expressed in naïve, central memory (CM) and effector memory (EM) T cells but not in terminal effector (TE) T cells.

As mentioned above, the T cells in the population of lymphocytes preferably comprise a low number of terminal effector T cells. Accordingly, in certain examples, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the CD3+ T cells in the population of lymphocytes express the cell surface marker CD27. In other examples, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the CD3+ T cells in the population of lymphocytes express the cell surface marker CD28. In other examples, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the CD3+ T cells in the population of lymphocytes express the cell surface markers CD27 and CD28.

The skilled person is aware of methods to determine the percentage of CD27 and/or CD28 positive cells in a population of cells. For example, the percentage of CD27 and/or CD28 positive cells in a population of cells may be determined by flow cytometry. Antibodies directed against CD27 and CD28 are known in the art and are commercially available.

In a particular example, the disclosure relates to the method according to the disclosure, wherein less than 10% of said T cell portion are positive for at least one of the markers from the group consisting of: CD45RA, CD57 and KLRG1.

That is, the CD3+ T cells in the population of lymphocytes may further by characterized by the absence of one or more senescence markers.

In certain examples, it is preferred that less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes are positive for the cell surface marker CD45RA.

The term "CD45RA", as used herein, refers to isoform RA of the cluster of differentiation 45, or protein tyrosine phosphatase, receptor type, C (PTPRC). CD45RA, preferably in combination with CD57 and KLRG1, is widely accepted as a marker for terminal differentiation of CD8+ memory T cells. The percentage of CD45RA positive cells in a population of lymphocytes is preferably determined by flow cytometry using antibodies directed against CD45RA.

In certain examples, it is preferred that less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes are positive for the cell surface marker CD57.

The CD57 antigen (alternatively HNK-1, LEU-7, or L2) is routinely used to identify terminally differentiated ‘senescent’ cells with reduced proliferative capacity and altered functional properties. The percentage of CD57 positive cells in a population of lymphocytes is preferably determined by flow cytometry using antibodies directed against CD57.

In certain examples, it is preferred that less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes are positive for the cell surface marker KLRG1.

Killer cell lectin-like receptor subfamily G member 1 (KLRG1) is a protein that in humans is encoded by the KLRG1 gene. KLRG1 is expressed on NK cells and antigen-experienced T cells and has been postulated to be a marker of senescence. The percentage of KLRG1positive cells in a population of lymphocytes is preferably determined by flow cytometry using antibodies directed against KLRG1.

In certain examples, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes are positive for at least one of the cell surface markers CD45RA, CD57 and/or KLRG1. In certain examples, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes are positive for two of the cell surface markers CD45RA, CD57 and/or KLRG1. In certain examples, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes are positive for all three of the cell surface markers CD45RA, CD57 and/or KLRG1.

In certain examples, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes are positive for at least one of the cell surface markers CD45RA and/or CD57. In certain examples, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes are double positive for CD45RA and CD57.

In certain examples, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes are positive for at least one of the cell surface markers KLRG1 and/or CD57. In certain examples, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes are double positive for KLRG1 and CD57.

In certain examples, more than 50%, more than 60%, more than 70%, more than 80%, more than 85%, more than 90% or more than 95% of the CD3+ T cells in the population of lymphocytes are negative for at least one of the cell surface markers CD45RA, CD57 and/or KLRG1. In certain examples, more than 50%, more than 60%, more than 70%, more than 80%, more than 85%, more than 90% or more than 95% of the CD3+ T cells in the population of lymphocytes are negative for two of the cell surface markers CD45RA, CD57 and/or KLRG1. In certain examples, more than 80%, more than 85%, more than 90% or more than 95% of the CD3+ T cells in the population of lymphocytes are double negative for CD57 and KLRG1. In certain examples, more than 80%, more than 85%, more than 90% or more than 95% of the CD3+ T cells in the population of lymphocytes are triple negative for CD45RA, CD57 and KLRG1.

In a particular example, the disclosure relates to a population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and at least 80% are double negative for CD57 and KLRG1.

In a particular example, the disclosure relates to the population of lymphocytes according to the disclosure, wherein said T cell portion has an average telomere length of at least 5 kb, at least 6 kb, at least 7 kb, at least 8 kb, at least 9 kb. The skilled person is aware that the average telomere length depends on the starting material. For example, the average telomere length depends on the age of the patient from which the starting material has been obtained.

Alternatively, or in addition to the senescence markers CD45RA, CD57 and KLRG1, the population of lymphocytes according to the disclosure may be characterized based on the average telomere length of the CD3+ T cells comprised in the population of lymphocytes. It is known in the art that the onset of replicative senescence is regulated by the length of telomeres, which are specialized structures at chromosome ends that progressively become shorter with each DNA replication cycle. Shortening of telomeres beyond a critical length induces p53-mediated growth arrest and senescence.

Methods for determining the average telomere length in the cells of a cell population are known in the art and have been described for example by Huang et al. (Scientific Reports volume 7, Article number: 6785 (2017)).

In certain examples, the CD3+ T cells in the population of lymphocytes may be characterized by an average telomere length of at least 5 kb, at least 6 kb, at least 7 kb, at least 8 kb, at least 9 kb or at least 10 kb.

Instead of characterizing the lymphocytes based on the average length of all telomeres of the CD3+ T cells comprised in the population of lymphocytes, the CD3+ T cells may also be characterized based on the average length of the shortest 20% of telomeres. That is, in certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein the shortest 20% of telomeres in said T cell portion has an average telomere length of at least 1 kb, at least 1.5 kb, at least 2 kb, at least 2.5 kb, at least 3 kb.

