

CO-CULTURE

FIELD

The invention relates to methods for culturing immune cells. In particular, the invention
5 relates to methods for producing a population of immune cells comprising co-culture of
immune cells with antigen presenting cells (APCs) in a dynamic suspension. Also provided
are systems for use in such methods.

BACKGROUND

10 Cell therapies using adoptive transfer of active immune cells are a powerful clinical
tool. Common forms of such therapies involve chimeric antigen receptor T (CAR T) cells,
natural killer (NK) cells, T cell receptor-engineered T (TCR-T) cells, tumour-infiltrating
lymphocytes (TILs), regulatory T (Treg) cells, and cytokine-induced killer (CIK) cells. However,
to be clinically viable, it is necessary for such mature active immune cells to be produced in
15 sufficient numbers to be administered to patients.

The maturation of immature or naïve immune cells requires the presence of antigen
presenting cells (APCs), as the APCs provide stimulation which activates the immature or
naïve immune cells. Therefore manufacturing of active immune cells typically comprises co-
culturing immature or naïve immune cells with APCs to facilitate the stimulation of the
20 immature or naïve immune cells by the APCs.

Current manufacturing processes for active immune cells involve the co-culture of
naïve or immature immune cells with antigen presenting cells (APCs) under static conditions.
The consensus is that such static conditions are required for the immune cells and APCs to
be kept in close proximity, such that the APCs can provide the necessary stimulation in order
25 to activate the immune cells. Current bioreactors for the production of immune cells, such as
the G-Rex® and manufacturing protocols emphasise the importance for static culture and
stable interactions between immune cells and APCs for the production of mature active (e.g.
as taught in Gotti *et al.* Cytotherapy (2022) 24:334-343, Lubin *et al.* (2023) and WO
2019/190579, each of which is herein incorporated by reference).

30

SUMMARY

The present inventors have recognised that current manufacturing processes
for active immune cells are associated with concerns such as length of “vein to vein” time,
cost, sterility issues and other factors.

35 It is an object of the present invention to overcome one or more of these problems,
and to provide manufacturing methods and systems for the production of populations of
immune cells, particularly active immune cells, that are appropriate for commercial scale

manufacturing and are GMP-compliant to facilitate regulatory approval for use in human patients.

The present inventors have surprisingly shown that a population of immune cells can be effectively co-cultured with APCs in a dynamic suspension. In particular, that populations of immune cells, particularly active immune cells, can be produced by co-culturing immune cells with APCs in a dynamic suspension, and that the efficacy of such methods are at least equivalent to those produced by the current "gold-standard" static co-culture methods. In particular, the inventors have shown that T cells can be co-cultured with dendritic cells (DCs) in a dynamic suspension, and that the number of reactive CD4⁺ and CD8⁺ T cells was equivalent to those produced using convention static co-culture.

The ability to produce a population of immune cells using a dynamic suspension has numerous advantages. In particular, dynamic co-culture can improve the quality and consistency of the immune cells, as dynamic culture reduces environmental variability, resulting in reproducibility of the process and improved consistency of the end product. Dynamic co-culture can also facilitate scale-up/scale-out: of the process, as such scalable hardware and software provides a significant reduction in the cost and effort of scaling. Dynamic co-culture can also reduce risk, as the co-culture process can be more precisely controlled and regulated, whilst the number of human interventions is reduced, decreasing the risk of batch loss. Also, dynamic co-culture can improve flexibility, as continuous monitoring enables online, in-situ responses so that the system can be dynamically adjusted to compensate for variations in process and/or starting materials.

Accordingly, the present invention provides a method for producing a population of immune cells, wherein said method comprises co-culture of isolated immune cells with antigen presenting cells (APCs) in a dynamic suspension.

Said dynamic suspension may result from agitation of the co-culture or from fluid flow, preferably from agitation of the co-culture. The agitation may be: (a) continuous; and/or (b) intermittent. Said intermittent agitation of the co-culture may comprise agitation for between about 5 minutes to about 30 minutes every 1 to 5 hours. Optionally said intermittent agitation is for: (a) between about 5 minutes to about 15 minutes, preferably about 10 minutes, every hour; or (b) between about 5 minutes to about 15 minutes, preferably about 10 minutes, every 3 hours.

According to a method of the invention: (a) a first period of the co-culture may be subjected to intermittent agitation, wherein said first period is optionally between about 0 to about 2 days, preferably between about 1 to about 2 days; and/or (b) said first period of co-culture may be followed by a second period of co-culture which is subjected to constant agitation, wherein said second period is optionally between about 2 to about 21 days, preferably between about 2 to about 10 days.

According to the invention, the agitation may be: (a) mechanical agitation; (b) rocking motion agitation; (c) vertical wheel agitation; or (d) pneumatic agitation. Preferably the isolated immune cells and APCs may be co-cultured under mechanical agitation and more preferably a co-culture may be carried out in a stirred tank bioreactor.

- 5 The agitation may be mechanical agitation, optionally at: (a) an RPM of between about 20 RPM to about 80 RPM, preferably between about 20 RPM to about 75 RPM, more preferably between about 25 RPM to about 70 RPM; or (b) an RPM of between about 70 RPM to about 150 RPM, preferably between about 70 RPM to about 100 RPM.

The immune cells may be T cells, optionally tumour infiltrating lymphocytes (TILs).

- 10 The T cells may be: (a) CD8⁺ T cells; (b) CD4⁺ T cells, optionally Th1, Th2, Th17, Tfh and/or Th9 cells; (c) NKT cells, optionally invariant NKT cells; and/or (d) regulatory T cell (Treg) cells.

The APCs may be: (a) dendritic cells (DCs); (b) B cells; and/or (c) macrophages.

Said antigen may be a tumour antigen, preferably a neoantigen, more preferably a clonal neoantigen.

- 15 Preferably the immune cells are T cells, most preferably TIL, and the APCs are DCs.

One or more of the following parameters may be monitored in a method of the invention, and monitored and maintained at a desired set point using a feedback mechanism: (a) pH; (b) dissolved oxygen (DO); (c) temperature; (d) gas mix of O₂, N₂, CO₂ and/or compressed air; and/or (e) the concentration of one or more nutrient and/or metabolite.

- 20 Optionally said one or more parameter may be monitored and maintained using a control loop mechanism.

In a method of the invention, fresh medium may be introduced into the co-culture: (a) at least once, twice, three times, four times, five times, six times or more during the method; and/or (b) every 2 to 3 days during the method.

- 25 In a method of the invention, the number of immune cells and/or APCs may be determined: (a) at least once, twice, three times, four times, five times, six times or more during the method; and/or (b) every 2 to 3 days during the method. Optionally the number of immune cells and/or APCs may be determined using flow cytometry.

- 30 In a method of the invention, the immune cells may be seeded at the start of the co-culture the immune cells: (a) at about 1x10⁶ immune cells/mL; and/or (b) in a ratio of about 10:1 immune cells: APCs.

- 35 The method of the invention may further comprise step of non-specifically expanding the immune cells before co-culturing with the APCs, wherein optionally: (a) the immune cells are T cells; and/or (b) the step of non-specifically expanding the immune cells comprises culturing the immune cells with one or more of IL-2, IL-21, IL-15, anti-CD3 antibodies, anti-CD28 antibodies and anti-CD2 antibodies, preferably all of IL-2, IL-21, IL-15, anti-CD3 antibodies, anti-CD28 antibodies and anti-CD2 antibodies.

In a method of the invention, the co-cultured cells may be harvested after a total duration of co-culture of between about 2 days to about 21 days, preferably of between about 2 days to about 10 days, and the immune cells may be used to re-seed the culture vessel, wherein optionally the immune cells may be reseeded at a density of about 1×10^6 immune cells/mL in the absence of APCs.

Following co-culture, the immune cells may be cultured in the absence of the APCs for a further period of time, wherein optionally: (a) in said further period of time the immune cells are cultured in a dynamic suspension; and/or (b) said further period is optionally between about 2 to about 21 days, preferably between about 2 to about 10 days.

The dynamic suspension of the immune cells may result from agitation of the immune cells, wherein optionally the agitation is continuous or intermittent.

Intermittent agitation of the immune cells may comprise agitation for between about 5 minutes to about 30 minutes every 1 to 5 hours, wherein optionally: (a) the agitation is for between about 5 minutes to about 15 minutes, preferably about 10 minutes, every hour; or (b) the agitation is for between about 5 minutes to about 15 minutes, preferably about 10 minutes, every 3 hours.

The agitation of the immune cells may be: (a) mechanical agitation; (b) rocking motion agitation; (c) vertical wheel agitation; or (d) pneumatic agitation; wherein preferably the isolated immune cells and APCs are co-cultured under mechanical agitation and more preferably wherein a co-culture is carried out in a stirred tank bioreactor.

Agitation of the immune cells may be mechanical agitation at: (a) an RPM of between about 20 RPM to about 80 RPM, preferably between about 20 RPM to about 75 RPM, more preferably between about 25 RPM to about 70 RPM; or (b) an RPM of between about 70 RPM to about 200 RPM, preferably between about 70 RPM to about 150 RPM or between about 70 RPM to about 100 RPM.

A method of the invention may comprise:

(a) (i) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 25 RPM followed by no agitation for about 1 hour for a first period of about 24 hours; (ii) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 50 RPM followed by no agitation for about 1 hour for a second period of about 24 hours; (iii) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 70 RPM for a third period of about 1 week; optionally (iv) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and (v) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a fourth period of about 1 week;

or

(b) (i) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 25 RPM followed by no agitation for about 3 hours for a first period of about 24 hours; (ii) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 50 RPM followed by no agitation for about 3 hours for a second period of about 24 hours; (iii) culturing the isolated immune cells and APCs with constant agitation at an RPM of about 70 RPM for a third period of about 1 week; optionally (iv) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and (v) co-culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a fourth period of about 1 week;

10 or

(c) (i) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 100 RPM followed by no agitation for about 1 hour for a first period of about 48 hours; (ii) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 100 RPM for a second period of about 1 week; optionally (iii) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and (iv) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a third period of about 1 week;

or

(d)(i) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 100 RPM followed by no agitation for about 3 hours for a first period of about 48 hours; (ii) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 100 RPM for a second period of about 1 week; optionally (iii) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and (iv) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a third period of about 1 week;

or

(e) (i) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 100 RPM for a first period of about 9 days; optionally (ii) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and (iii) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a second period of about 1 week.

A method of the invention may be carried out using a stirred tank bioreactor, optionally with a marine impeller.

A method of the invention may result in a yield of immune cells which is at least equivalent to that produced by a corresponding method in which the isolated immune cells are co-cultured with APCs without agitation.

The invention further provides a method of producing a cell therapy product, comprising: (a) producing a population of immune cells according to a method as described herein; (b) optionally isolating and/or purifying the immune cell population; and (c) formulating the immune cell population with a pharmaceutically acceptable carrier.

5 The invention also provides an immune cell population obtained or obtainable by the method of the invention, an immune cell composition comprising said immune cell population or a cell therapy product obtained or obtainable the invention, wherein preferably said population, composition or product comprises at least about 10×10^6 reactive immune cells, or at least about 0.2%-5%, 5%-10%, 10-20%, 20-30%, 30-40%, 40-50 %, 50-70% or 70-100%
10 reactive immune cells. The immune cells in said immune cell population, immune cell composition or cell therapy product may preferably be antigen-specific T cells.

 The invention further provides an immune cell population or composition as described herein for use in treating or preventing cancer in a subject, wherein preferably said cancer is bladder cancer, gastric, oesophageal, breast cancer, colorectal cancer, cervical cancer,
15 ovarian cancer, endometrial cancer, kidney cancer (renal cell), lung cancer (small cell, non-small cell and mesothelioma), brain cancer (e.g. gliomas, astrocytomas, glioblastomas), melanoma, lymphoma, small bowel cancers (duodenal and jejunal), leukemia, pancreatic cancer, hepatobiliary tumours, germ cell cancers, prostate cancer, head and neck cancers, thyroid cancer or sarcomas, and wherein more preferably the subject is a human.

20 The invention also provides a system for the production of a population of immune cells, the system comprising: (a) a suspension bioreactor; (b) isolated immune cells; (c) APCs; (d) a culture medium; and (e) at least one sensor; wherein: the isolated immune cells are in co-culture with the APCs in a dynamic suspension; and optionally wherein the at least one sensor capable of monitoring one or more of the following parameters: (i) pH; (ii) dissolved
25 oxygen (DO); (iii) temperature; (iv) gas mix of O_2 , N_2 , CO_2 and/or compressed air; and/or the concentration of one or more nutrient and/or metabolite. Said dynamic may suspension result from agitation of the co-culture of isolated immune cells and APCs. Optionally said agitation is selected from: (a) mechanical agitation; (b) rocking motion agitation; (c) vertical wheel agitation; or (d) pneumatic agitation; wherein preferably the isolated immune cells and APCs
30 are co-cultured under mechanical agitation and more preferably wherein the agitated bioreactor is a stirred tank bioreactor. Alternatively or in addition, in said system, said at least one sensor may be comprised in a proportional–integral–derivative controller allowing for said one or more parameter to be monitored and maintained using a control loop mechanism.

35

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Schematic showing different experimental protocols for a range of DASbox conditions as exemplified herein.

5

Figure 2: Plot showing % of reactive CD8⁺ T cells across the different DASbox conditions when compared with the GMF or GSD controls, either at day 9 or day 16. The dotted line shows the base line level of required reactivity. Purple crosses = day 9 data points, red crosses = day 16 data points. For D0 the base line reactivity is 92.13%. Variance at day 9 was in a range of approx. 20% around this base line. Variance at day 16 was in a range of approx. 15% around this base line.

10

Figure 3: Graph showing the total number of CD8⁺ T cells produced by the different DASbox conditions and the GMF and GSD controls at day 9 and day 16.

15

Figure 4: Graph showing the number of reactive CD8⁺ T cells produced by the different DASbox conditions and the GMF and GSD controls at day 9 and day 16.

Figure 5: Graph showing the overall reactive CD8⁺ T cells population produced by the different DASbox conditions and the GMF and GSD controls at day 9 and day 16.

20

Figure 6: Plot showing % of reactive CD4⁺ T cells across the different DASbox conditions when compared with the GMF or GSD controls, either at day 9 or day 16. The dotted line shows the base line level of required reactivity. Purple crosses = day 9 data points, red crosses = day 16 data points. For D0 the base line reactivity is 12.96.1%. Variance at day 9 was in a range of approx. 25% around this base line. Variance at day 16 was in a range of approx. 15% around this base line.

25

Figure 7: Graph showing the total number of CD4⁺ T cells produced by the different DASbox conditions and the GMF and GSD controls at day 9 and day 16.

30

Figure 8: Graph showing the number of reactive CD4⁺ T cells produced by the different DASbox conditions and the GMF and GSD controls at day 9 and day 16.

Figure 9: Graph showing the overall reactive CD4⁺ T cells population produced by the different DASbox conditions and the GMF and GSD controls at day 9 and day 16.

35

Figure 10: Schematic showing one of the controls exemplified, namely the “hybrid control” wherein a G-Rex100M was set up for the first 48 hours and cells were subsequently re-seeded into a DASbox vessel.

- 5 **Figure 11:** Schematic showing experimental protocol and a “hybrid control” to test the expansion and reactivity of patient-derived material, as exemplified herein.

Figures 12a and 12b: Graphs showing cell growth in a co-culture (D0-9), comparing the growth in a dynamic suspension (AMBR) versus a static system (G-Rex). Expansion data is shown measuring (a) CD3+ cells and (b) Fold expansion, using patient-derived material, as
10 exemplified herein. Cells were harvested at day 9 (D9_H) and reseeded (D9_S), without APCs.

Figures 13a and 13b: Graphs showing cell growth in a co-culture (D0-D9), comparing growth
15 in a dynamic suspension (AMBR) of patient-derived material versus cells from the HD (healthy donor) model. This expansion data is shown measuring (a) CD3+ cells and (b) Fold expansion, as exemplified herein. Cells were harvested at day 9 (D9_H) and reseeded (D9_S), without APCs.

20 **Figures 14a and 14b:** Graphs showing a reactivity data of patient-derived material co-cultured in a dynamic suspension (a) line plot and (b) bar graph formats, both measuring percentage of reactive CD8+ cells (TNFa+ and IFNg+).

DETAILED DESCRIPTION

25

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY,
30 20 ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide the skilled person with a general dictionary of many of the terms used in this disclosure. The meaning and scope of the terms should be clear; however, in the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition.

35 It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. In particular, any

methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of this disclosure.

The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and
5 examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be
10 applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and application to provide yet further embodiments of the disclosure. Moreover, due to biological functional equivalency considerations, some changes can be made in protein
15 structure without affecting the biological or chemical action in kind or amount. These and other changes can be made to the disclosure in light of the detailed description. All such modifications are intended to be included within the scope of the appended claims.

When applied to a cell, the term "isolated" in the context of the present invention denotes that the cell has been removed from its natural milieu and is thus free of other
20 extraneous or unwanted cells or cell types and is in a form suitable for use within the manufacturing methods described herein and/or for administration to patients. Such isolated cells are those that are separated from their natural environment.

Numeric ranges are inclusive of the numbers defining the range. Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the
25 lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within this disclosure. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either,
30 neither or both limits are included in the smaller ranges is also encompassed within this disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in this disclosure.

The terms "increased", "increase", "enhance", or "activate" are all used herein to mean
35 an increase by a statically significant amount. The terms "increased", "increase", "enhance", or "activate" can mean an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or

at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level. In the context of a yield or titre, an "increase" is an observable or statistically significant increase in such level.

The terms "decreased", "reduced", "reduction", or "inhibit" are all used herein to mean a decrease by a statistically significant amount. The terms "reduce," "reduction" or "decrease" or "inhibit" typically means a decrease by at least 10% as compared to a reference level (e.g. the absence of a given treatment) and can include, for example, a decrease by at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% , or more. As used herein, "reduction" or "inhibition" encompasses a complete inhibition or reduction as compared to a reference level. "Complete inhibition" is a 100% inhibition (i.e. abrogation) as compared to a reference level.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an immune cell" includes a plurality of such cells and reference to "the immune cell" includes reference to one or more immune cells and equivalents thereof known to those skilled in the art, and so forth. Furthermore, the use of the term "including", as well as other forms, such as "includes" and "included", is not limiting.