In a particular example, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 10% of said T cell portion secrete at least one protein from the group consisting of: TNF-α, IL-4, IL-5, Granzyme B and Perforin.

In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 15% of said T cell portion secrete at least one protein from the group consisting of: TNF-α, IL-4 and IL-5.

In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 10% of said T cell portion secrete at least one protein from the group consisting of: TNF-α, IL-4 and IL-5.

In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 15% of said T cell portion secrete TNF-α and wherein less

than 10% of said T cell portion secrete at least one protein from the group consisting of: IL-4 and IL-5.

Alternatively, or in addition to the senescence markers CD45RA, CD57 and KLRG1, the population of lymphocytes according to the disclosure may be characterized based on the secretion profile of the CD3+ T cells comprised in the population of lymphocytes. It is known in the art that terminal effector T cells secrete different proteins than less differentiated T cells. Accordingly, senescence of the cells in a population of cells may be determined based on the proteins that are secreted by the cells in the population.

In certain examples, the CD3+ T cells in the population of lymphocytes may be characterized in that less than 15% of these CD3+ T cells secrete TNF-α. In certain examples, the CD3+ T cells in the population of lymphocytes may be characterized in that less than 1% of these CD3+ T cells secrete TNF-α. Tumor necrosis factor (TNF, cachexin, or cachectin; often called tumor necrosis factor alpha or TNF-α) is a cytokine – a small protein used by the immune system for cell signaling. TNF-α is predominantly secreted by terminal effector T cells, but not by naïve T cells and central memory T cells.

In certain examples, less than 15%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes secrete detectable mounts of TNF-α.

In certain examples, the CD3+ T cells in the population of lymphocytes may be characterized in that less than 15% of these CD3+ T cells secrete IL-4. In certain examples, the CD3+ T cells in the population of lymphocytes may be characterized in that less than 10% of these CD3+ T cells secrete IL-4. Interleukin 4 (IL-4) has many biological roles, including the stimulation of activated B cell and T cell proliferation, and the differentiation of B cells into plasma cells. IL-4 is predominantly secreted by terminal effector T cells, but not by naïve T cells and central memory T cells.

In certain examples, less than 15%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes secrete detectable mounts of IL-4.

In certain examples, the CD3+ T cells in the population of lymphocytes may be characterized in that less than 15% of these CD3+ T cells secrete IL-5. In certain examples, the CD3+ T cells in the population of lymphocytes may be characterized in that less than 10% of these CD3+ T cells secrete IL-5. Through binding to the interleukin-5 receptor, interleukin 5 stimulates B cell growth and increases immunoglobulin secretion - primarily IgA. It is also a key mediator in eosinophil activation. IL-5 is predominantly secreted by terminal effector T cells, but not by naïve T cells and central memory T cells.

In certain examples, less than 15%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes secrete detectable mounts of IL-5.

In certain examples, the CD3+ T cells in the population of lymphocytes may be characterized in that less than 10% of these CD3+ T cells secrete Granzyme B. Granzyme B (GrB) is a serine protease most commonly found in the granules of natural killer cells (NK cells) and cytotoxic T cells. It is secreted by these cells along with the pore forming protein perforin to mediate apoptosis in target cells. Granzyme B is predominantly secreted by terminal effector T cells and effector memory T cells, but not by naïve T cells and central memory T cells.

In certain examples, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes secrete detectable mounts of Granzyme B.

In certain examples, the CD3+ T cells in the population of lymphocytes may be characterized in that less than 10% of these CD3+ T cells secrete Perforin. Perforin is a pore forming cytolytic protein found in the granules of cytotoxic T lymphocytes (CTLs) and natural

killer cells (NK cells). Upon degranulation, perforin binds to the target cell's plasma membrane, and oligomerizes in a Ca2+ dependent manner to form pores on the target cell. The pore formed allows for the passive diffusion of a family of pro-apoptotic proteases, known as the granzymes, into the target cell. Perforin is predominantly secreted by terminal effector T cells, but not by naïve T cells and central memory T cells.

In certain examples, less than 10%, less than 9%, less than 8%, less than 7%, less than 6% or less than 5% of the CD3+ T cells in the population of lymphocytes secrete detectable mounts of Perforin.

In certain examples, larger fractions of the cell population may express Granzyme B and/or Perforin. It is thus preferred herein, that less than 15% or, more preferably, less than 10% of the T cell portion comprised in the population of cells secrete at least one protein, at least two proteins or all proteins from the group consisting of: TNF-α, IL-4 and IL-5.

In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 10% of said T cell portion secrete at least one protein from the group consisting of: TNF-α, IL-4, IL-5, Granzyme B and Perforin. In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 10% of said T cell portion secrete two of the proteins from the group consisting of: TNF-α, IL-4, IL-5, Granzyme B and Perforin. In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 10% of said T cell portion secrete three of the proteins from the group consisting of: TNF-α, IL-4, IL-5, Granzyme B and Perforin. In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 10% of said T cell portion secrete four of the proteins from the group consisting of: TNF-α, IL-4, IL-5, Granzyme B and Perforin. In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 10% of said T cell portion secrete all of the proteins from the group consisting of: TNF-α, IL-4, IL-5, Granzyme B and Perforin.

In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 10% of said T cell portion secrete at least one of the proteins from the group consisting of: TNF-α, IL-4 and IL-5. In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 10% of said T cell portion secrete at least two of the proteins from the group consisting of: TNF-α, IL-4 and IL-5. In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein less than 10% of said T cell portion secrete all of the proteins from the group consisting of: TNF-α, IL-4 and IL-5.