"About" may generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Exemplary degrees of error are within 20 percent (%), typically, within 10%, and more typically, within 5% of a given value or range of values. Preferably, the term "about" shall be understood herein as plus or minus ($\pm$) 5%, preferably $\pm$ 4%, $\pm$ 3%, $\pm$ 2%, $\pm$ 1%, $\pm$ 0.5%, $\pm$ 0.1%, of the numerical value of the number with which it is being used.

The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the invention.

As used herein the term "consisting essentially of" refers to those elements required for a given invention. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that invention (i.e. inactive or non-immunogenic ingredients).

Embodiments described herein as “comprising” one or more features may also be considered as disclosure of the corresponding embodiments “consisting of” and/or “consisting essentially of” such features.

Concentrations, amounts, volumes, percentages and other numerical values may be presented herein in a range format. It is also to be understood that such range format is used merely for convenience and brevity and should be interpreted flexibly to include not only the numerical values explicitly recited as the limits of the range but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly recited.

The terms “individual”, “subject”, and “patient”, are used interchangeably herein to refer to a mammalian subject for whom diagnosis, prognosis, disease monitoring, treatment, therapy, and/or therapy optimisation is desired. The mammal can be (without limitation) a human, non-human primate, mouse, rat, dog, cat, horse, or cow. In a preferred embodiment, the individual, subject, or patient is a human. An “individual” may be an adult, juvenile or infant. An “individual” may be male or female.

A “subject in need” of treatment for a particular condition can be an individual having that condition, diagnosed as having that condition, or at risk of developing that condition.

A subject can be one who has been previously diagnosed with or identified as suffering from or having a condition in need of treatment or one or more complications related to such a condition, and optionally, have already undergone treatment for a condition as defined herein or the one or more complications related to said condition. Alternatively, a subject can also be one who has not been previously diagnosed as having a condition as defined herein or one or more complications related to said condition. For example, an individual can be one who exhibits one or more risk factors for a condition, or one or more complications related to said condition or a subject who does not exhibit risk factors.

As defined herein “treatment” refers to reducing, alleviating or eliminating one or more symptoms of the disease which is being treated, relative to the symptoms prior to treatment.

“Prevention” (or prophylaxis) refers to delaying or preventing the onset of the symptoms of the disease. Prevention may be absolute (such that no disease occurs) or may be effective only in some individuals or for a limited amount of time.

As used herein, the term “healthy individual” refers to an individual or group of individuals who are in a healthy state, e.g. individuals who have not shown any symptoms of the disease, have not been diagnosed with the disease and/or are not likely to develop the disease e.g. cancer or any other disease described herein). Preferably said healthy individual(s) is not on medication affecting cancer and has not been diagnosed with any other disease. The one or more healthy individuals may have a similar sex, age, and/or body mass index (BMI) as compared with the test individual. Application of standard statistical methods

used in medicine permits determination of normal levels of expression in healthy individuals, and significant deviations from such normal levels.

As used herein, the term "population" refers to two or more cells of the specified cell type(s). A population may be homogenous, i.e. may contain a single cell type, e.g. a T cell population. A population may be heterogenous, i.e. may contain two or more cell types, e.g. T cells and APCs.

As used herein, the term "peripheral blood cells" refer to the cellular components of blood, including red blood cells, white blood cells, and platelets, which are found within the circulating pool of blood.

As used herein, the terms "haematopoietic progenitor cells", "haematopoietic precursor cells" and "HPCs" refer to cells which are committed to a haematopoietic lineage but are capable of further haematopoietic differentiation and include haematopoietic stem cells, multipotential haematopoietic stem cells (haematoblasts), myeloid progenitors, megakaryocyte progenitors, erythrocyte progenitors, and lymphoid progenitors. Haematopoietic stem cells (HSCs) are multipotent stem cells that give rise to all the blood cell types including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cells). Haematopoietic progenitor cells typically express CD34. The haematopoietic progenitor cells may co-express CD133 and/or CD38. Haematopoietic precursor cells include CD34+ / CD45+ haematopoietic precursor cells and CD34+ / CD45+ / CD43+ haematopoietic precursor cells.

As used herein, the terms "common lymphoid progenitor cells" and "CLPs" refer to lymphoid progenitor cells which give rise to T cells, B cells and NK cells. Human bone marrow CLPs are typically CD34⁺CD38⁺Neprilysin⁺, and cord blood CLPs are typically CD34⁺CD38⁺CD7⁺.

As used herein, the term "expansion" or "expanding" means increasing the number of T cells by inducing their proliferation. Immune cells (e.g. T cells) may be expanded by ex vivo culture in conditions which provide mitogenic stimuli for said immune cells (e.g. T cells).

As used herein, the term "antigen-specific expansion" refers to a step of increasing the number of immune cells (e.g. T cells) in the presence of antigen. The presence of antigen leads to an increase in, or expansion of, immune cells (e.g. T cells) with specificity to said antigen within the overall population. The aim of this antigen-specific expansion is to preferentially or selectively expand immune cells (e.g. T cells) that bind and respond to one or more antigens. The conditions of antigen-specific expansion may be controlled in order to minimise any non-specific expansion of the immune cells (e.g. T cells). An antigen-specific expansion step increases the proportion or percentage of immune cells (e.g. T cells) specific

to said antigen within the overall population of immune cells (e.g. T cells), i.e. compared to the proportion or percentage of immune cells (e.g. T cells) not specific to said antigen.

An "antigen" as referred to herein is a molecule which itself, or a part thereof, is capable of stimulating an immune response, when presented to the immune system or immune cells in an appropriate manner.

As used herein, "cell therapy product" means refers to biological medicinal product which contains or consists of cells or tissues. Cell therapy products have properties for, or are used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of the cells or tissues of said product. Examples of cell therapy products include cellular immunotherapies, cancer vaccines, and other types of both autologous and allogeneic cells for certain therapeutic indications, including haemopoietic stem cells and adult stem cells, induced pluripotent stem cells and embryonic stem cells.

As used herein, "pharmaceutically acceptable carrier or diluent" means any substance suitable for use in administering to an animal. Certain such carriers enable pharmaceutical compositions to be formulated as, for example, tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspension and lozenges for the oral ingestion by a subject. In certain embodiments, a pharmaceutically acceptable carrier or diluent is sterile water, sterile saline, sterile buffer solution or sterile artificial cerebrospinal fluid.

For example, the term "pharmaceutically acceptable" may refer to salts, excipients, carriers, diluents, etc. approved by a regulatory agency of the Federal or a state government, or listed in the U.S. Pharmacopeia, European Pharmacopeia, or other generally recognized pharmacopeia.

Other definitions of terms may appear throughout the specification. Before the exemplary embodiments are described in more detail, it is to be understood that this disclosure is not limited to particular embodiments described, and as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be defined only by the appended claims.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that such publications constitute prior art to the claims appended hereto. All references cited in this specification are herewith incorporated by reference with respect to their entire disclosure content and the disclosure content specifically mentioned in this specification.

Disclosure related to the various methods of the invention are intended to be applied equally to other methods, therapeutic uses or methods, and *vice versa*.

Immune Cells

The invention relates to methods for producing immune cells or populations thereof. As described herein, said methods comprise the co-culture of immune cells with APCs.

The immune cells may be T cells and/or Natural Killer (NK) cells, as described herein.

- 5 Preferably the immune cells are T cells, such as tumour infiltrating lymphocytes (TILs). The T cells may be CD8⁺ T cells, CD4⁺T cells (optionally Th1, Th2, Th17, Tfh and/or Th9 cells), NKT cells (optionally invariant NKT cells) and/or regulatory T cell (Treg) cells, or any combination thereof.

- 10 As used herein, unless expressly stated to the contrary, the term “immune cells” encompasses natural immune cells, induced or adaptive immune cells and immune cells that have been created using recombinant DNA technology.

- An immune cell may be characterized on the basis of its marker expression profile (i.e. by the presence of one or more cell marker and/or the absence of one more cell marker). Methods for determining the presence of cell markers are well-known in the art and include, 15 for example, flow cytometry.

- The immune cells co-cultured with APCs may be isolated immune cells. Thus, the immune cells may be isolated from a sample prior to co-culturing in a method of the invention. Said sample may be obtained from the individual to be treated using the population of immune cells produced by a method of the invention, i.e. the method may be used to produce a 20 population of autologous immune cells. Alternatively, the sample may be obtained from an individual other than the individual to be treated using the immune cells produced by a method of the invention, i.e. the method may be used to produce a population of allogenic immune cells. Wherein the immune cells are allogenic, they may be matched (e.g. HLA matched) to the individual to be treated.

- 25 The sample may be obtained from any suitable tissue or bodily fluid. By way of non-limiting example, the sample may be taken from a tumour, peripheral blood (e.g. peripheral blood mononuclear cells or PBMC), bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue or from other tissues of an individual.

- 30 The immune cells may be produced from haematopoietic progenitor cells obtained from a sample. Said sample may be a sample obtained from the individual to be treated using the population of immune cells produced by a method of the invention (to produce autologous immune cells) or a sample from an individual other than the individual to be treated using the immune cells produced by a method of the invention (to produce allogenic immune cells). In 35 the latter case, the sample may be obtained from a blood or tissue bank. In embodiments where the methods of the invention produce T cells or NK cells, the haematopoietic progenitor cells are typically hematopoietic progenitor cells (HPCs) or common lymphoid progenitor cells

(CLPs), which can then be differentiated to T cells or NK cells for use in a method of the invention.

The immune cells (e.g. T cells) may be modified immune cells, for example genetically modified immune cells as described herein.

5

T cells

Preferably the methods of the invention are used to produce populations of T cells.

T cells (also referred to as T lymphocytes) express a T cell receptor (TCR) and a co-receptor which may be cluster of differentiation 4 (CD4) or cluster of differentiation 8 (CD8). T cells are present in the peripheral blood, lymph nodes, tissues and tumours. As used herein, the term "T cell" encompasses any type of T cell, such as an alpha beta T cell (e.g. CD8⁺ or CD4⁺), a gamma delta T cell, a NK T cell or a Treg cell.

The T cells produced by a method of the invention may be CD8⁺ T cells, CD4⁺ T cells, NKT cells and/or regulatory T cells, or any combination thereof. Preferably the T cells produced by a method of the invention are CD8⁺ T cells and/or CD4⁺ T cells, particularly CD4⁺ T cells. In other words, a T cell population produced by a method of the invention may comprise or consist of CD8⁺ T cells and/or CD4⁺ T cells, particularly CD4⁺ T cells.

The T cell population may be generated from T cells in a sample isolated from an individual, optionally an individual with a tumour. The sample may be taken from a tumour, peripheral blood (e.g. peripheral blood mononuclear cells or PBMC), bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue or from other tissues of the subject.

Suitable techniques are known in the art to isolate T cells from a sample. By way of non-limiting example, T cells can be obtained from a sample of blood collected from a subject using any number of techniques known to the skilled person. For example, density gradient separation techniques, such as FICOLL™ separation, and/or apheresis, such as leukapheresis, may be employed. Additional methods of isolating T cells for a T cell therapy are disclosed in U.S. Patent Publication No. 2013/0287748, which is herein incorporated by reference in its entirety.

30

CD3⁺

T cells express CD3 and so may be described as CD3 positive or CD3⁺. All T cells express CD3⁺ and this co-receptor is involved in activating both CD8⁺ T cells and CD4⁺ T cells, which together comprise the CD3⁺ T cell population.

35

CD8⁺ T cells

CD8⁺ T cells express CD8, and so may be described as CD8 positive or CD8⁺. CD8⁺ T cells are involved in MHC I-restricted interactions.

CD8⁺ T cells encompass both cytotoxic CD8⁺ T cells and CD8⁺ suppressor T cells. For the avoidance of doubt, the term “CD8⁺ T cells” and any disclosure herein relating to “CD8⁺ T cells” encompasses both cytotoxic CD8⁺ T cells and CD8⁺ suppressor T cells subtypes unless expressly stated to the contrary.

Cytotoxic CD8⁺ T cells are major killers of pathogens and neoplastic cells, with CD4⁺ T cells playing important roles in the maintenance of the CD8⁺ response and prevention of exhaustion. On activation, cytotoxic CD8⁺ T secreted death-inducing granules containing granzymes, perforin, cathepsin C and granulysin which can either fuse with the target-cell membrane, or be taken up by the target cell by endocytosis. Cytotoxic CD8⁺ T cells can also secrete cytokines, primarily TNF- α and IFN- γ , which have anti-tumour and anti-viral microbial effects. In addition, as ligand (FASL) is expressed on CD8⁺ T cells and its ligation by Fas receptors on target cells activates death domains (Fas-associated protein with death domains (FADD)), which, in turn, activate caspases and endonucleases, leading to the fragmentation of target-cell DNA. These expressed proteins and other factors can be used as markers for CD8⁺ T cells.

CD8⁺ suppressor T cells encompasses multiple distinct CD8⁺ T cell populations that are functionally characterized by downregulating immune responses. Commonly CD8⁺ suppressor cells are Foxp3⁺ and/or CD122⁺. Exemplary CD8⁺ suppressor cell phenotypes are CD8⁺CD25⁺CTLA-4⁺Foxp3⁺ and CD8⁺CD122⁺.

CD4⁺ T cells

CD4⁺ T cells express CD4, and so may be described as CD4 positive or CD4⁺. CD4⁺ T cells are involved in MHC II-restricted interactions.

CD4⁺ T cells encompass multiple different subtypes, including T-helper 1 (Th1), T-helper 2 (Th2) cells, T-helper 17 (Th17), follicular helper T cell (Tfh), T-helper 9 (Th9) and regulatory T cells (Treg) cells. For the avoidance of doubt, the term “CD4⁺ T cells” and any disclosure herein relating to “CD4⁺ T cells” encompasses any and all CD4⁺ T cell subtypes unless expressly stated to the contrary.

The initial step of differentiation of the naïve CD4⁺ cells is the antigenic stimulation as a result of interaction of TCR and CD4 as co-receptor with antigen-MHC II complex, presented by APCs. TCR coupled with CD3 activation consequently induces a network of downstream signalling pathways, that eventually lead to naïve cell proliferation and differentiation into specific effector cells.

Differentiation of naïve CD4⁺ cells to Th1 cells is driven by IFN γ and IL-12. Th1 cells are involved with the elimination of intracellular pathogens and are associated with organ-

specific autoimmunity. They mainly secrete IFN γ , lymphotoxin α (Lfa), and IL2. IFN γ is a commonly used marker for Th1 cells.

Differentiation of naïve CD4⁺ cells to Th2 cells is driven by IL-4 and IL-2. Th2 cells are involved in the immune response to extracellular parasites, including helminths, and play
5 major role in induction and persistence of asthma as well as other allergic diseases. The key effector-cytokines include IL4, IL5, IL9, IL13, IL10, IL25, and amphiregulin. IL-4 is a commonly used marker for Th2 cells.

Differentiation of naïve CD4⁺ cells to Th17 cells is driven by IL-21, IL-6, IL-23 and TGF- β . Th17 cells are involved in the immune response against extracellular bacteria and fungi.
10 They are also involved in the generation of autoimmune diseases. The key effector cytokines include IL17A, IL17F, IL21, and IL22. IL-17 is a commonly used marker for Th17 cells.

Differentiation of naïve CD4⁺ cells to Tfh cells is driven by IL-21 and IL-6. Tfh cells play a significant role in mediating humoral immunity through interaction with B-lymphocytes. Tfh secrete IFN γ , IL-4 and/or IL-10. CXCR5, ICOS and/or PD-1 are commonly used markers for
15 Tfh cells.

Differentiation of naïve CD4⁺ cells to Th9 cells is driven by IL-4 and TGF- β . Th9 cells secrete IL-9 and are involved in the immune response to parasitic infections. IL-9 production, together with a lack of IL-4, IL-5, and /or IL-13 production, is commonly used as a marker for Th9 cells.

20 Tregs are discussed further below.

Tumour infiltrating lymphocytes (TILs)

The immune cells or immune cell population, particularly T cells or a T cell population, may be generated from a sample from a tumour. In other words, the immune cell population,
25 particularly the T cell population may be isolated from a sample obtained from the tumour of an individual to be treated. Such immune cells are referred to as tumour infiltrating immune cells, and in particular when said immune cells are T cells, they are referred to herein as 'tumour infiltrating lymphocytes' (TIL). TIL are T cells that have infiltrated tumour tissue.

The isolated T cells in the method according to the invention may be TIL.

30 Isolation of biopsies and samples from tumours is common practice in the art and may be performed according to any suitable method and such methods will be known to one skilled in the art.

The tumour may be a solid tumour or a non-solid tumour.

Immune cells (e.g. T cells) may be isolated from a tumour sample using methods which
35 are well known in the art. For example, TIL may be isolated by culturing resected tumour fragments or tumour single-cell suspensions in medium containing IL-2. T cells may be purified

from single cell suspensions generated from samples on the basis of expression of CD3, CD4 or CD8. T cells may be enriched from samples by passage through a density gradient.

Gamma delta T cells

5 Gamma delta T cells ($\gamma\delta$ T cells) are typically CD8⁺ and CD4⁺ and express TCR chains encoded by the gamma and delta gene loci. $\gamma\delta$ T cells may also express Fc γ RIII/CD16 and Toll-like receptors. $\gamma\delta$ T cells do not typically require antigen presentation by MHC I or MHC II for activation, but instead are believed to be involved in the recognition of lipid antigens.

$\gamma\delta$ T cells have been shown to have roles in both innate and adaptive immune
10 responses. $\gamma\delta$ T cells display broad functional plasticity following recognition of infected/transformed cells by production of cytokines (IFN- γ , TNF- α , IL-17) and chemokines (RANTES, IP-10, lymphotactin), cytotoxicity of infected or transformed target cells (perforin, granzymes, TRAIL), and interaction with other cells including epithelial cells, monocytes, dendritic cells, neutrophils, and B cells.

15

NK T cells (NKT cells)

 NKT cells comprise a unique subset of CD1d-restricted T cells with characteristics of both NK- and T cells that can be subdivided into functional subsets.

 NK T cells recognise lipids and glycolipids presented by CD1d, rather than peptides
20 presented by class I or II MHCs. As such, NKT cells are important in recognizing glycolipid antigens. Upon activation, NK T cells are able to produce large quantities of IFN- γ , IL-4, and GM-CSF, as well as multiple other cytokines and chemokines (such as IL-2, IL-13, IL-17, IL-21, and TNF- α).

 iNKT cells are able to respond rapidly to danger signals and pro-inflammatory
25 cytokines, and are able to act via a range of effector functions, such as NK transactivation, T cell activation and differentiation, B cell activation, dendritic cell activation and cross-presentation activity, and macrophage activation.