Within the present disclosure, a cell is determined to secrete a specific protein, if detectable amounts of said protein can be identified in an ELISpot assay. The enzyme-linked immunospot (ELISpot) assay is a highly sensitive immunoassay that measures the frequency of cytokine-secreting cells at the single-cell level. In this assay, cells are cultured on a surface coated with a specific capture antibody in the presence or absence of stimuli. Proteins, such as cytokines, that are secreted by the cells will be captured by the specific antibodies on the surface. After an appropriate incubation time, cells are removed and the secreted molecule is detected using a detection antibody in a similar procedure to that employed by the ELISA. The detection antibody is either biotinylated and followed by a streptavidin-enzyme conjugate or the antibody is directly conjugated to an enzyme. By using a substrate with a precipitating rather than a soluble product, the end result is visible spots on the surface. Each spot corresponds to an individual cytokine-secreting cell. The ELISpot assay captures the presence of cytokines immediately after secretion, in contrast to measurements that are skewed by receptor binding or protease degradation. The assay is considered as one of the most sensitive cellular assays available. The limit of detection typically achieved can be 1 in 100,000 cells. The high sensitivity of the assay makes it particularly useful for studies of the small population of cells found in specific immune responses. ELISpot assays for determining the percentage of cells that secrete IFN-γ, TNF-α, IL-4, IL-5, Granzyme B and Perforin are known in the art.

Alternatively or in addition, the secretion of these proteins can be approximated by flow cytometry. For this, T cells have to be fixated and permeabilized such that antibodies can be

used for quantifying the intracellular pools of the respective proteins. Methods for quantifying the intracellular pools of TNF-α, IL-4, IL-5, Granzyme B and/or Perforin are known in the art.

In a particular example, the disclosure relates to the population of lymphocytes according to the disclosure, wherein at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the T cells in said T cell portion are CD8+ T cells.

That is, it is preferred that the majority of T cells in the population of lymphocytes are CD8+ T cells. As used herein, the term "CD8+ T cell" has its general meaning in the art and refers to a subset of T cells which express CD8 on their surface. They are MHC class I-restricted, and function as cytotoxic T cells. "CD8+ T cells" are also called cytotoxic T lymphocytes (CTL), T-killer cells, cytolytic T cells, or killer T cells. CD8 antigens are members of the immunoglobulin supergene family and are associative recognition elements in major histocompatibility complex class I-restricted interactions. As used herein, the term "tumor-infiltrating CD8+ T cell" refers to the pool of CD8+ T cells of the patient that have left the blood stream and have migrated into a tumor.

Preferably, the second largest portion of T cells in the population of lymphocytes are CD4+ T cells. As used herein, the term “CD4+ T cell” refers to a T cell that presents the co-receptor CD4 on its surface. CD4 is a transmembrane glycoprotein that serves as a co-receptor for T cell receptor (TCR), which can recognize a specific antigen. In certain examples, CD4+ T cells are T helper cells. T helper cells (TH cells) assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages. Helper T cells become activated when they are presented with peptide antigens by MHC class II molecules, which are expressed on the surface of antigen-presenting cells (APCs). Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response. These cells can differentiate into one of several subtypes, including THI, TH2, TH3, TH17, TH9, or TFH, which secrete different cytokines to facilitate different types of immune responses. Signaling from the APC directs T cells into particular subtypes. In certain examples, CD4+ T cells are

regulatory T cells. Regulatory T cells play an essential role in the dampening of immune responses, in the prevention of autoimmune diseases and in oral tolerance.

In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein up to 50%, up to 40%, up to 30%, up to 20% or up to 10% of the T cells in said T cell portion are CD4+ T cells.

In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein the ratio between CD8+ T cells and CD4+ T cells in said T cell portion is between 1:1 and 10:1. In certain examples, the disclosure relates to the population of lymphocytes according to the disclosure, wherein the ratio between CD8+ T cells and CD4+ T cells in said T cell portion is 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 or 10:1 or more than 10:1.

The skilled person is aware of methods to determine the percentage of CD4+ and/or CD8+ T cells in a population of lymphocytes. For example, the percentage of CD4+ and/or CD8+ T cells in a population of lymphocytes may be determined by flow cytometry using antibodies directed against CD4 and/or CD8, respectively.

In a particular example, the disclosure relates to the population of lymphocytes according to the disclosure, wherein at least two T cells in said T cell portion are directed against different antigens.

That is, the T cells comprised in the population of lymphocytes preferably recognize more than one antigen. Obtaining the population of lymphocytes according to the disclosure comprises a step of contacting these lymphocytes with a pool of different antigenic peptides. Thus, it is envisioned that primarily the T cells that recognize an antigen from the pool of antigens are expanded. The pool of antigenic peptides may comprise more than 50, more than 100, more than 200, more than 300, more than 400, more than 500 or more than 1000 different antigenic peptides. Accordingly, in certain examples, the T cell portion comprised in the population of lymphocytes may comprise at least 2, at least 5, at least 10, at least 25, at least 50, at least 75, at least 100, at least 150, at least 200 or at least 300 T cells, wherein each T cell is directed

against a different antigen. Non-limiting examples of antigens that may be recognized by the T cells comprised in the population of lymphocytes are provided herein.

It is preferred that that the population of lymphocytes comprises a number of cells that is suitable for use in adoptive cell transfer (ACT) therapy in humans. That is, the population of lymphocytes according to the disclosure comprises at least 10^6, 10^7, 10^8, 10^9 of 10^{10} CD3+ T cells. Preferably, the population of lymphocytes according to the disclosure comprises between 10^6 and 10^{10} CD3+ T cells, preferably between 10^7 and 10^9 T cells. In certain examples, the population of lymphocytes according to the disclosure comprises at least 10 x 10^8 T cells.

In a particular example, the disclosure relates to a population of lymphocytes for allogenic cell transfer in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and less than 10% are triple positive for CD45RA, CD57 and KLRG1.

In a particular example, the disclosure relates to a population of lymphocytes for allogenic cell transfer in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and less than 10% are triple positive for CD45RA, CD57 and KLRG1.

In a particular example, the disclosure relates to a population of lymphocytes for adoptive cell transfer therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and less than 10% are positive for CD45RA and CD57.

In a particular example, the disclosure relates to a population of lymphocytes for adoptive cell transfer therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and less than 10% are positive for CD45RA and CD57.

In a particular example, the disclosure relates to a population of lymphocytes for adoptive cell transfer therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and less than 10% are positive for CD57 and KLRG1.

In a particular example, the disclosure relates to a population of lymphocytes for adoptive cell transfer therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and less than 10% are positive for CD57 and KLRG1.

In a particular example, the disclosure relates to a population of lymphocytes for adoptive cell transfer therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and more than 80% are negative for CD57 and KLRG1.

In a particular example, the disclosure relates to a population of lymphocytes for adoptive cell transfer therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and more than 80% are negative for CD57 and KLRG1.

Preferably, the population of lymphocytes according to the disclosure is suitable for use in autologous cell therapy. Autologous cell therapy is a therapeutic intervention that uses an individual's cells, which are cultured and expanded outside the body, and reintroduced into the donor. Advantages of such an approach include the minimization of risks from systemic immunological reactions, bio-incompatibility, and disease transmission associated with non-autologous grafts or cells from the individual. It is preferred that the cells comprised in the population of lymphocytes according to the disclosure have been obtained by expanding T cells of an individual ex vivo and are subsequently infused back into the same individual.

Thus, in a particular example, the disclosure relates to a population of lymphocytes for autologous cell therapy in humans, the population of lymphocytes comprising at least 90%

CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and less than 10% are triple positive for CD45RA, CD57 and KLRG1.

Thus, in a particular example, the disclosure relates to a population of lymphocytes for autologous cell therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and less than 10% are triple positive for CD45RA, CD57 and KLRG1.

In a particular example, the disclosure relates to a population of lymphocytes for autologous cell therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and less than 10% are double positive for CD45RA and CD57.

In a particular example, the disclosure relates to a population of lymphocytes for autologous cell therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and less than 10% are double positive for CD45RA and CD57.

In a particular example, the disclosure relates to a population of lymphocytes for autologous cell therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and less than 10% are double positive for CD57 and KLRG1.

In a particular example, the disclosure relates to a population of lymphocytes for autologous cell therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at

least 20% are CD27/CD28 double positive and less than 10% are double positive for CD57 and KLRG1.

In a particular example, the disclosure relates to a population of lymphocytes for autologous cell therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and more than 80% are double negative for KLRG1 and CD57.

In a particular example, the disclosure relates to a population of lymphocytes for autologous cell therapy in humans, the population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and more than 80% are double negative for KLRG1 and CD57.

The disclosure also provides methods for producing a population of lymphocytes specific for one or more antigens as defined herein, said method comprising a single culture phase, wherein said single phase comprises (a) culturing a tissue or blood sample from a subject in the presence of said one or more antigens, which sample is known or suspected to contain lymphocytes; or (b) culturing the lymphocytes in the presence of said one or more antigens, which lymphocytes are isolated from a tissue or blood sample from a subject.

In certain examples, said culturing is continued until said T cell population of at least 10×10^8 cells is achieved. At all times the lymphocytes and/or population of T cells are maintained at temperatures greater than 0 °C during said single culture phase.

In certain examples, said culturing is continued until said T cell population of at least 1×10^7 cells is achieved. At all times the lymphocytes and/or population of T cells are maintained at temperatures greater than 0 °C during said single culture phase.

In certain examples, the sample comprising the lymphocytes and/or the T cells population are maintained at temperatures greater than 0°C subsequent to isolation from the subject and prior to culture. However, it is to be understood that frozen samples may also be used in the method of the present disclosure.

Previous expansion protocols for autologous tumor-infiltrating lymphocytes (TILs) consist of two phases. In an initial pre-REP phase, TILs are expanded for 3-5 weeks. In a subsequent REP phase, the TILs obtained in the pre-REP phase are rapidly expanded for an additional two weeks. Between the pre-REP and the REP phase, the TILs are typically cryopreserved. The disadvantage of this long cultivation period, including the optional cryopreservation step, is that a large fraction of the lymphocytes in the final product are terminal effector cells which rapidly die after infusion into the patient.

It is thus an aim of the present disclosure to establish an expansion protocol for lymphocytes with which high cell numbers (10^7 cells and more) can be reached in two to eight weeks, preferably in two to six weeks, more preferably in two to four weeks, and without the need of a cryopreservation step. Thus, in a preferred example, the lymphocytes are kept at temperatures greater than 0°C throughout the entire culturing process.