 NK T cells are most commonly found in the liver, but are also found in the thymus, spleen, peripheral blood, bone marrow and adipose tissue.

30 Type I NKT cells (also known as invariant NKT cells or iNKT cells) express an invariant TCR alpha chain and one of a small number of TCR beta chains. In humans, the highly conserved TCR expressed by iNKT cells is Va24-Ja18 paired with Vb11.

 Type II NKT cells express a wider range of TCR alpha chains.

35 Regulatory T cells (Treg cells)

 The terms "regulatory T lymphocyte" or "Treg cell" or "Treg," as used interchangeably herein and are intended to have its standard definition as used in the art. Treg cells are a

specialised subpopulation of T cells that act in a "regulatory" way to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens.

Tregs have sometimes been referred to suppressor T-cells. Treg cells are characterized by expression of the forkhead family transcription factor Foxp3 (forkhead box p3). They may also express CD4 or CD8 surface proteins. Alternatively or in addition, Treg cell may express CD25. Thus, Treg cells may be Foxp3⁺CD25⁺, Foxp3⁺CD25⁻, or Foxp3⁻CD25⁺. The Treg may be CD4⁺CD25⁺FOXP3⁺, CD4⁺CD25⁺CD127⁻, or CD4⁺CD25⁺FOXP3⁺CD127^{low}. Naturally-occurring Treg cells (CD4⁺CD25⁺Foxp3⁺) arise like all other T cells in the thymus. In contrast, adaptive Treg cells (also known as Tr1 cells or Th3 cells) may originate during a normal immune response. Antigen-specific activation of human effector T-cells leads to inducible expression of Foxp3 in a subgroup of the activated effector cells, and this subgroup can develop a regulatory (Treg) phenotype. One way to induce Tregs is by prolonged exposure of T effector cells to TGF- β . T-cells may also be converted to Treg cells by transfection or transduction of the Foxp3 gene into a mixed population of T-cells. A T-cell that is caused to express Foxp3 adopts the Treg phenotype and such recombinant Tregs are also defined herein as "Tregs".

Natural Killer cells

In some embodiments, the immune cells may be Natural Killer (NK) cells.

NK cells exhibit the highest level of cytotoxic activity within the immune system. NK cells are similar to B cells and T cells, but lack specific cell surface antigen receptors. Instead, NK cells have activatory and inhibitory receptors that recognise motifs.

NK cells circulate in the blood and the peripheral lymphoid organs such as lymph nodes and spleen. They can become activated by cytokines or upon encountering target cells. The recognition and elimination of target cells is based on balancing between inhibitory and activatory signals. Activatory signals are generated by activatory receptors (NKG2D, NKp46, NKp30) binding to ligands, which can be present not only on cancerous, pathogen-infected and damaged cells, but also on healthy cells. On the other hand, inhibitory signals are generated when inhibitory receptors (KIR, CD94/NKG2A) on NK cells bind to Major Histocompatibility Complex (MHC) Class I molecules that are normally present on all healthy cells. MHC Class I molecules on target cells are absent or greatly downregulated, making them ideal NK cell targets. This allowed NK cells to distinguish between target and healthy cells. In order for NK cells to recognise and kill target cells, overall activatory signals must be greater than inhibitory signals.

NK cells recognise and kill cancerous, pathogen-infected and damaged cells without prior sensitisation, making them part of the innate immune response. For example, NK cells

provide an early response to virus infection, occurring prior to T cell killing of infected cells. NK cells can kill target cells within minutes. NK cells also secrete cytokines and “weaponise” other parts of the immune system. For example, NK cells promote T cell effector function and enhance antibody-directed cellular cytotoxicity (ADCC).

5 An NK cell may be defined in terms of its marker expression, its function/activity, or a combination thereof. Such definitions are standard in the art and methods are known by which marker expression and/or NK cell activity may be assessed. Thus, one of skill in the art would readily be able to categorise a cell as an NK cell using standard methodology and definitions.

For example, NK cells may be CD56⁺, CD45⁺, and/or CD16⁺. NK cells of the invention
10 may be CD3⁻ and/or CD19⁻.

Unlike other immune cells as described herein, NK cells do not require co-culture with APCs for activation. However, NK cells for cell therapy products are typically produced in a co-culture system. Rather than co-culture with APCs, NK cells may be co-cultured with supporting cells.

15 As used herein, the term “supporting cells” refers to a cell which supports the expansion and/or activation of an immune cell, such as an NK cell. A supporting cell may preferably be a stromal cell (also referred to as a mesenchymal stromal cell or MSC). Stromal cells are connective tissue cells and may be from any organ. Non-limiting examples of stromal cells which may be used as supporting cells in the present invention are MSC, fibroblasts or
20 pericytes, with MSC, fibroblasts and/or fibroblast-like cells being preferred. Examples of commercially available cell lines which can be used as supporting cells include M2-10B4 (a fibroblast-like cell line from ATCC), OP9 cells (an MSC cell line) and EL08, particularly EL08.1D2.

For the avoidance of doubt, any and all disclosure herein in relation to methods of the
25 invention involving co-culture of immune cells with APCs applies equally and without reservation to methods of producing NK cells involving the co-culture of NK cells with supporting cells. Thus, the invention provides a method for producing NK cells or a population thereof, wherein said method comprises co-culture of isolated NK cells with supporting cells in a dynamic suspension. By way of non-limiting example, the invention provides a method for
30 producing NK cells or a population thereof, wherein said method comprises co-culture of isolated NK cells with stromal cells (e.g. EL08 cells) in a dynamic suspension. Typically the dynamic suspension results from agitation of the co-culture, as described herein.

Antigen Presenting Cells

35 An antigen-presenting cell (APC) or accessory cell is a cell that displays antigen complexed with MHCs on their surfaces; this process is known as antigen presentation. T cells may recognise these complexes using their T cell receptors (TCRs).

In one aspect, the APCs have been loaded with antigen. Loading of antigen may be achieved by methods known in the art. For example, antigen may be loaded by pulsing the antigen presenting cells (APCs) with peptide or by genetic modification. In the context of the present invention, the term “antigen” refers to one or more antigens.

5 Methods for loading APCs with antigens by pulsing the APCs are known in the art. For example, a protocol for loading APCs by pulsing with peptides comprising an identified mutation is described in Leko *et al.* (J Immunol. 2019, 202: 3458-3467).

 Typically the APCs are loaded with antigen and co-culture of said loaded APCs with isolated immune cells results in antigen-specific expansion of the immune cells. As used
10 herein “antigen-specific expansion” refers to increasing the number or proportion of immune cells (e.g. T cells) specific to a particular antigen within a population of immune cells (e.g. T cells). Optionally, antigen-specific expansion may also be preceded by a non-specific T cell pre-expansion step as described herein.

 APCs may be selected from dendritic cells (DCs), B cells (also referred to as B
15 lymphocytes) and/or macrophages.

 Preferably the APC is a DC. DCs may be derived from monocytes isolated from blood to produce monocyte-derived dendritic cells (MoDCs). DCs may be produced from a blood sample obtained from the same individual as is used to obtain the immune cells i.e., autologous DCs. Preferably the DCs are autologous MoDCs. Standard methods in the art
20 may be used to produce dendritic cells from isolated monocytes. For example, a protocol for obtaining PBMC-derived DCs is described in Leko *et al.* (J. Immunol. 2019, 202: 3458-3467). Further, DC purification/isolation kits are commercially available, such as e.g. EasySep™ DC enrichment kits from StemCell™ Technologies. In addition, CD14 Microbeads and associated protocols are available from Miltenyi Biotec (available at [https://www.miltenyibiotec.com/GB-](https://www.miltenyibiotec.com/GB-en/products/cd14-microbeads-human.html#130-050-201)
25 [en/products/cd14-microbeads-human.html#130-050-201](https://www.miltenyibiotec.com/GB-en/products/cd14-microbeads-human.html#130-050-201)).

 The APC may be a B cell. The B cell may be expanded from blood, for example a blood sample obtained from the same individual as is used to produce the immune cells i.e., are autologous B cells.

 B cells may be expanded from CD19⁺ cells isolated from a blood sample. Any suitable
30 method may be used to isolate CD19⁺, such as positive or negative selection using immunomagnetic particles coated with anti-CD19 antibodies. CD19 purification/isolation reagents and kits are commercially available, such as e.g. CD19 MicroBeads or B Cell Isolation Kit II, human (Miltenyi Biotec) and EasySep™ Human CD19 Positive Selection Kit (StemCell™ Technologies). Another approach is to use positive selection for CD20 or CD22,
35 for example using CD20 or CD22 MicroBeads 20 (Miltenyi Biotec).

 Standard methods known in the art may be used to produce B cells from isolated CD19⁺ monocytes or directly from blood samples or PBMCs. For example, a protocol for B

cell expansion is described in Kotsiou et al. (Blood 2016, 128:72-81) using CD40L, F(ab')₂ fragment goat anti-IgA + IgG + IgM, CpG and IL-4. Another typical method is culture with CD40L expressing feeder cells as taught by Su et al (J Immunol 2016, 197:4163-4176). B cell expansion kits are commercially available, such as e.g. ImmunoCult™ Human B Cell Expansion Kit from StemCell™ Technologies and B Cell Expansion Kit, Human from Miltenyi Biotec.

Isolated CD19⁺ cells may be cultured with IL-4, CD40L and CpG to expand B cells. Exemplary methods for B cell expansion, and in particular further concentrations of IL-4, CD40L and CpG, are further described in WO 2022/269250, which is herein incorporated by reference in its entirety.

The B cell expansion medium may comprise IL-4 at a concentration of about 10 to 100 ng/ml, for example about 25 to 75 ng/ml. In some preferred embodiments, the B cell expansion medium comprises about 50 ng/ml of IL-4.

Alternatively or in addition, the B cell expansion medium may comprise CD40L at a concentration of about 0.5 to about 50 IU/mL. In some preferred embodiments, the CD40L is present at a concentration of about 12 IU/mL.

Alternatively or in addition, the B cell expansion medium may comprise CpG at a concentration of about 0.1 to about 10 µg/mL. In some preferred embodiments, the CD40L is present at a concentration of about 4.6 µg/ml.

The APC may be a macrophage. Macrophages may be derived from monocytes isolated from blood, which can then be differentiated to produce monocyte-derived macrophages (MoMΦs). Macrophages may be produced from a blood sample obtained from the same individual as is used to produce the immune cells i.e. autologous macrophages. Preferably the macrophages are autologous MoMΦs. Standard methods in the art may be used to produce macrophages from isolated monocytes. For example, a protocol for obtaining PBMC-derived macrophages is described in Rios et al. (Hypertension. Methods in Molecular Biology, vol 1527. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-6625-7_24). Further, macrophage purification/isolation kits are commercially available, such as e.g. EasySep™ DC enrichment kits from StemCell™ Technologies.

The APCs may be used at a ratio of from about 2: 1 to about 1: 100, such as about 1:1, 1:2, 1:3, 1:4, 1:5, 1:10, 1:20, 1:50 or 1:75 APCs to immune cells (APC:immune cell). Preferably the ratio of APC:immune cell is between about 1:5 to about 1:20, particularly about 1:10. Thus, the ratio of immune cells:APCs is preferably between about 5:1 to about 20:1, particularly about 10:1.

The APCs may be loaded as described herein with antigens in the form of peptides containing one or more identified mutations as single stimulants or as pools of stimulating peptides, such as e.g. peptides comprising mutations identified as neoantigens. For example,

Leko et al. describes a protocol comprising loading APCs with antigens by incubating the APCs with pools of up to 12 individual peptides each comprising an identified point mutation flanked on both sides by 12 wild type amino acids.

Immature APCs, e.g. immature DCs may be loaded with peptide and then matured.

5 Alternatively or in addition, mature APCs, e.g. mature DCs may be loaded with peptide. Thus, APCs, e.g. DCs, may be loaded with peptide twice, both when immature and mature.

Alternatively, methods for loading APCs with antigens by modifying the APCs to express the antigen are known in the art. For example, the APCs may be modified to express an antigen sequence by transfecting the APCs with mRNA encoding the antigen sequence.

10 The mRNA encoding the antigen sequence may be in the form of a minigene or tandem minigene. The APCs may be transfected with mRNA encoding peptides comprising identified mutations as constructs or as constructs encoding for multiple such peptides. For example, Leko et al. describes a protocol comprising loading APCs with antigens by electroporating the APCs with tandem minigene RNA comprising up to 12 minigenes, each comprising the coding
15 sequence for a mutated amino acid flanked bilaterally by a sequence encoding 12 wild type amino acids.

The APC may be a cell capable of presenting the relevant peptide, for example in the correct HLA context. Such a cell may be an autologous cell expressing an autologous HLA molecule, or a non-autologous cell expressing an array of matched HLAs. An artificial APC
20 may be irradiated.

The term "peptide" is used in the normal sense to mean a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the amino and carboxyl groups of adjacent amino acids. The term includes modified peptides and synthetic peptide analogues.

25 The peptide may be made using chemical methods (Peptide Chemistry, A practical 35 Textbook. Mikos Bodansky, Springer-Verlag, Berlin.). For example, peptides can be synthesized by solid phase techniques (Roberge JY et al (1995) Science 269: 202-204), cleaved from the resin, and purified by preparative high performance liquid chromatography (e.g., Creighton (1983) Proteins Structures And Molecular Principles, WH Freeman and Co,
30 New York NY). Automated synthesis may be achieved, for example, using the ABI 431A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The peptide may alternatively be made by recombinant means, or by cleavage from the polypeptide which is or comprises the antigen. The composition of a peptide may be
35 confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure).

As is well known in the art, antigens are presented to T cells in the context of antigen-derived peptides bound by major histocompatibility molecules (MHC). Methods of predicting

whether a peptide is likely to bind to a particular MHC molecule, and hence function as an antigen for presenting to T cells, are known in the art. For example, as explained below, MHC binding of peptides may be predicted using the netMHC (Lundegaard et al.) and netMHCpan (Jurtz et al.) algorithms. Thus, APCs may be loaded with peptides that are predicted using any
5 such method as likely to be presented by one or more MHC molecules of relevance. Instead or in addition to this, APCs may be loaded with antigen using a plurality of candidate peptides each comprising a mutation of interest and differing from each other by the location of the mutation of interest in the peptide.

Equivalent prediction means can be used to identify non-peptide antigens (e.g. carbohydrate or nucleic acid) that may be presented by APCs to immune cells which recognise such non-peptide antigens.

MHC class I proteins form a functional receptor on most nucleated cells of the body. There are 3 major MHC class I genes in HLA: HLA-A, HLA-B, HLA-C and three minor genes HLA-E, HLA-F and HLA-G. β 2-microglobulin binds with major and minor gene subunits to
15 produce a heterodimer.

Peptides that bind to MHC class I molecules are typically 7 to 13, more usually 8 to 11 amino acids in length. The binding of the peptide is stabilised at its two ends by contacts between atoms in the main chain of the peptide and invariant sites in the peptide-binding groove of all MHC class I molecules. There are invariant sites at both ends of the groove which
20 bind the amino and carboxy termini of the peptide. Variations in peptide length are accommodated by a kinking in the peptide backbone, often at proline or glycine residues that allow the required flexibility.

There are 3 major and 2 minor MHC class II proteins encoded by the HLA locus. The genes of the class II combine to form heterodimeric ($\alpha\beta$) protein receptors that are typically
25 expressed on the surface of APCs.

Peptides which bind to MHC class II molecules are typically between 8 and 20 amino acids in length, more usually between 10 and 17 amino acids in length and can be longer (for example up to 40 amino acids). These peptides lie in an extended conformation along the MHC II peptide-binding groove which (unlike the MHC class I peptide-binding groove) is open
30 at both ends. The peptide is held in place mainly by main-chain atom contacts with conserved residues that line the peptide-binding groove.

The peptide may comprise a mutation (e.g. a non-silent amino acid substitution encoded by a SNV) at any residue position within the peptide. By way of example, a peptide which is capable of binding to an MHC class I molecule is typically 7 to 13 amino acids in
35 length. As such, the amino acid substitution may be present at position 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 in a peptide comprising thirteen amino acids.

In one aspect, longer peptides, for example peptides that are 27, 28, 29, 30 or 31 amino acids long, may be used to stimulate both CD4⁺ and CD8⁺ cells. The mutation may be at any position in the peptide. In one aspect, the mutation is at or near the centre of the peptide, e.g. at position 12, 13, 14, 15 or 16.

5 Any suitable number of (peptide or non-peptide) antigens may be presented via APCs in a method of the invention, for example from 10 to 300 antigens, such as 25 to 250, 50 to 200, 70 to 185, or 100 to 150 antigens, such as about 10, 20, 50, 75, 100, 125, 150, 175, 200 or 250 antigens. Thus, any suitable number of (peptide or non-peptide) antigens may be used to drive antigen-specific expansion of immune cells in a method of the invention, for example
10 from 10 to 300 antigens, such as 25 to 250, 50 to 200, 70 to 185, or 100 to 150 antigens, such as about 10, 20, 50, 75, 100, 125, 150, 175, 200 or 250 antigens.

Antigens

In some embodiments, the isolated immune cell population may be co-cultured with
15 APCs loaded with an antigen which may be a tumour antigen. More preferably, the antigen may be a tumour specific antigen such as a neoantigen, more preferably a clonal neoantigen. Thus, the immune cells described here (e.g. T cells) may target tumour antigens. More preferably, the immune cells (e.g. T cells) described herein may target neoantigens, more preferably clonal neoantigens.

20 The tumour antigen which may be targeted by an immune cell (e.g. T cell) produced according to the present invention is not particularly limited.

Tumour antigens are well-known in the art and include the following: CEA, immature laminin receptor, TAG-72, HPV E6 and E7, BING-4, calcium-activated chloride channel 2, cyclin-B1, 9D7, Ep-CAM, 15 EphA3, Her2/neu, telomerase, mesothelin, SAP-1, survivin,
25 BAGE family, CAGE family, GAGE family, MAGE family, SAGE family, XAGE family, NY-ESO-1/LAGE-1, PRAME, SSX-2, Melan-A/MART-1, gp100/pmel17, tyrosinase, TRP-1/-2, P.polypeptide, MC1 R, prostate-specific antigen, beta-catenin, BRCA 1/2, CDK4, CML66, fibronectin, MART-2, p53, ras, TGF-betaRII and MUC1. Other examples of tumour antigens are described in WO 2022/269250, which is herein incorporated by reference in its entirety.