The method of the present disclosure is characterized in that the cells are cultured in a "conditioned culture medium". That is, certain parameters of the culture medium are monitored throughout the entire process and are adjusted to predefined values if necessary. Suitable parameters of the culture medium that are monitored and/or adjusted throughout the method of the disclosure are disclosed elsewhere herein. With that, optimal growth conditions can be maintained throughout the entire process.

The method of the disclosure is further characterized in that it comprises a step of "dynamic culturing". Dynamic culturing requires that the cells are cultured in a continuous flow of culture medium. Dynamic culturing comprises both circulation, where conditioned culture medium is circulated withing the growth chamber, and perfusion, where culture medium from the growth chamber is replaced with fresh culture medium.

The methods of expansion of the desired T cell populations from the sample, e.g. comprising lymphocytes and/or T cells, comprises the presentation of one or more antigens to the T cells within the sample to be cultured. The antigens may be presented by any means known in the art and/or described herein suitable to induce expansion of the T cells specifically recognizing the one or more antigens. As an exemplary non-limiting example, the one or more soluble antigens can be continuously provided in the culture medium (e.g. to maintain a steady state concentration or a desired range of concentration(s)) or may be included for one or more specific periods less than the entire culture phase. The soluble antigens can also be introduced at one or more discreet time points of the culture phase. Additionally or alternatively, the soluble antigens can be presented to the lymphocyte samples and/or T cells during the culture by antigen-presenting cells (APCs) as is disclosed herein. It is preferred that the APCs are B cells. The APCs can be engineered to present the one or more desired antigens by any means known in the art or described herein. Alternatively or in addition, The APCs can be contacted with antigenic peptides by any means known in the art or described herein.

In certain examples, the one or more antigens that are added in the culturing step are comprised in a tumor sample. That is, the tumor sample itself may simultaneously serve as a source of lymphocytes and as a source of antigens. In such examples, the tumor samples may be co-cultured with an APC in the absence of antigenic peptides.

The APCs may be recombinantly engineered to express the one or more antigens of interest either transiently or constitutively. Recombinant engineering can be achieved by any means known in the art or described herein and, preferably, is achieved by transduction using a viral vector or transfection using plasmids or mRNAs.

However, it is preferred herein that the APCs, in particular the B cells, are contacted with antigenic peptides that have been chemically synthesized, as described in more detail below.

The antigens may be one or more known antigen characterizing a disease or cancer, or may be determined by assessing a patient sample to determine one or more neoantigens. For that,

patient cells may be collected by biopsy and analyzed by mass spectrometry or scRNAseq to identify the neoantigens. The sequences available from these methods may then be analyzed using a proprietary algorithm to identify and select the relevant neoantigens.

The population of lymphocytes, isolated lymphocytes and/or the methods of their production and use are provided not only as tools for the treatment of disease (e.g. for use as a medicament or in the development and manufacture of a medicament) but will be also be understood to have applicability as model systems for investigating disease therapies. Accordingly, while the lymphocytes of the disclosure as disclosed herein are preferably human lymphocytes, more preferably primary human lymphocytes (e.g. including NK cells and T cells), and most preferably primary human T cells (e.g. including CD3+ T cells, CD4+ T cells, CD8+ T cells, γδ T cells), also provided are lymphocytes derived from lymphocyte cell lines (whether of human or non-human origin) as well as lymphocytes that are primary cells of non-human origin, for example and not being limited to, primary lymphocytes and lymphocytes derived from mice, rats, monkeys, apes, cats and dogs.

From the more preferred primary human lymphocytes, the most preferred is a primary human T cell. Therefore, the disclosure also provides populations of primary human T cells characterized by at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and less than 10% are triple positive for CD45RA, CD57 and KLRG1.

Alternatively, the disclosure also provides populations of primary human T cells characterized by at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and less than 10% are triple positive for CD45RA, CD57 and KLRG1.

Further, the disclosure also provides populations of primary human T cells characterized by at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 50% are CD27/CD28 double positive and more than 80% are double positive for CD57 and KLRG1.

Further, the disclosure also provides populations of primary human T cells characterized by at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and more than 80% are double positive for CD57 and KLRG1.

The lymphocyte population provided herein or produced according to the methods provided herein, whether human or not and whether primary or not, can be comprised of any lymphocyte class or subclass known in the art or described herein known or believed useful for adoptive cell therapies and/or known or believed to be of use in an *in vitro* or *in vivo* model systems. Non-limiting examples of lymphocyte classes encompassed by the disclosure include populations of lymphocytes comprising T cells (include CD3+ T cells, CD4+ T cells, CD8+ T cells, γδ T cells, invariant T cells), as well as B cells, Macrophages, and NK cells, as well as combinations thereof.

The populations of cells provided herein and/or for use in the methods of their production (e.g. APCs, preferably B cells) includes genetically engineered cells that may either be a directly genetically engineered cell, i.e. a cell that has been directly subject to genetic engineering methods, or may be a cell derived from such an engineered cell, e.g. a daughter cell or progeny of a cell that was directly genetically engineered. Any suitable genetic engineering method can be used, including but not limited to lipofection, CRISPR/CAS, calcium phosphate transfection, sleeping beauty transposons, PEG mediated transfection, and transduction with viral vectors (e.g. lentiviral vectors). Exogenous nucleic acid molecules may be introduced into cells as linear molecules and/or as circular molecules (e.g. plasmids, miniplasmids or mRNAs). In non-limiting examples, one or more of the lymphocytes within the lymphocyte population of the disclosure can be engineered to express one or more immunomodulators such as OX40L, 4-1BBL, CD80, CD86, CD83, CD70, CD40L, GITR-L, CD127L, CD30L (CD153), LIGHT, BTLA, ICOS-L (CD275), SLAM (CD150), CD662L, interleukin-12, interleukin-7, interleukin-15, interleukin-17, interleukin-21, interleukin-4, Bcl6, Bcl-XL, BCL-2, MCL1, STAT-5, and/or activators of one or more signaling pathways (e.g. the JAK/STAT pathway, the Akt/PBK signaling pathway, the BCR signaling pathway, and/or the BAFF/BAFFR signaling pathway). Similarly, one or

more APC of use in the methods disclosed herein may be engineered to express one or more known antigens or one or more neoantigens determined from a patient sample.