30 The immune cells (e.g. T cells) may target neoantigens. A "neoantigen" is a tumour-specific antigen which arises as a consequence of a mutation within a cancer cell. Thus, a neoantigen is not expressed (or expressed at a significantly lower level) by healthy (i.e. non-tumour) cells in a subject. A neoantigen may be processed to generate distinct peptides which can be recognised by immune cells (e.g. T cells) when presented in the context of MHC
35 molecules. As described herein, neoantigens may be used as the basis for cancer immunotherapies. References herein to "neoantigens" are intended to include also peptides

derived from neoantigens. The term "neoantigen" as used herein is intended to encompass any part of a neoantigen that is immunogenic.

The binding of a neoantigen to a particular MHC molecule (encoded by a particular HLA allele) may be predicted using methods which are known in the art. Examples of methods
5 for predicting MHC binding include those described by Lundegaard et al., O'Donnel et al., and Bullik-Sullivan et al. For example, MHC binding of neoantigens may be predicted using the netMHC (Lundegaard et al.) and netMHCpan (Jurtz et al.) algorithms. Binding of a neoantigen to a particular MHC molecule is a prerequisite for the neoantigen to be presented by said MHC molecule on the cell surface. Further discussion of neoantigens, and particularly the types of
10 mutations which can cause neoantigens are described in WO 2022/269250, which is herein incorporated by reference in its entirety.

Preferably, the immune cells (e.g. T cells) may target clonal neoantigens. A "clonal neoantigen" (also sometimes referred to as a "truncal neoantigen") is a neoantigen arising from a clonal mutation. A "clonal mutation" (sometimes referred to as a "truncal mutation") is
15 a mutation that is present in essentially every tumour cell in one or more samples from a subject (or that can be assumed to be present in essentially every tumour cell from which the tumour genetic material in the sample(s) is derived). Thus, a clonal mutation may be a mutation that is present in every tumour cell in one or more samples from a subject. For example, a clonal mutation may be a mutation which occurs early in tumorigenesis.

20 A "subclonal neoantigen" (also sometimes referred to as a "branched neoantigen") is a neoantigen arising from a subclonal mutation. A "subclonal mutation" (also sometimes referred to as a "branch mutation") is a mutation that is present in a subset or a proportion of cells in one or more tumour samples from a subject (or that can be assumed to be present in a subset of the tumour cells from which the tumour genetic material in the sample(s) is
25 derived). For example, a subclonal mutation may be the result of a mutation occurring in a particular tumour cell later in tumorigenesis, which is found only in cells descended from that cell.

The wording "essentially every tumour cell" in relation to one or more samples of a subject may refer to at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at
30 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 tumour cells in the one or more samples or the subject.

As such, a clonal neoantigen is a neoantigen which is expressed effectively throughout a tumour. A subclonal neoantigen is a neoantigen that is expressed in a subset or a proportion of cells or regions in a tumour. 'Expressed effectively throughout a tumour' may mean that the
35 clonal neoantigen is expressed in all regions of the tumour from which samples are analysed.

It will be appreciated that a determination that a mutation is 'encoded (or expressed) within essentially every tumour cell' refers to a statistical calculation and is therefore subject to statistical analysis and thresholds.

Likewise, a determination that a clonal neoantigen is 'expressed effectively throughout a tumour' refers to a statistical calculation and is therefore subject to statistical analysis and thresholds.

Various methods for determining whether a neoantigen is "clonal" are known in the art. Any suitable method may be used to identify a clonal neoantigen, for example as described in Landau et al. (Cell. 2013 Feb 14;152(4):714-26); MacGranahan et al. (Science 2016 March 25;351(6280):1463-1469); or Roth et al. (Nat Methods. 2014 April; 11(4): 396-398).

Further discussion of clonal neoantigens, and methods for determining whether a neoantigen is a clonal neoantigen are described in WO 2022/269250, which is herein incorporated by reference in its entirety.

Alternatively, the APCs may present a non-tumour specific antigen. Non-tumour specific antigens may include autoantigens important in autoimmune disease. Non-tumour specific antigens may also include donor derived antigens important in the context of transplantation, for example in relation to graft rejection, graft vs host disease (GVHD).

Dynamic suspension

The prevailing teaching in the art is that co-culture of immune cells with APCs must be under static conditions (and typically in adherent culture), to facilitate stable interactions between the APCs and immune cells in order for stimulation of the immune cells by the APCs to occur.

As exemplified herein, the present inventors have surprisingly shown that populations of immune cells particularly T cells, can be produced efficiently by co-culturing isolated immune cells (particularly T cells) with APCs in a dynamic suspension.

Accordingly, the invention relates to methods for culturing immune cells, and particularly for producing populations of immune cells, wherein isolated immune cells are co-cultured with APCs, wherein said co-culture occurs in a dynamic suspension. The immune cells may be any as described herein.

Suspension culture refers to a type of cell culture in which cells are suspended within a culture medium. Dynamic suspension culture refers to suspension culture wherein the cells are in motion whilst suspended in a culture medium. A dynamic suspension is one in which the static phase is reduced or eliminated, i.e. in which the number or proportion of immune cells and/or APC which have settled out of suspension is reduced or eliminated.

Typically in a dynamic suspension, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or more, up to 100% of the immune

cells and/or APCs are in suspension. In other words, less than 20%, less than 15%, less than 10%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1% or less, down to 0% of the immune cells and/or APCs have settled out of the suspension culture.

As discussed in more detail below, the volumetric power input may be set such that
5 the % of immune cells and/or APCs remaining in suspension is above a desired threshold or %.

It is within the routine practice of one of ordinary skill in the art to determine suitable volumetric power inputs to achieve a desired dynamic suspension for a given co-culture of immune cells and APCs.

Typically a method of the invention does not comprise an initial static co-culture phase
10 prior to the co-culture of the immune cells and APCs in a dynamic suspension. Thus, the co-culture in a dynamic suspension may take place from day 0, such as from seeding the bioreactor with the immune cells and/or APCs. Beginning the dynamic suspension from day 0, and particularly from seeding of the bioreactor eliminates an initial static co-culture phase.

The dynamic suspension may result from agitation (continuous or intermittent) or by fluid flow,
15 as described herein.

Agitation

Typically the dynamic suspension results from agitation of the co-culture. Said
agitation may be achieved by any appropriate means. By way of non-limiting example,
20 agitation may be mechanical agitation; rocking motion agitation; vertical wheel agitation; and/or pneumatic agitation. Suitable bioreactors capable of generating such different types of agitation are well-known in the art, with commercially available bioreactors available.

As described herein, conventional methods for the production of a population of
immune cells rely on co-culture of immune cells with APCs under static conditions. Such
25 conventional methods may comprise an initial stirring or mixing step to distribute the immune cells and APCs at the start of the co-culture, to facilitate contact between the immune cells and the APCs. However, such initial stirring or mixing steps are not agitation according to the present invention, and do not result in a dynamic suspension. For example, such initial mixing or stirring steps typically occur only once at the start of the co-culture, and typically for a short
30 period of time (e.g. about 5 minutes or less, about 4 minutes or less, about 3 minutes or less, about 2 minutes or less, about 1 minute or less, about 30 seconds or less). Alternatively or in addition, the volumetric power input of such stirring or mixing is typically not particularly limited, provided that that the immune cells and APCs are mixed by the step. For example, the cell suspension may be mixed manually using a Stripette®. A method of the invention may include
35 a mixing step, e.g. at the start of the co-culture and/or following reseeding of the immune cells as described herein. Alternatively, a method of the invention may not comprise a mixing step,

but rather rely on the dynamic suspension to distribute the immune cells and APCs within the bioreactor used for the method.

Agitation according to the invention may be continuous and/or intermittent.

By continuous agitation, it is meant that the agitation is uninterrupted. Agitation may
5 be defined as continuous for a defined period of time, such as for at least about 1 day, at least about 2 days, at least about 3 days, at least about 4 days, at least about 5 days, at least about 6 days, at least about 7 days, at least about 8 days, at least about 9 days, at least about 10 days, at least about 11 days, at least about 12 days, at least about 13 days, at least about 14 days, at least about 15 days, at least about 16 days, at least about 17 days, at least about 18
10 days, at least about 19 days, or at least about 20 days, up to the total length of the method (or co-culture step thereof).

The agitation may be continuous for at least about 1 day, at least about 2 days, at least about 3 days, at least about 4 days, at least about 5 days, at least about 6 days, at least about 7 days, at least about 8 days, at least about 9 days, at least about 10 days, at least about 11
15 days, at least about 12 days, at least about 13 days, at least about 14 days, at least about 15 days, at least about 16 days, at least about 17 days, at least about 18 days, at least about 19 days, or at least about 20 days, up to the total length of the method (or co-culture step thereof).

By intermittent agitation, it is meant that the agitation occurs at intervals. Said intervals may be regular or irregular, preferably regular. By regular intervals, it is meant that all intervals
20 within a method or method step are the same length. By irregular intervals it is meant that at least one interval within a method or method step is of a different length to the other intervals.

An interval will comprise a duration of agitation and a duration where agitation is absent. The intervals (comprising agitation and non-agitation) may be of any appropriate duration. For example, an interval may be for about 30 minutes, about 1 hour, about 90
25 minutes, about 2 hours, about 3 hours, about 4 hours or more. By way of non-limiting example, regular intervals may be about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours or more.

In a given interval, the duration of the agitation may be greater than the duration without agitation. By way of non-limiting example, agitation may be for 45 minutes and then culture
30 without agitation for 15 minutes. In a given interval, the duration of the agitation may be equal to the duration without agitation. By way of non-limiting example, agitation may be for 30 minutes and then culture without agitation for 30 minutes. Typically in a given interval, the duration of the agitation may be less than the duration without agitation. By way of non-limiting example, agitation may be for 10 minutes and then culture without agitation for 45 minutes.

35 The duration of the agitation and non-agitation within interval may be constant, or may vary between intervals. Where intervals are regular, having constant durations of agitation results in the durations of non-agitation being of different lengths. Typically the duration of

agitation and non-agitation within intervals is constant. Preferably the intervals are regular and the duration of agitation and non-agitation within intervals is constant.

Typically, where an agitation is intermittent, the intervals are regular and between about 1 hour to about 5 hours, such as between about 1 hour to about 4 hours, preferably between
5 about 1 hour to about 3 hours, more preferably between about 1 hour to about 2 hours in length.

Intermittent agitation may comprise intervals with durations of agitation of between about 5 minutes to about 60 minutes, such as between about 5 minutes to about 45 minutes, preferably between about 5 minutes to about 30 minutes, more preferably between about 5
10 minutes to about 15 minutes.

Intermittent agitation may comprise agitation for between about 5 minutes to about 60 minutes, as between about 5 minutes to about 45 minutes, preferably between about 5 minutes to about 30 minutes, more preferably between about 5 minutes to about 15 minutes, every 1 to 5 hours.

15 Intermittent agitation may comprise agitation for between about 5 minutes to about 60 minutes, as between about 5 minutes to about 45 minutes, preferably between about 5 minutes to about 30 minutes, more preferably between about 5 minutes to about 15 minutes, every 1 to 4 hours.

Intermittent agitation may comprise agitation for between about 5 minutes to about 60
20 minutes, as between about 5 minutes to about 45 minutes, preferably between about 5 minutes to about 30 minutes, more preferably between about 5 minutes to about 15 minutes, every 1 to 3 hours.

Intermittent agitation may comprise agitation for between about 5 minutes to about 60 minutes, as between about 5 minutes to about 45 minutes, preferably between about 5
25 minutes to about 30 minutes, more preferably between about 5 minutes to about 15 minutes, every 1 to 2 hours.

Intermittent agitation may comprise agitation for between about 5 minutes to about 30 minutes every 1 to 5 hours.

Intermittent agitation may comprise agitation for between about 5 minutes to about 30
30 minutes every 1 to 4 hours.

Intermittent agitation may comprise agitation for between about 5 minutes to about 30 minutes every 1 to 3 hours.

Intermittent agitation may comprise agitation for between about 5 minutes to about 30 minutes every 1 to 2 hours.

35 Intermittent agitation may comprise agitation for between about 5 minutes to about 15 minutes every 1 to 5 hours.

Intermittent agitation may comprise agitation for between about 5 minutes to about 15 minutes every 1 to 4 hours.

Intermittent agitation may comprise agitation for between about 5 minutes to about 15 minutes every 1 to 3 hours.

5 Intermittent agitation may comprise agitation for between about 5 minutes to about 15 minutes every 1 to 2 hours.

Typically intermittent agitation according to the present invention comprises agitation for between about 5 minutes to about 30 minutes every 1 to 3 hours. Preferably intermittent agitation is for between about 5 minutes to about 15 minutes (such as between about 5
10 minutes to about 10 minutes, about 5 minutes, about 6 minutes, about 7 minutes, about 8 minutes, about 9 minutes, about 10 minutes, about 11 minutes, about 12 minutes, about 13 minutes, about 14 minutes or about 15 minutes) (i) every hour; or (ii) every 3 hours. More preferably intermittent agitation is for about 10 minutes (i) every hour; or (ii) every 3 hours.

Alternatively or in addition to the volumetric power input being set to ensure that the %
15 of immune cells and/or APCs remaining in suspension is above a desired threshold or % (as described herein), the intermittent agitation according to the invention may also be set so as to ensure that the % of immune cells and/or APCs remaining in suspension is above a desired threshold or %. It is within the routine practice of one of ordinary skill in the art to determine suitable intervals of intermittent agitation to achieve a desired dynamic suspension for a given
20 co-culture of immune cells and APCs.

Agitation according to the invention may comprises a first period of continuous agitation and a second period of intermittent agitation. Preferably, agitation according to the invention may comprises a first period of intermittent agitation and a second period of continuous agitation.

25 A first period of intermittent or continuous agitation (preferably intermittent agitation) may be between about 0 days to about 3 days, such as between about 0 days to about 2 days, preferably between about 1 day to about 2 days.

A second period of intermittent or continuous agitation (preferably continuous agitation) may be between about 0 days to about 21 days, such as between about 1 days to about 21
30 days, between about 2 days to about 21 days, between about 2 days to about 14 days, preferably between about 2 day to about 10 days (e.g. about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days or about 10 days).

In some preferred embodiments, agitation according to the invention may comprise (a) a first period of intermittent or continuous agitation (preferably intermittent agitation) of
35 between about 0 days to about 3 days, such as between about 0 days to about 2 days, preferably between about 1 day to about 2 days; and (b) a second period of intermittent or continuous agitation (preferably continuous agitation) of between about 0 days to about 21

days, such as between about 1 days to about 21 days, between about 2 days to about 21 days, between about 2 days to about 14 days, preferably between about 2 days to about 10 days (e.g. about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days or about 10 days).

5 In some preferred embodiments, a method of the invention may comprise co-culture of immune cells and APCs for: (a) a first period of intermittent or continuous agitation (preferably intermittent agitation) of between about 0 days to about 3 days, such as between about 0 days to about 2 days, preferably between about 1 day to about 2 days; and (b) a
10 second period of intermittent or continuous agitation (preferably continuous agitation) of between about 0 days to about 21 days, such as between about 1 days to about 21 days, between about 2 days to about 21 days, between about 2 days to about 14 days, preferably between about 2 days to about 10 days (e.g. about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days or about 10 days).

15 In some particularly preferred embodiments, a method of the invention comprises a first period of co-culture which is subjected to intermittent agitation followed by a second period of co-culture which is subjected to constant agitation. Said first period is optionally between about 0 to about 2 days, preferably between about 1 to about 2 days; and/or said second period is optionally between about 2 to about 21 days, preferably between about 2 to about 10 days.

20 As described herein, typically a method of the invention does not comprise an initial static co-culture phase prior to the co-culture of the immune cells and APCs in a dynamic suspension. This dynamic suspension may be achieved by continuous agitation. Thus, the continuous agitation of the co-culture may take place from day 0, such as from seeding the bioreactor with the immune cells and/or APCs. Beginning continuous agitation from day 0,
25 and particularly from seeding of the bioreactor eliminates an initial static co-culture phase.

Alternatively or in addition as described herein, the dynamic suspension may be achieved by intermittent agitation. Thus, the intermittent agitation of the co-culture may take place from day 0, such as from seeding the bioreactor with the immune cells and/or APCs. Beginning intermittent agitation from day 0, and particularly from seeding of the bioreactor
30 eliminates an initial static co-culture phase. For the avoidance of doubt, intermittent agitation does not comprise a static phase.

Volumetric Power Input

Volumetric power input (VPI) is the power required to maintain the motion of the culture
35 medium and cells in a culture vessel (e.g. bioreactor). In the art, VPI is commonly referred to as Power/Volume, i.e. P/V and the two terms (VPI and P/V) can be used interchangeably.

Volumetric power input is a fundamental engineering principle and as such forms part of the common general knowledge in the bioprocessing space. VPI can be calculated using the power input (PI) and volume of a given reactor.

PI can itself be calculated from the density of the culture medium, the impeller diameter, and the power number of the impeller. Power number can be obtained by using the power number curves that correspond to the impeller type, or calculated using known correlations. Many handbooks and textbooks are available which describe how power numbers, PI and VPI may be calculated. By way of non-limiting example, Bioprocess engineering principles by Pauline M. Doran (2nd ed., Academic Press, Elsevier, Amsterdam), which is herein incorporated by reference in its entirety, particularly Chapter 7 which describes fluid flow and mixing, and provides calculations and power number curves for various impeller types. Therefore, it is within the routine practice of one of ordinary skill in the art to calculate such values.

The VPI may be set such that the % of immune cells and/or APCs remaining in suspension is above a desired threshold or %. It is within the routine practice of one of ordinary skill in the art to determine suitable VPIs to achieve a desired dynamic suspension for a given co-culture of immune cells and APCs.

Numerous factors affect VPI, such as the density of the culture medium, the impeller diameter, the power number of the impeller and the volume of the bioreactor.

It is within the routine practice of one of ordinary skill in the art to determine the necessary VPI to achieve a dynamic suspension according to the present invention. Standard formula and equations are known in the art for calculating VPI. Non-limiting examples include those described in Bioprocess engineering principles by Pauline M. Doran, which is herein incorporated by reference in its entirety.

Wherein the agitation is mechanical agitation, the speed of rotation of the impeller may be changed to achieve the desired VPI.

For mechanical agitation, an impeller may be set to rotate at a low RPM. As used herein, the term "low RPM" means an RPM of between about 20 RPM to about 80 RPM (e.g. about 20 RPM, about 25 RPM, about 30 RPM, about 35 RPM, about 40 RPM, about 45 RPM, about 50 RPM, about 55 RPM, about 60 RPM, about 65 RPM, about 70 RPM, about 75 RPM or about 80 RPM), such as between about 20 RPM to about 75 RPM, between about 20 RPM to about 70 RPM, between about 20 RPM to about 65 RPM, between about 20 RPM to about 60 RPM, between about 20 RPM to about 55 RPM, between about 25 RPM to about 55 RPM, or between about 25 RPM to about 50 RPM, preferably between about 20 RPM to about 75 RPM, more preferably between about 25 RPM to about 70 RPM.

Alternatively, an impeller may be set to rotate at a high RPM. As used herein, the term "high RPM" means an RPM of between about 70 RPM to about 200 RPM (e.g. about 70 RPM,

about 75 RPM, about 80 RPM, about 85 RPM, about 90 RPM, about 95 RPM, about 100 RPM, about 105 RPM, about 110 RPM, about 115 RPM, about 120 RPM, about 125 RPM, about 130 RPM, about 135 RPM, about 140 RPM, about 145 RPM, about 150 RPM, about 155 RPM, about 160 RPM, about 165 RPM, about 170 RPM, about 175 RPM, about 180 RPM, about 185 RPM, about 190 RPM, about 195 RPM or about 200 RPM,), such as between about 70 RPM to about 175 RPM, between about 70 RPM to about 150 RPM, between about 70 RPM to about 125 RPM, between about 70 RPM to about 120 RPM, between about 70 RPM to about 110 RPM, between about 70 RPM to about 100 RPM, preferably between about 70 RPM to about 150 RPM or between about 70 RPM to about 100 RPM.

The above RPMs were calculated in the Examples in a BioBlu Single Use DASBox 0.3C at 100mL. Using routine calculations and common general knowledge, one of ordinary skill in the art would be able to determine corresponding RPM values for other bioreactors without undue burden. For example, as discussed in more detail below, RPM may be calculated based on the desired VPI value.

Agitation speed (RPM) for mechanical agitation (as in a stirred tank bioreactor) may be calculated as follows:

$$1) \text{ Volumetric Power Input } (W.m^{-3}) = \frac{\text{Power Input}}{\text{Vessel Volume } (m^3)}$$

$$2) \text{ Power Input} = \text{Volumetric Power Input } (W.m^{-3}) \times \text{Vessel Volume } (m^3)$$

$$3) N_{calc}^3 (tr.s^{-1}) = \frac{\text{Power Input } (W)}{\text{Power Number} \times \text{Fluid Density } (kg.m^{-3}) \times \text{Impeller Diameter}^5 (m^5)}$$

$$4) N_{calc} (tr.s^{-1}) = \left(\frac{\text{Power Input } (W)}{\text{Power Number} \times \text{Fluid Density } (kg.m^{-3}) \times \text{Impeller Diameter}^5 (m^5)} \right)^{0.33}$$

$$5) \text{ Agitation Speed } (N) = N_{calc} (tr.s^{-1}) \times 60$$

A low VPI may be used, typically to provide a low RPM. As used herein, the term “low VPI” means an VPI of between about 0.1 to about 1.9 (e.g. about 0.1, about 0.2, about 0.3, about 0.4, about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8 or about 1.9), such as between about 0.1 to about 0.6.

A high VPI may be used, typically to provide a high RPM. As used herein, the term “high VPI” means an VPI of between about 1.9 to about 4.6 (e.g. about 1.9, about 2.0, about 2.5, about 3.0, about 3.5, about 4.0, about 4.5, or about 4.6).

By way of non-limiting example, for a reactor volume of 100mL, exemplary RPM to VPI correlations are as follows:

25 RPM corresponds to a VPI of 0.1

50 RPM corresponds to a VPI of 0.6

5 75 RPM corresponds to a VPI of 1.9

100 RPM corresponds to a VPI of 4.6

200 RPM corresponds to a VPI of 36.6

Although the fundamental nature of the agitation (in terms of fluid dynamics) differs
10 between that of mechanical agitation (as in a stirred tank bioreactor) and other forms of agitation, such as rocking agitation, methods and calculations for scaling the VPI from one form of agitation to another are described in the art. By way of non-limiting example, the PI of stirred tank reactors and rocking motion reactors is compared in Bai *et al.* (2019) Chem. Eng. Sci. 209: 115183, which is herein incorporated by reference in its entirety, and found to be
15 comparable. Other parameters which can be used to scale between stirred tank reactors and rocking motion reactors include mixing time (as described in Bartczak *et al.* (2022), Chem. Eng. J. 431(4): 133288, herein incorporated by reference) and oxygen mass transfer coefficient (kLa) (as described in Engineering Characterisation of a Rocked Bag Bioreactor for Improved Process Development and Scale-Up (2017) by Douglas Marsh, available
20 <https://discovery.ucl.ac.uk/id/eprint/1534658>).

Furthermore, if required VPI values for alternative forms of agitation can readily be determined empirically by the skilled person without undue burden, using standard techniques and/or information supplied by the manufacturer of the relevant bioreactor system.

25 Mechanical agitation

In some preferred embodiments the agitation is mechanical agitation.

Mechanically agitated bioreactors typically agitate the culture medium using a mechanically driven impeller. Common types of impeller include marine impellers (also referred to as pitch-blade impellers), radial flow creators (also referred to as radial flow
30 impellers), angled pitch-blade impellers and helical impellers.

Marine impellers generate an axial flow as the cell suspension is pumped in one direction and sucked from the opposite side parallel to the axis shaft. Marine impellers are known for their shear sensitivity and efficient mixing at low impeller tip speeds. Thus, in some preferred embodiments, the dynamic suspension results from mechanical agitation using a
35 marine impeller. Non-limiting examples of suitable bioreactors with mechanical agitation include the DASbox® bioreactor system from Eppendorf, the Ambr® series of bioreactors from Sartorius, the DynaDrive series of bioreactors from ThermoFisher Scientific, Eppendorf BioBlu

bioreactors (e.g. the 0.3C, 3L, 10L or 50L reactor), Mobius® CellReady bioreactors (e.g. 50L and 200L) from Merck Millipore, the Univessel® bioreactor series and the MA-series of mechanically agitated bioreactors from Bioreactor Sciences. Preferably a stirred tank bioreactor (STR) is used in a method of the invention. STR provide mechanical agitation, typically by a marine impeller, such as the DASbox® system, an Ambr® bioreactor, an Eppendorf BioBlu bioreactor or a DynaDrive® bioreactor. In some embodiments, a combination of impellers may be used in a stirred tank bioreactor. For example, three marine impellers and one radial impeller may be used. Radial flow creators pump the fluid radially outwards and suck it in from both sides of the impeller in an axial flow direction.

Angled pitch-blade impellers result in a mixed flow, where the vertically angled blades move the liquid in axial as well as radial directions.

Helical impellers produce distributed flow and provide equally distributed shear plane as well as gradients in all directions.

Alternatively, rather than by use of a mechanically driven impeller, mechanically agitated bioreactors may agitate the culture medium using an externally mounted motor, e.g., such as a spinner with a mount supporting the bioreactor vessel, such that the vessel itself is rotated. An example of such a bioreactor is the LimGROW bioreactor, which is scalable and provides a one-time use consumable product.

Rocking motion agitation

Rocking motion agitation (also referred to as wave agitation) generates a wave motion by mechanically rocking of a culture-containing chamber back and forth. These waves provide mixing and mass transfer, resulting in a suitable environment for suspension culture cells.

Such rocking motion bioreactors typically comprise a chamber that is partially filled with medium and inoculated with cells. The remaining part of the chamber is filled with air. The air is continuously passaged through the head space during the cultivation mixing and mass transfers are achieved by rocking the chamber back and forth. This rocking motion generates waves at the liquid air interface, greatly enhancing oxygen transfer.

Non-limiting examples of suitable bioreactors with rocking motion agitation include Biostat® RM bioreactors and CultiBag RM bioreactors from Sartorius, AppliFlex bioreactors from Applikon, Wave Bioreactors from GE Healthcare and the Thermo Scientific HyPerforma Rocker Bioreactor.

Vertical wheel agitation

In a bioreactor with vertical wheel agitation, the culture medium moves in a lemniscate pattern throughout the entire volume of the chamber (typically U-shaped). Typically a vertical wheel impeller has a geometry of peripheral paddles and oppositely-oriented axial vanes,

which combine radial mixing in the vertical plane and axial mixing in the horizontal plane. Non-limiting examples of suitable bioreactors with vertical wheel agitation include the PBS Biotech Vertical-Wheel bioreactors.

5 Pneumatic agitation

Pneumatic agitation typically involves the introduction of compressed air or gas, which results in aeration, mixing and fluid circulation with the bioreactor chamber without moving mechanical parts. The compressed air or gas is usually introduced at the bottom of a bioreactor vessel through nozzles, perforated plates, or a ring sparger.

10 There are two main types of pneumatically agitated bioreactors: airlift and bubble-column bioreactors, which are generally of low shear stress and simple design and construction. Airlift bioreactors incorporate a vertical division or loop (either internal or external to the reactor chamber), allowing circulation of the culture medium. In a bubble-column bioreactor, the compressed air or gas is simply bubbled into the reaction chamber. Typically,
15 the height-to-diameter ratio in pneumatically agitated bioreactors is high. In bubble columns, air is bubbled at the base of the column and medium is agitated with this.

Non-limiting examples of suitable bioreactors with pneumatic agitation include Air Lift bioreactors from Electrolab Biotech.

20 **Flow**

Alternatively, dynamic suspension may be achieved by fluid flow within a bioreactor system, such as a hollow fibre bioreactor. Said flow may be continuous or intermittent, typically continuous.

A hollow fibre bioreactor is a high-density continuous-perfusion culture system
25 comprising as a key element a chamber or cartridge containing a plurality of semi-permeable hollow fibres in a parallel array and with at least one inlet port and at least one outlet port. Culture medium entering the chamber or cartridge flows through the interior of the fibres allowing nutrients, gases, and waste products to diffuse both ways across the fibre walls.

Hollow fibre bioreactors are commercially available. Non-limiting examples include the
30 Quantum® bioreactor system by Terumo BCT and hollow fibre bioreactors from FiberCellSystems.

As discussed above in relation to dynamic suspensions resulting from agitation, methods of the invention in which a dynamic suspension results from fluid flow may include a mixing step, e.g. at the start of the co-culture and/or following reseedling of the immune cells
35 as described herein. Alternatively, such methods of the invention may not comprise a mixing step, but rather rely on the dynamic suspension to distribute the immune cells and APCs within the bioreactor used for the method.

Methods for producing immune cells

The invention relates to methods for culturing immune cells and/or producing populations of immune cells, wherein said method comprises co-culture of isolated immune cells with APCs in a dynamic suspension.

As described herein, the prevailing teaching in the art is that co-culture of immune cells with APCs must be done under static conditions, as this is required for the immune cells and APCs to be kept in close proximity in order to form stable interactions facilitating stimulation by the APCs and activation of the immune cells.

In contrast, the present inventors have surprisingly demonstrated that co-culturing immune cells and APCs in a dynamic suspension is at least as efficient as the current “gold-standard” static co-culture methods, whilst providing numerous advantages.

Without being bound by theory, it is believed that the immune cells are still able to come into contact with APCs in a dynamic suspension, such that the APCs are capable of stimulating the immune cells to drive immune cell expansion. By “come into contact” it is meant that immune cells and APCs are in direct contact for a sufficient time for activatory signals from the APCs to stimulate immune cell activation. For example, contacting may be for up to 48 hours, for up to 36 hours, for up to 24 hours, for up to 12 hours, for up to 8 hours, for up to 4 hours, for up to 2 hours, for up to 1 hour, or for up to 30 minutes. An immune cell and APC may be in direct contact for the entirety of a period of contact, or may only be in direct contact for a fraction of a period of contact.

By way of non-limiting example, it has been reported that T cells require contact with APCs (by formation of an immunological synapse between TCR and MHC) in the region of from about 1 minute to about 1 hour to trigger signalling within the T cell leading to activation, such as between about 1 minute to about 30 minutes, or between about 10 minutes to about 30 minutes. The contact time may occur via a single immune cell – APC contact, or may be cumulative (e.g. multiple contacts of shorter duration giving a total contact time which is sufficient to trigger activation).

During the course of a dynamic suspension, an immune cell may come into contact with one or more APCs one or more times. Any given immune cell may come into contact with the same APC or with different APCs.

When producing a cell therapy product the starting material is typically a sample derived from a subject as described herein. Said sample is typically heterogeneous, i.e. it contains multiple different cell types (e.g. at least 2, at least 3, at least 4, at least 5 or more cell types). During a method of the invention, the constituents of the cells within a co-culture may change over time. For example, a method of the invention may begin with a heterogeneous sample or population in co-culture, and over the course of the method one or

more immune cell type may increase and/or one or more different cell type may decrease. The final product of a method may be a homogeneous immune cell population (e.g. a TIL population) or a population enriched for or consisting of only desired immune cell types (e.g. CD4⁺ T cells and CD8⁺ T cells). Thus, the methods of the invention may be described interchangeably as methods for the production of immune cells and methods for the production of populations of immune cells.

Co-culturing of immune cells (e.g. T cells) with APCs results in active immune cells, as described herein. In other words, the immune cells produced by methods of the invention will typically respond to restimulation with an antigen. Thus, the methods of the invention typically produce active immune cells. Accordingly, the methods of the invention may be described interchangeably as methods for the production of active immune cells and methods for the production of populations of active immune cells.

The immune cells may be any immune cells as described herein. The APCs may be any APCs as described herein, provided that said APCs provided that the APCs provide one or more stimulatory or activatory signal to said immune cells.

By way of non-limiting example, the immune cells may be T cells or a specific subtype of T cells as described herein (e.g. CD4⁺ T cells and/or CD8⁺ T cells), and the APCs may be DCs. By way of a further non-limiting example, the immune cells may be T cells or a specific subtype of T cells as described herein (e.g. CD4⁺ T cells and/or CD8⁺ T cells), and the APCs may be B cells. By way of a further non-limiting example, the immune cells may be TILs and the APCs may be DCs.

Thus, the invention provides a method for culturing T cells, wherein said method comprises co-culture of isolated T cells with DCs in a dynamic suspension. The invention also provides a method for producing a population of T cells, wherein said method comprises co-culture of isolated T cells with DCs in a dynamic suspension. Said T cells may be any T cell type or combination thereof, such as those described herein. By way of non-limiting example, the invention also provides a method for culturing CD4⁺ T cells and/or CD8⁺ T cells, wherein said method comprises co-culture of isolated CD4⁺ T cells and/or CD8⁺ T cells with DCs in a dynamic suspension. By way of a further non-limiting example, the invention provides a method for producing a population of CD4⁺ T cells and/or CD8⁺ T cells, wherein said method comprises co-culture of isolated CD4⁺ T cells and/or CD8⁺ T cells with DCs in a dynamic suspension. By way of a further non-limiting example, the invention provides a method for culturing TILs, wherein said method comprises co-culture of isolated TILs cells with DCs in a dynamic suspension. By way of further non-limiting example, the invention provides a producing a population of TILs, wherein said method comprises co-culture of isolated CD4⁺ T cells and/or CD8⁺ T cells with DCs in a dynamic suspension.

Typically the dynamic suspension results from agitation of the co-culture. Said agitation may be continuous and/or intermittent agitation as described herein. By way of non-limiting example, intermittent agitation may comprise agitation for between about 5 minutes to about 30 minutes every 1 to 5 hours, such as: (i) for between about 5 minutes to about 15 minutes, preferably about 10 minutes, every hour; or (ii) for between about 5 minutes to about 15 minutes, preferably about 10 minutes, every 3 hours.

For the avoidance of doubt, any continuous and/or intermittent agitation described herein may be used in combination with any immune cell and/or APC type described herein. For example, the invention provides a method for culturing T cells or producing a population thereof, wherein said method comprises co-culture of isolated T cells with DCs in a dynamic suspension, wherein said dynamic suspension results from agitation, particularly continuous and/or intermittent agitation. By way of further non-limiting example, the invention also provides a method for culturing CD4⁺ T cells and/or CD8⁺ T cells or producing a population thereof, wherein said method comprises co-culture of isolated CD4⁺ T cells and/or CD8⁺ T cells with DCs in a dynamic suspension wherein (a) a first period of the co-culture is subjected to intermittent agitation, wherein said first period is optionally between about 0 to about 2 days, preferably between about 1 to about 2 days; and (b) said first period of co-culture is followed by a second period of co-culture which is subjected to constant agitation, wherein said second period is optionally between about 2 to about 21 days, preferably between about 7 to about 21 days, more preferably between about 10 to about 18 days, even more preferably between about 14 to about 16 days. By way of a further non-limiting example, the invention also provides a method for culturing TILs or producing a population thereof, wherein said method comprises co-culture of isolated TILs with DCs in a dynamic suspension wherein (a) a first period of the co-culture is subjected to intermittent agitation, wherein said first period is optionally between about 0 to about 2 days, preferably between about 1 to about 2 days; and (b) said first period of co-culture is followed by a second period of co-culture which is subjected to constant agitation, wherein said second period is optionally between about 2 to about 21 days, preferably between about 7 to about 21 days, more preferably between about 10 to about 18 days, even more preferably between about 14 to about 16 days.

Culturing of immune cells and/or APCs in dynamic suspension is potentially associated with numerous advantages. Typically, dynamic suspension cultures are amenable to repeated or continuous monitoring. This enables conditions within the bioreactor to be monitored in real-time, and for adjustments to be made to ensure that one or more culture parameter is maintained within a desired range or at a desired value.

Thus, the methods of the invention may comprise monitoring one or more of the following parameters: (a) pH; (b) dissolved oxygen (DO); (c) temperature; (d) gas mix of O₂, N₂, CO₂ and/or compressed air; and/or (e) nutrient and/or metabolite concentrations. Any

combination of these parameters may be monitored, for example, any 1, 2, 3, 4 or all 5 of these parameters may be monitored. Preferably, a method of the invention may comprise monitoring: (a) pH; (b) dissolved oxygen (DO); (c) temperature; and (d) nutrient and/or metabolite concentrations.