It is preferred that the APCs, in particular the B cells, are engineered to express one or more of the immunomodulators OXO40L, 4-1BB and/or interleukin-12.

In certain examples, the APCs, in particular the B cells, are engineered to express OXO40L and 4-1BB.

In certain examples, the APCs, in particular the B cells, are engineered to express OXO40L and interleukin-12.

In certain examples, the APCs, in particular the B cells, are engineered to express 4-1BB and interleukin-12.

In certain examples, the APCs, in particular the B cells, are engineered to express OXO40L, 4-1BB and interleukin-12.

Nucleic acids encoding the above-mentioned immunomodulators may be introduced into the APC's, in particular the B cells, by any method known in the art and/or disclosed herein. Preferably, mRNAs encoding the above-mentioned immunomodulators are introduced into the APC's, in particular the B cells, by means of transfection to transiently express the encoded proteins.

The lymphocytes and populations of lymphocytes of the disclosure, preferably human lymphocytes, more preferably primary human lymphocytes, and most preferably primary human T cells, are envisioned for use in therapy and may be autologous (*i.e.*, the donor from which the cells were derived and recipient are the same subject) or may be allogenic (*i.e.,* the donor from which the cells were derived is different from the recipient). Where autologous, any appropriate source can be used as known in the art or described herein, including but not limited to a tumor environment whether solid (such as for tumor-infiltrating lymphocytes

(TILs)) or circulating tumor cells; and peripheral blood (such as for PBMCs). Preferably, the lymphocytes in the population of lymphocytes have been obtained by expanding TILs ex vivo.

Where the cells are allogenic, they may be further genetically engineered or prepared such that they are not alloreactive. As understood in the art, and as used herein, non-alloreactive indicates that the cells have been engineered (*e.g.*, genetically engineered) such that they are rendered incapable of being recognized as or recognizing allogenic cells (cells of foreign origin). Similarly, the genetically engineered lymphocytes of the disclosure can be additionally or alternatively engineered so as to prevent their own recognition by the recipient's immune system. As a non-limiting example in this respect, the lymphocytes of the disclosure may have disruption or deletion of the endogenous major histocompatibility complex (MHC). Such cells may have diminished or eliminated expression of the endogenous MHC, preventing or diminishing activation of the recipient's immune system against the autologous cells.

As understood in the art, such non-alloreactive cells are incapable of reacting to cells of a foreign host. Therefore, non-alloreactive cells derived from third-party donors may become universal, *i.e.* recipient independent. As explained above, the non-alloreactive cells may also comprise additional engineering rendering them incapable of eliciting an immune response and/or of being recognized by the recipient's immune system, preventing them from being rejected. Such cells that are non-alloreactive and/or that are incapable of eliciting an immune response or being recognized by the recipient's immune system may also be termed "off the shelf" cells as is known in the art. Lymphocytes can be rendered non-alloreactive and/or incapable of eliciting or being recognized by an immune system by any means known in the art or described herein. In a non-limiting example, in the context of T cells non-alloreactive cells can have reduced or eliminated expression of the endogenous T cell receptor (TCR) when compared to an unmodified control cell. Such non-alloreactive T cells may comprise modified or deleted genes involved in self-recognition, such as but not limited to, those encoding components of the TCR including, for example, the alpha and/or beta chain. Similarly, the genetically engineered lymphocytes disclosed herein can additionally or alternatively have reduced or eliminated expression of the endogenous MHC when compared to an unmodified control cell. Such lymphocytes may comprise any modifications or gene deletions known in

the art or described herein to minimize or eliminate antigen presentation, in particular, so as to avoid immunogenic surveillance and elimination in the recipient. As noted, non-alloreactive cells which optionally avoid immune surveillance are widely referenced in the art as “off the shelf” cells and the terms are used interchangeably herein. Such non-alloreactive / off the shelf leucocytes may be obtained from repositories. The genetic modifications to reduce or eliminate alloreactivity (i.e. to render the cell non-alloreactive) and/or to reduce or eliminate self-antigen presentation (i.e. so as to prevent them from eliciting an immune response or being recognized by the recipient’s immune system), as known in the art or described herein can be performed before, concurrently with, or subsequent to any other genetic engineering in the context of the present disclosure.

The disclosure also encompasses a population of lymphocytes, preferably human lymphocytes obtainable by any method disclosed herein.

The disclosure provides a method of immunotherapy for treating a disease comprising the use of the cells or population of cells as disclosed herein. Accordingly, provided is a population of lymphocytes (preferably human leucocytes, more preferentially primary human lymphocytes, and most preferentially primary human T cells) as described herein for use as a medicament. The disclosure also provides the population of lymphocytes as disclosed herein within a pharmaceutically acceptable carrier in the form of a pharmaceutical composition. The medicament and pharmaceutical compositions as disclosed herein are, in particular, of use in adoptive cell therapies.