5 One or more sensor may be used to monitor said one or more parameter. Where more than one parameter is monitored, individual sensors may be used to monitor each parameter. Alternatively, sensors may be capable of monitoring multiple parameters, e.g. pH and DO; pH and temperature; or gas mix, pH and temperature, etc.

Optimal values or ranges for one or more of these parameters may depend on a
10 number of factors, such as the immune cell type, the APC types, the type of agitation, the type of culture vessel (e.g. bioreactor), the specific cell culture medium and/or supplements used etc. It is within the routine practice of one of ordinary skill in the art to determine optimal ranges for any/all of these parameters for a given method of the invention.

For example, pH may be maintained within a range of between about 7 to about 7.4.
15 For example, DO may be maintained within a range of between about 10% to about 100% (e.g. about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90% or about 100%), typically between about 20% to about 90% (e.g. about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80% or about 90%).

For example, temperature may be maintained within a range of between about 36.5°C
20 to about 37.5°C, preferably 37.0°C.

For example, the gas mix may be a mix of oxygen, nitrogen and CO₂, and may be maintained with oxygen in a range of between about 4.2% to about 21.0%, CO₂ of between about 0% to about 5% and with balanced nitrogen.

The one or more parameter to be monitored may be monitored and maintained at a
25 desired set point using a feedback mechanism. Thus one or more of: (a) pH; (b) DO; (c) temperature; (d) gas mix of O₂, N₂, CO₂ and/or compressed air; and/or (e) nutrient and/or metabolite concentrations may be monitored, and if a parameter is determined to be outside a desired range/value, suitable adjustments may be made to restore said parameter to the desired range or value. For example, DO may be controlled by using a blend of oxygen which
30 is passed through the headspace of the bioreactor, wherein increasing the proportion of oxygen in the blend increases the DO. By way of further non-limiting example, CO₂ and/or a base (e.g. sodium bicarbonate) may be used to control pH.

Said one or more parameter may be monitored and maintained using a control loop mechanism. A control loop mechanism typically comprises or consists of a sensor, a controller
35 function, and a final control element (FCE) which controls the process necessary to automatically adjust the value of a given parameter to equal the value of a desired set-point or to fall within the desired range. Control loops may be open (wherein the controller function

is independent of the parameter) or closed (wherein the controller function is dependent on the parameter). Preferably the control loop is a closed control loop.

At the start of a method of the invention (or co-culture step thereof), a culture vessel (e.g. bioreactor) will typically be seeded with a defined number of immune cells (e.g. T cells) and/or APCs (e.g. DCs). Appropriate numbers of immune cells (e.g. T cells) and/or APCs (e.g. DCs) may be readily selected by one of ordinary skill in the art without undue burden. By way of non-limiting example, commercial bioreactors often have standard operating conditions and parameters (SOC and SOP) published by the manufacturer, which can include initial cell seeding numbers or density. The number of immune cells (e.g. T cells) and/or APCs (e.g. DCs) may be counted by any appropriate technique, such as cell counting (e.g. using a haemocytometer or automated image-based cell counters, such as Cedex HiRes (Roche CustomBiotech, Germany), Vi-CELL (Beckman-Coulter, USA), Eve (NanoEntek, Korea)) or flow cytometry, preferably flow cytometry.

At the start of a method of the invention (or co-culture step thereof), the immune cells (e.g. T cells) may be seeded at between about 0.5×10^6 cells/mL to about 5×10^6 cells/mL, such as between about 0.5×10^6 cells/mL to about 2.5×10^6 cells/mL, between about 0.5×10^6 cells/mL to about 2×10^6 cells/mL, between about 0.5×10^6 cells/mL to about 1.5×10^6 cells/mL, or between about 0.5×10^6 cells/mL to about 1×10^6 cells/mL. Typically the immune cells (e.g. T cells) are seeded at between about 0.5×10^6 cells/mL to about 1.5×10^6 cells/mL, or between about 0.5×10^6 cells/mL to about 1×10^6 cells/mL. Preferably the immune cells (e.g. T cells) may be seeded at about 0.25×10^6 cells/mL, 0.5×10^6 cells/mL, 0.6×10^6 cells/mL, 0.7×10^6 cells/mL, 0.8×10^6 cells/mL, 0.9×10^6 cells/mL, 1×10^6 cells/mL, 1.1×10^6 cells/mL, 1.2×10^6 cells/mL, 1.3×10^6 cells/mL, 1.4×10^6 cells/mL, or 1.5×10^6 cells/mL, most preferably at about 1×10^6 cells/mL.

Alternatively or in addition, at the start of a method of the invention (or co-culture step thereof), the immune cells (e.g. T cells) may be seeded at a ratio of between about 20:1 immune cells:APCs to about 1:1 immune cells:APCs, such as between about 15:1 immune cells:APCs to about 1:1 immune cells:APCs, between about 20:1 immune cells:APCs to about 5:1 immune cells:APCs, between about 15:1 immune cells:APCs to about 5:1 immune cells:APCs, between about 10:1 immune cells:APCs to about 1:1 immune cells:APCs, or between about 10:1 immune cells:APCs to about 5:1 immune cells:APCs. Typically the immune cells (e.g. T cells) are seeded at between about 15:1 immune cells:APCs to about 5:1 immune cells:APCs, or between about 10:1 immune cells:APCs to about 5:1 immune cells:APCs. Preferably the immune cells (e.g. T cells) are seeded at a ratio of about 15:1 immune cells:APCs, about 14:1 immune cells:APCs, about 13:1 immune cells:APCs, about 12:1 immune cells:APCs, about 11:1 immune cells:APCs, about 10:1 immune cells:APCs, about 9:1 immune cells:APCs, about 8:1 immune cells:APCs, about 7:1 immune cells:APCs,

about 6:1 immune cells:APCs, about 5:1 immune cells:APCs, most preferably at a ratio of about 10:1 immune cells:APCs.

In general terms, as cells are cultured, nutrients within the culture medium will be used up and waste products will accumulate. Therefore, culture of cells generally requires introduction or replacement of culture medium to ensure continued/optimal cell growth. Accordingly, a method of the invention may comprise the introduction of fresh culture medium into the co-culture or replacement of the co-culture medium with fresh culture medium. The terms "medium" and "culture medium" are used interchangeably herein. As used herein, the terms "fresh medium" and "fresh culture medium" are used to refer to culture medium which has not previously been in contact with cells (e.g. immune cells and/or APCs). As such, fresh medium typically has not been depleted of nutrients and will contain no or minimal levels of waste products.

Accordingly, in a method of the present invention, fresh medium may be introduced to the culture vessel (e.g. bioreactor) at least once during the method. Typically fresh medium is introduced during the co-culture stage. Fresh medium may be introduced at least once, twice, three times, four times, five times, six times or more during the method (or co-culture step thereof). Alternatively or in addition, fresh medium may be introduced every 2 to 3 days or every other day during the method (or co-culture step thereof).

The number of immune cells (e.g. T cells) and/or APCs (e.g. DCs) may be determined at one or more time point during a method of the invention. The number of immune cells (e.g. T cells) and/or APCs (e.g. DCs) may be determined at least once, twice, three times, four times, five times, six times or more during the method (or co-culture step thereof). Alternatively or in addition the number of immune cells (e.g. T cells) and/or APCs (e.g. DCs) may be determined every 2 to 3 days during the method.

Any appropriate technique may be used to determine the number of immune cells (e.g. T cells) and/or APCs (e.g. DCs). Suitable techniques are well-known in the art and could be readily selected by one of ordinary skill without undue burden. By way of non-limiting example, the number of immune cells (e.g. T cells) and/or APCs (e.g. DCs) may be determined by cell counting (e.g. using a haemocytometer or automated image-based cell counters, such as Cedex HiRes (Roche CustomBiotech, Germany), Vi-CELL (Beckman-Coulter, USA), Eve (NanoEntek, Korea)), flow cytometry, in-situ microscopy, Raman spectroscopy and/or capacitance-based techniques (e.g. using Viamass by Sartorius Biotech GmbH) and non-invasive acoustic quantification based on ultrasonic pulsed Doppler (USPD). Preferably flow cytometry may be used to determine the number of immune cells (e.g. T cells) and/or APCs (e.g. DCs).

Once the number of immune cells (e.g. T cells) and/or APCs (e.g. DCs) has been determined, this information may be used to feedback into to the method. For example, the

number of immune cells (e.g. T cells) and/or APCs (e.g. DCs) may be used to determine when fresh medium is introduced and/or the timings of intermittent agitation (the length of intervals and/or the duration of agitation within intervals).

As discussed further below, once the number of immune cells (e.g. T cells) and/or APCs (e.g. DCs) has reached a threshold number, the immune cells (e.g. T cells) may be purified from the APCs (e.g. DCs) and used to reseed a culture vessel (e.g. bioreactor). The culture vessel (e.g. bioreactor) may be the same culture vessel (e.g. bioreactor) as used for the co-culture step, or a different culture vessel (e.g. bioreactor).

10 ***Additional method steps***

A method of the invention may comprise one or more additional step.

For example, a method of the invention may comprise any one or more of the following optional steps:

- a step of isolating immune cells (e.g. T cells) from a sample;
- 15 • a step of modifying, e.g. by gene editing, at least a portion of the immune cells (e.g. T cells);
- a step of isolating APCs (e.g. DCs) from a sample (which may be the same sample as used for the immune cells or a different sample, optionally from the same patient);
- a step of non-specifically expanding the immune cells (T cells) before co-culturing with the APCs (also referred to as a pre-expansion step);
- 20 • a final step of purifying and/or formulating the immune cells (e.g. T cells) at the end of the method; and/or
- a step of culturing the immune cells (e.g. T cells) in the absence of APCs.

25 ***Modifying the immune cells***

A method for producing immune cells (e.g. T cells) according to the present invention may further comprise a step of modifying, e.g. by gene-editing, at least a portion of the immune cells (e.g. T cells).

The immune cells (e.g. T cells) may be modified by gene-editing methods. Gene editing methods are known in the art, and may be selected from a CRISPR method, a TALE method, a zinc finger method, and a combination thereof.

Methods for gene-editing are described in WO2021/081378, which is herein incorporated by reference in its entirety.

Non-limiting examples of suitable modifications for immune cells (e.g. T cells) are described in WO 2022/269250, which is herein incorporated by reference in its entirety.

Pre-expansion step

In embodiments where in the method comprises a non-specific pre-expansion step prior to the co-culture, and wherein the immune cells are T cells, said pre-expansion step may comprise culturing the T cells in the presence of IL-2 and IL-21.

5 Such a pre-expansion step may further comprise culturing the T cells in the presence of anti-CD3 antibodies, anti-CD28 antibodies, anti-CD2 antibodies and/or IFN γ .

Such a pre-expansion step may comprise culturing the T cells in the presence of IL-2, IL-15, IL-21, anti-CD3 antibodies, anti-CD28 antibodies and anti-CD2 antibodies, as described in WO 2022/269250, which is herein incorporated by reference in its entirety.

10

Culturing immune cells in the absence of APCs

In some preferred embodiments, a method of the invention comprises co-culture of isolated immune cells with APCs in a dynamic suspension as a first step of the invention, and a subsequent step of culturing the immune cells (e.g. T cells) in the absence of APCs (e.g.

15 DCs).

Typically, in methods which comprise a subsequent step of culturing the immune cells (e.g. T cells) in the absence of APCs (e.g. DCs), the co-cultured immune cells (e.g. T cells) and APCs (e.g. DCs) are harvested once a threshold total number of immune cells / cell density (e.g. T cells) and APCs (e.g. DCs) has been reached, and the immune cells (e.g. T

20 cells) used to re-seed a culture vessel (e.g. bioreactor). Said culture vessel to be re-seeded may be by the same culture vessel (e.g. bioreactor) used for the co-culture step, or a different culture vessel (e.g. bioreactor).

Following harvesting of the immune cells (e.g. T cells) and APCs (e.g. DCs), it may be necessary to purify the immune cells (e.g. T cells) from the APCs (e.g. DCs) before re-seeding the culture vessel (e.g. bioreactor). Purification of the immune cells (e.g. T cells) may be by any appropriate technique, examples of which are known in the art and described herein. Alternatively, following the co-culture step the APCs may have died and fragmented, and so may no longer be present within the culture medium, such that it is not necessary to separate the immune cells (e.g. T cells) from the APCs (e.g. DCs) before re-seeding.

30 The threshold number of cells required before the immune cells (e.g. T cells) are purified from the APCs (e.g. DCs) may be given as the total number of immune cells (e.g. T cells) and APCs (e.g. DCs). The threshold number of cells required before the immune cells (e.g. T cells) are purified from the APCs (e.g. DCs) may be given as the number of immune cells (e.g. T cells) only (excluding APCs (e.g. DCs)).

35 Alternatively or in addition, the immune cells (e.g. T cells) may be reseeded to provide a desired cell density, such that the immune cells may be reseeded at between about 0.5×10^6 cells/mL to about 5×10^6 cells/mL, such as between about 0.5×10^6 cells/mL to about 2.5×10^6

cells/mL, between about 0.5×10^6 cells/mL to about 2×10^6 cells/mL, between about 0.5×10^6 cells/mL to about 1.5×10^6 cells/mL, or between about 0.5×10^6 cells/mL to about 1×10^6 cells/mL. Typically the immune cells (e.g. T cells) are reseeded at between about 0.5×10^6 cells/mL to about 1.5×10^6 cells/mL, or between about 0.5×10^6 cells/mL to about 1×10^6 cells/mL.

- 5 Preferably the immune cells (e.g. T cells) may be reseeded at about 0.5×10^6 cells/mL, 0.6×10^6 cells/mL, 0.7×10^6 cells/mL, 0.8×10^6 cells/mL, 0.9×10^6 cells/mL, 1×10^6 cells/mL, 1.1×10^6 cells/mL, 1.2×10^6 cells/mL, 1.3×10^6 cells/mL, 1.4×10^6 cells/mL, or 1.5×10^6 cells/mL, most preferably at about 1×10^6 cells/mL. The total number of immune cells reseeded typically depends on the volume of the reactor. By way of non-limiting example based on the above
- 10 cell densities, for a reactor volume of 100mL, the immune cells (e.g. T cells) may be reseeded at a total density of about 50×10^6 cells to about 500×10^6 cells, such as between about 50×10^6 cells to about 250×10^6 cells, between about 50×10^6 cells to about 200×10^6 cells, between about 50×10^6 cells to about 150×10^6 cells, or between about 50×10^6 cells to about 100×10^6 cells. Typically the immune cells (e.g. T cells) are reseeded at between about 50×10^6 cells to
- 15 about 150×10^6 cells, or between about 50×10^6 cells to about 100×10^6 cells in the absence of APCs. Preferably the immune cells (e.g. T cells) may be reseeded at about 50×10^6 cells, about 60×10^6 cells, about 70×10^6 cells, about 80×10^6 cells, about 90×10^6 cells, about 100×10^6 cells, about 110×10^6 cells, about 120×10^6 cells, about 130×10^6 cells, about 140×10^6 cells, or about 150×10^6 cells, most preferably at about 100×10^6 cells in the absence of APCs.

- 20 When a method of the invention comprises after the co-culture a subsequent step of culturing the immune cells in the absence of APCs for a further period of time, this culture of the immune cells is typically in a dynamic suspension.

- The dynamic suspension may be as defined herein in the context of the co-culture step. For the avoidance of doubt, unless expressly states to the contrary, any disclosure
- 25 herein describing and defining a dynamic suspension, including the disclosure of a dynamic suspension in the context of the co-culture applies equally and without reservation to a subsequent step of culturing the immune cells in the absence of APCs for a further period of time in a dynamic suspension.

- The further period of time in which the immune cells (e.g. T cells) are cultured in the
- 30 absence of APCs (e.g. DCs) may be at least about 1 day, at least about 2 days, at least about 3 days, at least about 4 days, at least about 5 days, at least about 6 days, at least about 7 days, at least about 8 days, at least about 9 days, at least about 10 days, at least about 11 days, at least about 12 days, at least about 13 days, at least about 14 days, at least about 15 days, at least about 16 days, at least about 17 days, at least about 18 days, at least about 19
- 35 days, or at least about 20 days, or more. Preferably, the further period of time in which the immune cells (e.g. T cells) are cultured in the absence of APCs (e.g. DCs) is between about 2 days to about 21 days, between about 2 days to about 14 day, preferably between about 2

days to about 10 days (e.g. about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days or about 10 days).

The dynamic suspension of the immune cells (e.g. T cells) are cultured in the absence of APCs (e.g. DCs) may result from agitation. Said agitation may be continuous agitation
5 and/or intermittent agitation. Preferably when a method of the invention comprises after the co-culture a subsequent step of culturing the immune cells in the absence of APCs for a further period of time, this culture of the immune cells may be subject to continuous agitation.

The continuous agitation may be as defined herein in the context of the co-culture step. For the avoidance of doubt, unless expressly states to the contrary, any disclosure herein
10 describing and defining continuous agitation, including the disclosure of continuous agitation in the context of the co-culture applies equally and without reservation to a subsequent step of culturing the immune cells in the absence of APCs for a further period of time under continuous agitation.

By way of non-limiting example, continuous agitation of the immune cells (e.g. T cells)
15 in the absence of APCs may be for at least about 1 day, at least about 2 days, at least about 3 days, at least about 4 days, at least about 5 days, at least about 6 days, at least about 7 days, at least about 8 days, at least about 9 days, at least about 10 days, at least about 11 days, at least about 12 days, at least about 13 days, at least about 14 days, at least about 15 days, at least about 16 days, at least about 17 days, at least about 18 days, at least about 19
20 days, or at least about 20 days, or more. Preferably, continuous agitation of the immune cells (e.g. T cells) in the absence of APCs may be for between about 2 days to about 21 days, between about 2 days to about 14 days, preferably between about 2 days to about 10 days (e.g. about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days or about 10 days).

25 This additional step of culturing the immune cells in the absence of APCs following the co-culture for a further period of time period of time may be subject to continuous agitation as an alternative or in addition to the co-culture being subject to continuous agitation. Thus, the co-culture may be subject to intermittent agitation and the additional step of culturing the immune cells in the absence of APCs following the co-culture for a further period of time period
30 of time may be subject to continuous agitation. Alternatively, the co-culture may be subject to a first period of intermittent agitation and a second period of continuous agitation and the additional step of culturing the immune cells in the absence of APCs following the co-culture for a further period of time period of time may be subject to continuous agitation. Further alternatively, the co-culture may be subject to continuous agitation and the additional step of
35 culturing the immune cells in the absence of APCs following the co-culture for a further period of time period of time may be subject to continuous agitation.