The population of lymphocytes, medicaments and/or pharmaceutical compositions of the disclosure are of use in the treatment of cancers regardless of tumor type, as well as in the treatment of viral diseases, bacterial diseases such as Tuberculosis (including antibiotic-resistant diseases), and parasitic diseases.

The population of lymphocytes, medicaments and/or pharmaceutical compositions of the disclosure can be used in combination with antineoplastic or immunomodulating agents such as, but not limited to Azacitidine, Capecitabine, Carmofur, Cladribine, Clofarabine, Cytarabine,

Decitabine, Floxuridine, Fludarabine, Fluorouracil, Gemcitabine, Mercaptopurine, Nelarabine, Pentostatin, Tegafur, Tioguanine, Methotrexate, Pemetrexed, Raltitrexed, Hydroxycarbamide, Irinotecan, Topotecan, Daunorubicin, Epirubicin, Idarubicin, Mitoxantrone, Valrubicin, Etoposide, Teniposide, Cabazitaxel, Docetaxel, Paclitaxel, Vinblastine, Vincristine, Vindesine, Vinflunine, Vinorelbine, Bendamustine, Busulfan, Carmustine, Chlorambucil, Chlormethine, Cyclophosphamide, Dacababazine, Fotemustine, Ifosfamide, Lomustine, Melphalan, Streptozotocin, Temozolomide, Carboplatin, Cisplatin, Nedaplatin, Oxaliplatin, Altretamine, Bleomycin, Bortezomib, Dactinomycin, Estramustine, Ixabepilone, Mytomycin, Alemtuzumab, Bevacizumab, Cetuximab, Denosumab, Gemtuzumab ozogamicin, Ibritumomab tiuxetan, Ipilimumab, Nivolumab, Ofatumumab, Panitumumab, Pembolizumab, Pertuzumab, Rituximab, Tositumomab, Trastuzumab, Afatinib, Aflibercept, Axitinib, bosutinib, Crizotinib, Dasatinib, Erlotinib, Gefitinib, Imatinib, Lapatinib, Nilotinib, Pazopanib, Ponatinib, Regorafenib, Ruxolitinib, Sorafenib, Sunitinib, Vandetanib, Everolimus, Temsirolimus, Alitretinoin, Bexarotene, Isotretinoin, Tamibarotene, Tretinoin, Lenalidomide, Pomalidomide, Thalidomide, Panobinostat, Romidepsin, Valproate, Vorinostat, Anagrelide, Arsenic trioxide, Aspariganse, BCG Vaccine, Denileukin diftitox, Vemurafenib, goserelin, Toremifene, Fulvestrant, bicalutamide, enzalutamide, apalutamide, darolutamide, anastrozole, letrozole, degarelix, abiraterone, filgrastim, molgramostin, pegfilgrastim, lipecfilgrastim, balugrastim, levacetylmethadol, interferon gamma, interferon alfa -2b, interferon alfa-n1, interferon beta-1a, peginterferon alfa-2b, peginterferonbeta-1a, ropeginterferon alfa-2v, tasonermin, histamine dihydrochloride, mifarmurtide, plerixafor, sipuleucel-T , dasiprotimut-T , muronab-CD3, mycophenolic acid, sirolimus, leflunomide, efalizumab, natalizumab, abatacept, exulizumab, ofatumumuab, fingolimd, eltrombopag, tofacitinib, teriflunomide, apremilast, vedolizumab, baricitinib, ozamimod, upacitinib, filgotinib, etanercept, infliximab, adalimumab, certolizumab pegol, golimumab, valdecoxib, anakinra, rilonacept, 38stekinumab, tocilizumab, canakinumab, secukinumab, Iopinavir, ritonavir, brodalumab, ixekizumab, sarilumab, tacrolimus, voclosporin, thalidomide, methotrexate, lenalidomide, pirfenidone, pomalidomide, dimethyl fumarate, darvadstrocel. As used herein combination with the population of lymphocytes, medicaments and/or pharmaceutical compositions of the disclosure does not indicate that the lymphocyte therapy and one or more additional medicaments need be administered together, e.g. in the same

CLAIMS

1. A method for expansion of a population of lymphocytes specific for one or more antigens in a controlled single culture vessel, the method comprising a step of

   a) culturing a tissue or blood sample from a subject in the presence of said one or more antigens, which sample is known or suspected to contain lymphocytes; or
   b) culturing lymphocytes in the presence of said one or more antigens, which lymphocytes are isolated from a tissue or blood sample from a subject;

   wherein the lymphocytes are cultured in a conditioned culture medium.

2. The method according to claim 1, wherein the conditioned culture medium is a culture medium in which at least one of the following parameters is monitored and adjusted if necessary: pH, dissolved oxygen (DO) concentration, glucose concentration, lactate concentration and/or temperature, in particular wherein the conditioned culture medium is a culture medium in which pH, dissolved oxygen (DO) concentration, glucose concentration, lactate concentration and temperature are monitored and adjusted if necessary.

3. The method according to claim 1 or 2, the method comprising a step of adjusting the culture volume to the expansion rate of the lymphocytes, in particular wherein the culture volume increases at least by a factor of 2, 3, 4, 5 or 6 during the expansion of the lymphocytes.

4. The method according to any one of claims 1 to 3, the method comprising a step of dynamic culturing the lymphocytes with the conditioned culture medium.

5. The method according to any one of claims 1 to 4, wherein the tissue sample is derived from a tumor, in particular wherein the tissue sample is a tumor sample, in particular wherein the tumor comprises at least one neoantigen.