The intermittent agitation may be as defined herein in the context of the co-culture step. For the avoidance of doubt, unless expressly states to the contrary, any disclosure herein describing and defining intermittent agitation, including the disclosure of intermittent agitation in the context of the co-culture applies equally and without reservation to a subsequent step of culturing the immune cells in the absence of APCs for a further period of time under intermittent agitation.

By way of non-limiting example, a subsequent step of culturing the immune cells in the absence of APCs for a further period of time under intermittent agitation may comprise agitation for between about 5 minutes to about 60 minutes, as between about 5 minutes to about 45 minutes, preferably between about 5 minutes to about 30 minutes, more preferably between about 5 minutes to about 15 minutes, every 1 to 5 hours. Typically said intermittent agitation may comprise agitation for between about 5 minutes to about 30 minutes every 1 to 3 hours. Preferably said intermittent agitation may comprise agitation for between about 5 minutes to about 15 minutes, more preferably about 10 minutes, every 1 to 3 hours. By way of example, said intermittent agitation may comprise agitation for between about 5 minutes to about 15 minutes, more preferably about 10 minutes, every 1 hour. By way of further example, said intermittent agitation may comprise agitation for between about 5 minutes to about 15 minutes, more preferably about 10 minutes, every 3 hours.

As discussed above in the context of co-culture, a subsequent step of culturing the immune cells in the absence of APCs for a further period of time may comprise a period of continuous agitation and followed by a period of intermittent agitation. A subsequent step of culturing the immune cells in the absence of APCs for a further period of time may comprise a period of continuous agitation followed by a period of intermittent agitation. Preferably a subsequent step of culturing the immune cells in the absence of APCs for a further period of time comprises continuous agitation without culturing the immune cells in the absence of APCs under intermittent agitation. In other words, preferably when a method of the invention comprises a step of culturing the immune cells in the absence of APCs for a further period of time, this culture of the immune cells is preferably subject to continuous agitation.

As discussed above in the context of co-culture, alternatively or in addition to the volumetric power input being set to ensure that the % of immune cells remaining in suspension is above a desired threshold or % (as described herein), the intermittent agitation according to the invention may also be set so as to ensure that the % of immune cells remaining in suspension is above a desired threshold or %. It is within the routine practice of one of ordinary skill in the art to determine suitable intervals of intermittent agitation to achieve a desired dynamic suspension for a given culture of immune cells.

The disclosure herein in relation to VPI herein, including the disclosure of VPI in the context of the co-culture step applies equally and without reservation to a subsequent step of

culturing the immune cells in the absence of APCs for a further period of time under intermittent agitation.

By way of non-limiting example, when the agitation of the immune cells in the absence of APCs is mechanical agitation, the speed of rotation of the impeller may be changed to achieve the desired VPI. For example, a low RPM of between about 20 RPM to about 70 RPM, preferably between about 20 RPM to about 60 RPM, more preferably between about 25 RPM to about 50 RPM may be used. Alternatively, a high RPM of between about 70 RPM to about 200 RPM preferably between about 70 RPM to about 150 RPM or between about 70 RPM to about 100 RPM may be used.

Typically a high RPM and/or VPI is used for the agitation when culturing the immune cells in the absence of APCs. For the avoidance of doubt, the disclosure of high RPM and/or high VPI as described herein in relation to agitation of the co-culture applies equally and without reservation for agitation of immune cells in the absence of APCs. By way of non-limiting example, a high VPI of between about 1.9 to about 36.6 or more (e.g. about 1.9, about 2.5, about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 36 or about 36.6), such as between about 1.9 to about 4.6 or more (e.g. about 1.9, about 2.0, about 2.5, about 3.0, about 3.5, about 4.0, about 4.5, or about 4.6), preferably about 4.6 VPI or about 36.6 may be used. If a higher VPI is used (e.g. to correspond to an RPM of up to 200 RPM in the BioBlu Single Use DASBox 0.3C at 100mL), then these can be readily calculated as described herein without undue skill or burden.

Any of the disclosure of different types of agitation discussed above, including the disclosure of different types of agitation in the context of the co-culture step applies equally and without reservation to a subsequent step of culturing the immune cells in the absence of APCs for a further period of time under intermittent agitation. Thus, mechanical agitation, rocking motion agitation, vertical wheel agitation or pneumatic agitation may be used. Preferably the agitation is mechanical agitation. The same type of agitation may be used for both the co-culture and the culturing the immune cells in the absence of APCs. The types of agitation used for the co-culture and the culturing the immune cells in the absence of APCs may be different. Preferably, the same type of agitation may be used for both the co-culture and the culturing the immune cells in the absence of APCs.

In some embodiments, the invention provides a method for producing a population of immune cells, wherein said method comprises: (a) co-culturing isolated immune cells with APCs with intermittent agitation for between about 5 minutes to about 15 minutes (e.g. for about 10 minutes) at an RPM of between about 25 to about 50 RPM (e.g. about 25 RPM) followed by no agitation for about 1 hour for a first period of about 24 hours; (b) co-culturing the isolated immune cells and APCs with intermittent agitation for between about 5 minutes to about 15 minutes (e.g. for about 10 minutes) at an RPM of between about 25 to about 50 RPM

(e.g. about 50 RPM) followed by no agitation for about 1 hour for a second period of about 24 hours; and (c) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of between about 25 to about 80 RPM (e.g. about 70 RPM) for a third period of about 1 week. After this third period, said method may further comprise (d) re-seeding the immune
5 cells in the absence of APCs, preferably to a density of between about 0.5×10^6 immune cells/mL to about 2×10^6 immune cells/mL (e.g. about 1×10^6 immune cells/mL); and (e) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of between about 70 to about 100 RPM (e.g. about 100 RPM) for a fourth period of about 1 week. Said immune cells may preferably be T cells, particularly TILs. Said APCs may be DCs.

10 Preferably, the invention provides a method for producing a population of immune cells, wherein said method comprises: (a) co-culturing isolated immune cells with APCs with intermittent agitation for about 10 minutes at an RPM of about 25 RPM followed by no agitation for about 1 hour for a first period of about 24 hours; (b) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 50 RPM followed
15 by no agitation for about 1 hour for a second period of about 24 hours; and (c) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 70 RPM for a third period of about 1 week. After this third period, said method may further comprise (d) re-seeding the immune cells in the absence of APCs, preferably to a density of about 1×10^6 immune cells/mL; and (e) culturing the isolated immune cells in the absence of APCs with
20 constant agitation at an RPM of about 100 RPM for a fourth period of about 1 week. Said immune cells may preferably be T cells, particularly TILs. Said APCs may be DCs.

In some embodiments, the invention provides a method for producing a population of immune cells, wherein said method comprises: (a) co-culturing the isolated immune cells and APCs with intermittent agitation for between about 5 minutes to about 15 minutes (e.g. for
25 about 10 minutes) at an RPM between about 25 to about 50 RPM (e.g. about 25 RPM) followed by no agitation for about 3 hours for a first period of about 24 hours; (b) co-culturing the isolated immune cells and APCs with intermittent agitation for between about 5 minutes to about 15 minutes (e.g. for about 10 minutes) at an RPM of between about 25 to about 50 RPM (e.g. about 50 RPM) followed by no agitation for about 3 hours for a second period of
30 about 24 hours; (c) culturing the isolated immune cells and APCs with constant agitation at an RPM of between about 25 to about 80 RPM (e.g. about 70 RPM) for a third period of about 1 week. After this third period, said method may further comprise (d) re-seeding the immune cells in the absence of APCs, preferably to a density of between about 0.5×10^6 immune cells/mL to about 2×10^6 immune cells/mL (e.g. about 1×10^6 immune cells/mL); and (e) co-
35 culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of between about 70 to about 100 RPM (e.g. about 100 RPM) for a fourth period of about 1 week. Said immune cells may preferably be T cells, particularly TILs. Said APCs may be DCs.

Preferably, the invention provides a method for producing a population of immune cells, wherein said method comprises: (a) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 25 RPM followed by no agitation for about 3 hours for a first period of about 24 hours; (b) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 50 RPM followed by no agitation for about 3 hours for a second period of about 24 hours; (c) culturing the isolated immune cells and APCs with constant agitation at an RPM of about 70 RPM for a third period of about 1 week. After this third period, said method may further comprise (d) re-seeding the immune cells in the absence of APCs, preferably to a density of about 1×10^6 cells/mL; and (e) co-culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a fourth period of about 1 week. Said immune cells may preferably be T cells, particularly TILs. Said APCs may be DCs.

In some embodiments, the invention provides a method for producing a population of immune cells, wherein said method comprises: (a) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of between about 70 to about 100 RPM (e.g. about 100 RPM) followed by no agitation for about 1 hour for a first period of about 48 hours; and (b) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of between about 70 to 100 RPM (e.g. about 100 RPM) for a second period of about 1 week. After this second period, said method may further comprise (c) re-seeding the immune cells in the absence of APCs, preferably to a density of between about 0.5×10^6 immune cells/mL to about 2×10^6 immune cells/mL (e.g. about 1×10^6 immune cells/mL); and (d) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of between about 70 to about 100 RPM (e.g. about 100 RPM) for a third period of about 1 week. Said immune cells may preferably be T cells, particularly TILs. Said APCs may be DCs.

Preferably, the invention provides a method for producing a population of immune cells, wherein said method comprises: (a) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 100 RPM followed by no agitation for about 1 hour for a first period of about 48 hours; and (b) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 100 RPM for a second period of about 1 week. After this second period, said method may further comprise (c) re-seeding the immune cells in the absence of APCs, preferably to a density of about 1×10^6 cells/mL; and (d) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a third period of about 1 week. Said immune cells may preferably be T cells, particularly TILs. Said APCs may be DCs.

In some embodiments, the invention provides a method for producing a population of immune cells, wherein said method comprises: (a) co-culturing the isolated immune cells and

APCs with intermittent agitation for about 10 minutes at an RPM of between about 70 to about 100 RPM (e.g. about 100 RPM) followed by no agitation for about 3 hours for a first period of about 48 hours; and (b) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of between about 70 to about 100 RPM (e.g. about 100 RPM) for a second
5 period of about 1 week. After this second period, said method may further comprise (c) re-seeding the immune cells in the absence of APCs, preferably to a density of between about 0.5×10^6 immune cells/mL to about 2×10^6 immune cells/mL (e.g. about 1×10^6 immune cells/mL); and (d) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of between about 70 to about 100 RPM (e.g. about 100 RPM) for a third period of
10 about 1 week. Said immune cells may preferably by T cells, particularly TILs. Said APCs may be DCs.

Preferably, the invention provides a method for producing a population of immune cells, wherein said method comprises: (a) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 100 RPM followed by no
15 agitation for about 3 hours for a first period of about 48 hours; and (b) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 100 RPM for a second period of about 1 week. After this second period, said method may further comprise (c) re-seeding the immune cells in the absence of APCs, preferably to a density of about 1×10^6 cells/mL; and (d) culturing the isolated immune cells in the absence of APCs with constant
20 agitation at an RPM of about 100 RPM for a third period of about 1 week. Said immune cells may preferably by T cells, particularly TILs. Said APCs may be DCs.

In some embodiments, the invention provides a method for producing a population of immune cells, wherein said method comprises: (a) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of between about 70 to about 100 RPM (e.g. about
25 100 RPM) for a first period of about 9 days. Wherein after this period, said method may further comprise (b) re-seeding the immune cells, preferably to a density of between about 0.5×10^6 immune cells/mL to about 2×10^6 immune cells/mL (e.g. about 1×10^6 immune cells/mL); and (c) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of between about 70 to about 100 RPM (e.g. about 100 RPM) for a second period of
30 about 1 week. Said immune cells may preferably by T cells, particularly TILs. Said APCs may be DCs.

Preferably, the invention provides a method for producing a population of immune cells, wherein said method comprises: (a) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 100 RPM for a first period of about 9 days. Wherein
35 after this period, said method may further comprise (b) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and (c) culturing the isolated immune cells in the absence

of APCs with constant agitation at an RPM of about 100 RPM for a second period of about 1 week. Said immune cells may preferably be T cells, particularly TILs. Said APCs may be DCs.

In some particularly preferred embodiments, a method of the invention is carried out using a stirred tank bioreactor, optionally with a marine impeller.

5 As described and exemplified herein, the methods of the invention potentially provide numerous advantages over the conventional "gold-standard" methods for the co-culture of immune cells and APCs, which require static culture.

For example, a method of the invention may provide a yield of immune cells that is at least equivalent to that produced by an appropriate control method. An appropriate control
10 method may be a corresponding method in which the isolated immune cells are co-cultured with APCs in a static culture and/or in which the isolated immune cells are co-cultured with APCs without agitation. Non-limiting examples of appropriate control methods include the GMF and GSD methods described in detail in the Examples herein.

As used herein, the term "equivalent" may be defined such that the use of a dynamic
15 suspension and/or agitation does not significantly decrease the yield of immune cells compared with the use of an appropriate control method. By way of non-limiting example, a method of the invention produces a yield of immune cells that is no more than 2-fold lower, no more than 1.5-fold lower, no more than 1.0-fold lower, no more than 0.5-fold lower, no more than 0.25-fold lower, or less than the yield of immune cells compared with the use of an
20 appropriate control method. The term "equivalent" may be defined such that the yield of immune cells produced by a method using a dynamic suspension is statistically unchanged (e.g. $p < 0.05$, $p < 0.01$) compared with the yield of immune cells produced by a method using appropriate control method.

Preferably, a method of the invention produces a yield of immune cells that is increased
25 compared with the yield of immune cells produced by an appropriate control method. The yield of immune cells may be at least 1.5-fold, at least 2-fold, or at least 2.5-fold greater than the immune cells produced by an appropriate control method.

Whether a method of the invention produces at least an equivalent yield or an increased yield of immune cells compared with an appropriate control method, the methods
30 of the invention may be associated with other advantages as a result of the dynamic suspension. By way of non-limiting example, the immune cells produced by a method of the invention may have improved quality and consistency compared with immune cells produced by an appropriate control method. Alternatively or in addition, methods of the invention may be easier to scale-up/scale-out compared with an appropriate control method and/or may
35 provide a significant reduction in the cost and effort of scaling. Alternatively or in addition, methods of the invention may be more precisely controlled and regulated compared with an appropriate control method, whilst the number of human interventions is reduced, decreasing

the risk of batch loss. Alternatively or in addition, methods of the invention are suitable for continuous monitoring, enabling online, in-situ responses so that the system can be dynamically adjusted to compensate for variations in process and/or starting materials.

5 ***Culture medium and supplements***

Any appropriate culture medium may be used for the co-culture of the immune cells (e.g. T cells) and APCs (e.g. DCs), and/or for the culture of immune cells (e.g. T cells) in the absence of APCs (e.g. DCs). The same culture medium may be used for the co-culture of the immune cells (e.g. T cells) and APCs (e.g. DCs), and for the culture of immune cells (e.g. T cells) in the absence of APCs (e.g. DCs). Alternatively, a different culture medium may be used for the co-culture of the immune cells (e.g. T cells) and APCs (e.g. DCs), and for the culture of immune cells (e.g. T cells) in the absence of APCs (e.g. DCs).

It is common in cell culture to supplement a culture medium with one or more additional components. By way of example, a culture medium used in a method of the invention may comprise one or more cytokine; serum (e.g. fetal bovine serum) or preferably serum replacements (e.g. human serum albumin or synthetic GMP-compliant equivalents); one or more antibody; amino acids; sodium pyruvate; and glutamine; or any combination thereof.

Cytokines

A culture medium used in a method of the invention may comprise one or more cytokine, such as those described herein.

In particular, a culture medium may comprise IL-2, particularly if the immune cells are T cells.

The term "IL-2" refers to the T cell growth factor known as interleukin-2 and includes all forms of IL-2 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars and variants thereof. For example, the term IL-2 encompasses human recombinant forms of IL-2 such as Aldesleukin (trade name PROLEUKIN®). Aldesleukin (des-alanyl-I, serine-125 human IL-2) is a nonglycosylated human recombinant form of IL-2 with a molecular weight of approximately 15 kDa. The term IL-2 also encompasses pegylated forms of IL-2, as described in WO 2012/065086.

The concentration of IL-2 used in the antigen-specific expansion step may be described as "lower" or "reduced", for example in comparison to the concentration of IL-2 used in a non-specific pre-expansion step, if included in a method of the invention.

Alternatively or in addition, a culture medium may comprise IL-15, particularly if the immune cells are T cells.

The term "IL-15" refers to the immunomodulatory cytokine interleukin-15 and includes

all forms of IL-15 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars and variants thereof. For example, the term IL-15 encompasses human recombinant forms of IL-15.

Further alternatively or in addition, a culture medium may comprise IL-21, particularly
5 if the immune cells are T cells.

The term "IL-21" refers to the immunomodulatory cytokine interleukin-21 and includes all forms of IL-21 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars and variants thereof. For example, the term IL-21 encompasses human recombinant forms of IL-21.

10 The use of IL-2, IL-15 and IL-21 in the culture of T cells, and suitable concentrations thereof, are described in WO 2022/266250, which is herein incorporated by reference in its entirety.

Serum replacement

15 Cells in in vitro culture are commonly supplemented with serum, for example human- or bovine-derived serum, in order to assist cell growth and maintenance. However, for GMP purposes in the production of therapeutic products intended for human administration, it is desirable not to include human- or bovine-derived serum if avoidable.

Alternatives to human or bovine-derived sera are commercially available in the form
20 of serum replacement, for example CTS™ Immune Cell SR (Gibco).

A further option for serum replacement is the use of platelet lysate. Platelet lysate is a substitute supplement for fetal bovine serum (FBS) in cell culture. It is obtained from blood platelets after freeze/thaw cycles that cause the platelets to lyse, releasing growth factors supportive of cell expansion. FBS-free cell culture media containing platelet lysate are
25 commercially available in GMP-quality and may be used in the manufacture of cell therapies. Platelet lysate may preferably be obtained from human blood, referred to herein as human platelet lysate (hPL).

Platelet lysate may be included in the cell culture medium as defined herein. Platelet lysate may be present at a concentration of about 1% to about 10%, for example about 5%.

30 The use of serum replacements, particularly hPL in the culture of T cells, and suitable concentrations thereof, are described in WO 2022/266250, which is herein incorporated by reference in its entirety.