6. The method according to any one of claims 1 to 5, wherein the lymphocytes comprise tumor-infiltrating lymphocytes, in particular wherein the tumor-infiltrating lymphocytes are T cells.

7. The method according to any one of claims 1 to 6, wherein one or more antigens are added to the culture medium in the form of peptides, in particular wherein the peptides are added to the culture medium at a concentration of 0.1 to 10 µg/ml.

8. The method according to any one of claim 1 to 7, wherein said culturing step comprises a step of co-culturing the lymphocytes with antigen-presenting cells (APCs), in particular wherein the antigen-presenting cells (APCs) are engineered to present one or more antigens; and/or wherein the antigen-presenting cells (APCs) comprise or are B cells, in particular wherein the B cells are obtained by apheresis and/or wherein the B cells are activated before addition to the lymphocytes, in particular wherein the B cells are activated with IL-4 and/or CD40L.

9. The method according to claim 8, wherein the antigen-presenting cells (APCs) are genetically engineered to express one or more transgene, in particular wherein the genetically engineered APCs have been obtained by transfecting the APCs with nucleic acids encoding the one or more transgene and/or wherein at least one of the one or more transgenes encodes an immunomodulator, in particular wherein the immunomodulator is selected from the group consisting of: OX40L, 4-1BBL, CD80, CD86, CD83, CD70, CD40L, GITR-L, CD127L, CD30L (CD153), LIGHT, BTLA, ICOS-L (CD275), SLAM (CD150), CD662L, interleukin-12, interleukin-7, interleukin-15, interleukin-17, interleukin-21, interleukin-4, Bcl6, BCLXL, BCL-2, MCL1, STAT-5, and

activators of one or more signaling pathways (e.g. the JAK/STAT pathway, the Akt/PKB signaling pathway, the BCR signaling pathway, and/or the BAFF/BAFFR signaling pathway), in particular wherein the immunomodulator is one or more of OX40L, 4-1BB and/or interleukin 12.

10. The method according to any one of claims 5 to 9, wherein the presence of at least one of the one or more antigens has been confirmed in the tumor sample comprising the lymphocytes prior to the culturing step; and/or wherein at least one of the one or more antigens is a neoantigen and wherein the presence of said neoantigen has been confirmed in the tumor sample comprising the lymphocytes prior to the culturing step, in particular wherein confirming the presence of at least one of the one or more antigens in the tumor sample comprises a step of sequencing genomic DNA that has been obtained from the tumor sample.

11. The method according to any one of claims 1 to 10, wherein the method comprises a step of activating the lymphocytes during culturing, in particular wherein the activation step comprises the addition of a CD3 agonist to the culture medium, in particular wherein the CD3 agonist is added to the culture medium after 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 days.

12. The method according to any one of claims 1 to 11, wherein the culture medium is supplemented with human AB serum and/or IL-2, and/or wherein said culturing is continued until said T cell population reaches at least 10^7 cells.

13. The method according to any one of claims 1 to 12 wherein said culturing is performed at temperatures of greater than 0 °C, and/or wherein said sample or said lymphocytes are maintained at temperatures greater than 0°C subsequent to isolation from said subject and prior to said culture.

14. A population of lymphocytes obtainable by the method of any one of claims 1 to 13.

15. A population of lymphocytes comprising at least 90% CD3+ T cells and less than 5% B cells, wherein at least 70% of said T cell portion are viable, at least 20% are CD27/CD28 double positive and less than 10% are triple positive for CD45RA, CD57 and KLRG1, in particular wherein said T cells are specific for one or more antigens.

16. The population of lymphocytes according to claim 34 or 35, wherein less than 15% of said T cell portion secrete at least one protein from the group consisting of: TNF-α, IL-4 and IL-5, and/or wherein at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the T cells in said T cell portion are $CD8^+$ T cells, and/or wherein at least two T cells in said T cell portion are directed against different antigens, in particular wherein at least one antigen is a neoantigen, and/or wherein said T cell portion comprises at least 10^7 T cells.

17. A pharmaceutical composition comprising the population of lymphocytes according to any one of claims 14 to 16, in particular wherein the lymphocytes are suspended in pharmaceutically acceptable buffer, in particular wherein the pharmaceutically acceptable buffer comprises about 0.9% NaCl and, optionally, up to 15% DMSO.

18. The population of lymphocytes according to any one of claims 14 to 16 or the pharmaceutical composition according to claim 17 for use as a medicament.

19. Use of the population of lymphocytes according to any one of claims 14 to 16 or the pharmaceutical composition according to claim 17 in the manufacture of a medicament for treating cancer, in particular wherein the treatment of cancer comprises a cancer therapy which is an adoptive cell therapy and/or wherein the cancer therapy is autologous cell therapy.

20. The use according to claim 18, wherein the medicament is to be infused into a subject suffering from cancer; optionally

wherein the population of cells has been obtained by:
a) surgically removing a tumor from a subject or taking a biopsy from a subject's tumor;

b) identifying at least one tumor antigen in the tumor sample obtained in step (a); and

c) expanding lymphocytes comprised in the tumor sample obtained in step (a) with the method according to any one of claims 1 to 13, wherein the lymphocytes are expanded in the presence of at least one tumor antigen that has been identified in step (b) to be present in the tumor sample;

in particular wherein the tumor antigen is a tumor-associated antigen or a tumor-specific antigen and/or wherein the lymphocytes comprise tumor-infiltrating lymphocytes (TILs), in particular wherein the TILs specifically recognize one or more tumor antigens, in particular wherein at least one tumor antigen is a neoantigen.