Antibodies

35 A culture medium used in a method of the invention may comprise one or more antibody, such as those described herein.

The term "CD3" refers to cluster of differentiation 3. CD3 is a protein complex and T cell co-receptor that is involved in T cell activation. It is composed of a CD3 γ chain, a CD3 δ chain, and two CD3 ϵ chains. These chains associate with the T cell receptor and the ζ -chain (zeta-chain) to generate an activation signal in T lymphocytes.

5 Binding of an anti-CD3 antibody to CD3 stimulates T-cell activation. Anti-CD3 30 antibodies are known in the art. For example, suitable anti-CD3 antibodies include, OKT3 (Muromab), TRX4 (Otelixizumab), PRV-031 (Teplizumab) and Visilizumab. Preferably, the anti-CD3 antibody may be OKT3.

10 The term "CD28" refers to Cluster of Differentiation 28. CD28 is constitutively expressed on naive T cells. Stimulation of CD28, for example by anti-CD28 antibodies, provides co-stimulatory signals required for T cell activation and survival. Suitable anti-CD28 antibodies are known in the art.

15 The term "CD2" refers to Cluster of Differentiation 2. CD2 is a cell adhesion molecule found on the surface of T cells and natural killer (NK) cells. In addition to its adhesive properties, CD2 also acts as a co-stimulatory molecule on T cells and NK cells. Suitable anti CD2 antibodies are known in the art.

20 Antibodies may be provided as soluble tetrameric antibody complexes. Binding of the tetrameric antibody complexes results in the crosslinking of cell surface ligands, thereby providing the required primary and co-stimulatory signals for immune cell (particularly T cell) activation. Such antibody complexes are designed to activate and expand human immune cells (particularly human T cells) in the absence of magnetic beads, feeder cells or antigen.

In one aspect, a CD3/CD28 tetrameric antibody complex is used. Such complex is commercially available (e.g. ImmunoCult™ Human CO3/CO28 T cell Activator from STEMCELL Technologies, Inc.).

25 The use of antibodies in the culture of T cells, and suitable concentrations thereof, are described in WO 2022/266250, which is herein incorporated by reference in its entirety.

Systems for producing immune cells

30 The invention also provides systems for culturing immune cells and/or the production of a population of immune cells, said systems being compatible with the methods of the invention.

Thus the invention provides a system comprising: (a) suspension bioreactor; (b) isolated immune cells; (c) APCs; (d) a culture medium; and (e) at least one sensor; wherein the isolated immune cells are in co-culture with the APCs in a dynamic suspension.

35 Examples of suitable bioreactors compatible suitable for use in a system of the invention are known in the art and are commercially available. Non-limiting examples are described herein. Preferably the suspension bioreactor is a stirred tank bioreactor.

Typically, the dynamic suspension within a system of the invention results from agitation of the co-culture of isolated immune cells and APCs. The agitation may be any type of agitation, such as those described herein. By way of non-limiting example, said agitation may be selected from: mechanical agitation; rocking motion agitation; vertical wheel agitation; or pneumatic agitation. Examples of suitable bioreactors compatible with and/or providing these different types of agitation are known in the art and are commercially available. Non-limiting examples of such bioreactors are described herein. Preferably the isolated immune cells and APCs are co-cultured under mechanical agitation.

The system may comprise at least one, at least two, at least three, at least four, at least five or more sensors. The system may comprise 1, 2, 3, 4, 5, 6, 7, 8 or more sensors.

The at least one sensor may be capable of monitoring one or more parameter which may affect the co-culture of the immune cells and APCs. Non-limiting examples of such parameters are described herein. In some preferred embodiments, the at least one sensor may be capable of monitoring one or more of the following parameters: (i) pH; (ii) DO; (iii) temperature; (iv) gas mix of O₂, N₂, CO₂ and/or compressed air; and/or (v) nutrient and/or metabolite concentrations.

Said at least one sensor may be comprised in a proportional–integral–derivative controller allowing for said one or more parameter to be monitored and maintained using a control loop mechanism. Such control loop mechanisms are described herein.

Immune cell populations

The invention also provides an immune cell population obtained or obtainable by the method of the invention. Said immune cell population may be a T cell population, particularly antigen-specific T cells. Preferably the immune cell population may be a TIL population.

Immune cell populations (e.g. T cell populations) produced in accordance with the present invention may be enriched with immune cells (e.g. T cells) that are specific to, i.e. target, a given antigen. That is, the immune cell (e.g. T cell) population that is produced in accordance with the present invention will have an increased number of immune cells (e.g. T cells) that target one or more given antigens. For example, the immune cell (e.g. T cell) population of the invention will typically have an increased number of immune cells (e.g. T cells) that target said antigen compared with the immune cells (e.g. T cells) in the sample isolated from the subject. That is to say, the composition of the immune cell (e.g. T cell) population will differ from that of a "native" immune cell (e.g. T cell) population (i.e. a population that has not undergone method described herein), in that the percentage or proportion of immune cells (e.g. T cells) that target said antigen will be increased.

An immune cell (e.g. T cell) population according to the invention may have at least about 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17,

18, 19, 20, 20 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100% T cells that target a given antigen or set of antigens. For example, the immune cell (e.g. T cell) population may have about 0.2%-5%, 5%-10%, 10-20%, 20-30%, 30-40%, 40-50 %, 50-70% or 70-100% immune cells (e.g. T cells) that target a given antigen or set of antigens. In one aspect, the
 5 immune cell (e.g. T cell) population has at least about 1, 2, 3, 4 or 5% immune cells (e.g. T cells) that target said antigen(s), for example at least about 2% or at least 2% immune cells (e.g. T cells) that target said antigen(s).

Alternatively put, the immune cell (e.g. T cell) population may have not more than about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97,
 10 98, 99, 99.1, 99.2, 99.3, 99.4, 99.5, 99.6, 99.7, 99.8% immune cells (e.g. T cells) that do not target a given antigen. For example, the immune cell (e.g. T cell) population may have not more than about 95%-20 99.8%, 90%-95%, 80-90%, 70-80%, 60-70%, 50-60 %, 30-50% or 0-30% immune cells (e.g. T cells) that do not target said antigen. In one aspect, the immune cell (e.g. T cell) population has not more than about 99, 98, 97, 96 or 95% immune cells (e.g.
 15 T cells) that do not target said antigen, for example not more than about 98% or 95% immune cells (e.g. T cells) that do not target said antigen.

An expanded population of antigen-reactive immune cells (e.g. T cells) may have a higher activity than a population of immune cells (e.g. T cells) not expanded, for example, using an antigen. Reference to "activity" may represent the response of the immune cell (e.g.
 20 T cell) population to restimulation with an antigenic peptide, e.g. a peptide corresponding to the peptide used for expansion, or a mix of antigen-derived peptides. Suitable methods for assaying the response are known in the art. For example, cytokine production may be measured (e.g. IL-2 or IFN γ production may be measured in the case of T cells). The reference to a "higher activity" includes, for example, a 1-5, 5-10, 10-20, 20-50, 50-100, 100-500, 500-
 25 1000-fold increase in activity. In one aspect, the activity may be more than 1000-fold higher.

The invention further provides a plurality or population, i.e. more than one, of immune cells (e.g. T cells) wherein the plurality of immune cells (e.g. T cells) comprises an immune cell (e.g. T cell) which recognises a given antigen and an immune cell (e.g. T cell) which recognises a different antigen. As such, the invention provides a plurality of immune cells (e.g.
 30 T cells) which recognise different antigens. Different immune cells (e.g. T cells) in the plurality or population may alternatively have different receptors (e.g. TCRs in the case of T cells) which recognise the same antigen.

Immune cells (e.g. T cells) produced by a method of the invention may have one or more desirable functional characteristic.

35 By way of non-limiting example, T cells may have increased CD25 expression, CD27 expression and/or IFN γ expression.

The term "CD25" refers to the Interleukin-2 receptor alpha chain (IL2RA). The interleukin 2 receptor alpha and beta (IL2RB) chains, together with the common gamma chain (IL2RG), constitute the high-affinity IL2 receptor. Homodimeric alpha chains (IL2RA) result in low-affinity receptor, while homodimeric beta (IL2RB) chains produce a medium-affinity receptor. CD25 is expressed with CD4 on regulatory T cells. CD27 is a member of the tumour necrosis factor receptor superfamily. CD27 binds CD70, resulting in differentiation and clonal expansion of T cells. CD27 plays a role in the generation of T cell memory.

By way of further non-limiting example, alternatively or in addition, T cells may have decreased CD57 expression. The CD57 antigen is present on subsets of peripheral blood mononuclear cells, NK lymphocytes and T lymphocytes. CD57 expression on human lymphocytes may indicate an inability to proliferate (senescence), though CD57 positive cells may also display high cytotoxic potential, memory-like features and potent effector functions.

Advantageous functional characteristics of T cells are described in WO 2022/266250, which is herein incorporated by reference in its entirety.

In embodiments where the immune cells are T cells, the T cell population may be all or primarily composed of CD8+ T cells, or all or primarily composed of a mixture of CD8+ T cells and CD4+ T cells or all or primarily composed of CD4+ T cells.

Helper 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. TH cells express CD4 on their surface (i.e. they are CD4+ T cells). TH cells become activated when they are presented with peptide antigens by MHC class II molecules on the surface of antigen presenting cells (APCs). These cells can differentiate into one of several subtypes, including TH1, TH2, TH3, TH 17, Th9, or TFH, which secrete different cytokines to facilitate different types of immune responses.

The present invention further provides an immune cell (e.g. T cell) composition which comprises a population of immune cells (e.g. T cells) according to the invention as described herein.

The immune cell (e.g. T cell) composition may be a pharmaceutical composition comprising a plurality of immune cells (e.g. T cells) as defined herein. The pharmaceutical composition may additionally comprise a pharmaceutically acceptable carrier, diluent or excipient. The pharmaceutical composition may optionally comprise one or more further pharmaceutically active polypeptides and/or compounds. Such a formulation may, for example, be in a form suitable for intravenous infusion.

Preferably said population or composition comprises at least about 10x10⁶ reactive immune cells, or at least about 0.2%-5%, 5%-10%, 10-20%, 20-30%, 30-40%, 40-50 %, 50-70% or 70-100% reactive immune cells. Said immune cell composition may be a T cell

composition, particularly an antigen-specific T cells composition. Preferably said composition is a TIL composition.

The invention also provides an immune cell population or composition of the invention for use in treating or preventing cancer in a subject. Preferably said cancer is bladder cancer, gastric, oesophageal, breast cancer, colorectal cancer, cervical cancer, ovarian cancer, endometrial cancer, kidney cancer (renal cell), lung cancer (small cell, non-small cell and mesothelioma), brain cancer (e.g. gliomas, astrocytomas, glioblastomas), melanoma, lymphoma, small bowel cancers (duodenal and jejunal), leukemia, pancreatic cancer, hepatobiliary tumours, germ cell cancers, prostate cancer, head and neck cancers, thyroid cancer or sarcomas, and wherein more preferably the subject is a human.

The invention also provides a method of treating or preventing cancer in a subject, said method comprising administering a therapeutically effective amount of an immune cell population or composition of the invention to a subject in need thereof. Preferably said cancer is bladder cancer, gastric, oesophageal, breast cancer, colorectal cancer, cervical cancer, ovarian cancer, endometrial cancer, kidney cancer (renal cell), lung cancer (small cell, non-small cell and mesothelioma), brain cancer (e.g. gliomas, astrocytomas, glioblastomas), melanoma, lymphoma, small bowel cancers (duodenal and jejunal), leukemia, pancreatic cancer, hepatobiliary tumours, germ cell cancers, prostate cancer, head and neck cancers, thyroid cancer or sarcomas, and wherein more preferably the subject is a human.

The invention also provides the use of an immune cell population or composition of the invention in the manufacture of a medicament for preventing or treating cancer. Preferably said cancer is bladder cancer, gastric, oesophageal, breast cancer, colorectal cancer, cervical cancer, ovarian cancer, endometrial cancer, kidney cancer (renal cell), lung cancer (small cell, non-small cell and mesothelioma), brain cancer (e.g. gliomas, astrocytomas, glioblastomas), melanoma, lymphoma, small bowel cancers (duodenal and jejunal), leukemia, pancreatic cancer, hepatobiliary tumours, germ cell cancers, prostate cancer, head and neck cancers, thyroid cancer or sarcomas, and wherein more preferably the subject is a human.

The invention also provides a method of producing a cell therapy product, said method comprising carrying out a method of culturing immune cells and/or producing a population of immune cells as described herein and formulating the immune cells and/or immune cell population with a pharmaceutically acceptable carrier to produce a cell therapy product. Said method may optionally comprise one or more step of isolating and/or purifying the immune cells and/or immune cell population prior to formulation. Suitable methods for isolating and/or purifying the immune cells/immune cell populations are known in the art, as are suitable carriers for formulating the immune cells to produce a cell therapy product.

EXAMPLES

The invention is now described with reference to the Examples below. These are not limiting on the scope of the invention, and a person skilled in the art would appreciate that suitable equivalents could be used within the scope of the present invention. Thus, the Examples may be considered component parts of the invention, and the individual aspects described therein may be considered as disclosed independently, or in any combination.

Example 1 – Dynamic suspension co-culture produces reactive CD8⁺ yields at least equivalent to those produced by static co-culture

To investigate whether a dynamic suspension co-culture can support growth of target immune cell types, an experiment was designed in which co-culture in a dynamic suspension i.e. in a BioBlu Single Use DASbox 0.3C at 100 ml system was used to produce reactive CD4⁺/CD8⁺ T cells, and was compared to a conventional static co-culture system i.e. in a G-Rex system (standard current system).

To enable comparison of the DASbox conditions to the G-Rex manufacturing process, a G-Rex10M MFG control (GMF) was set-up, representative of a standard static co-culture manufacture. The feeding regime in the G-Rex10M was a batch-fed approach every 2-3 days. Due to the volume limitations in the DASbox a new feeding regime was applied using different volumes to the standard feeding regime (batch-fed). Therefore, along with the MFG G-Rex10M, a secondary G-Rex10M implementing a scale-down model of the DASbox feeding regime (GSD) was used to provide a more direct comparison between the dynamic DASbox conditions and the static co-culture.

Healthy donor (HD) immature DCs (iDCs) and TIL-like cells are generated from leukapheresis material from healthy donors. The iDCs were differentiated from healthy donor isolated monocytes (CD14⁺). The TIL-like cells were generated from CD14⁺ cells performing an initial co-culture for 9-10 days using iDCs followed by another activation and expansion for another 6-7 days to mimic the phenotype of the TIL cells of a patient. In this experiment cryopreserved aliquots of HDiDCs and TIL-like-cells were thawed in parallel on Day -1 of co-culture and incubated at 37°C for 20-22 hrs. iDCs are differentiated into mature dendritic cells (mDCs) using a cytokine cocktail, before pulsing with commercially available peptides on Day 0 to form antigen-presenting cells (APCs). Finally, TIL-like-cells are co-cultured with the newly formed APCs and expanded for 16 days. TIL-like cells and iDCs used in this experiment, were seeded at 1×10^6 cells/mL at a 10:1 ratio (TIL-like cell to DC ratio).

On Day 0, the MFG and the GSD G-Rex10M started at 10×10^6 cells and the G-Rex100M and the DASbox at 10×10^7 cells. On day 9, the MFG control was seeded at 20×10^6 cells whereas the GSD control had a lower starting seed (10×10^6 cells as opposed to 20×10^6

cells), again to maintain the seeding density of 10×10^6 cells/mL. All the DASbox vessels at Day 9 were reseeded at 100×10^6 cells.

Healthy donor TIL-like model

5 Peripheral blood T cells from healthy donors were expanded in culture in the presence of dendritic cells (DC) and selected antigens, followed by one round of polyclonal expansion. Cells obtained after polyclonal expansion were selected as “TIL-like” cells based on the proliferative potential, polyfunctionality and expression of inhibitory receptors (e.g., PD-1, TIM-3, TIGIT). Cells were restimulated with antigen and their cytokine profile assessed by flow
10 cytometry.

Dynamic suspension co-cultures were carried out using a range of agitation conditions, as outlined in Figure 1.

At the end of the method, the number of CD8⁺ T cells was quantified, and the overall expansion of CD8⁺ reactive T cells was calculated as follows:

15

$$1. \frac{D9 \text{ Reactive CD8+Harvest Population}}{D0 \text{ Reactive CD8+Seed Population}} \times \frac{D16 \text{ Reactive CD8+Harvest Population}}{D9 \text{ reactive CD8+Seed Population}} = \text{Overall Fold Expansion}$$

$$2. \text{Overall Fold Expansion} \times D0 \text{ reactive CD8 Seed Population} = \text{Total reactive CD8 population}$$

20 (where D0= reactive CD8⁺ cell count on day 0; D9 = reactive CD8⁺ cell count on day 9; D16 = reactive CD8⁺ cell count on day 16)

These values were then compared with the GMF/GSD controls. From a commercial perspective, it is desirable for the dynamic co-culture/DASbox conditions to support reactive
25 CD4⁺/CD8⁺ expansion in-line with or better than the GMF and/or GSD controls. This potentially allows commercially viable numbers of cells to be produced, whilst providing the other advantages associated with dynamic suspension culture (e.g. continuous monitoring, real-time parameter adjustment, reduced costs, etc.).

30 As shown in Figure 2, there was no significant decrease in the number of reactive CD8⁺ T cells across the different DASbox conditions when compared with the GMF or GSD controls, either at day 9 or day 16.

As shown in Figure 3, at day 9, the total number of CD8⁺ T cells was observably increased in the constant (high) agitation condition, as well as the two intermittent (high) agitation conditions. Slight decreases were observed between the two low intermittent
35 agitation conditions and the GSD and GMF controls.

At day 16, the total number of CD8⁺ T cells was equivalent to the total number of CD8⁺ T cells produced in the GSD control, with a slight (but statistically insignificant) decrease compared with the GMF control (also shown in Figure 3). The total number of cells across the different DASbox conditions and controls was reduced at day 16 compared to day 9 as the cells were reseeded at day 9 in all cases.

The number of reactive CD8⁺ T cells was quantified across the different DASbox conditions and the GMF and GSD controls. As shown in Figure 4, at day 9, the number of reactive CD8⁺ T cells was observably increased in the constant (high) agitation condition, as well as the two intermittent (high) agitation conditions. Slight decreases were observed between the two low intermittent agitation conditions and the GSD and GMF controls.

At day 16, the total of reactive CD8⁺ T cells was not statistically different to the number of reactive CD8⁺ T cells produced in the GSD or GFM controls (also shown in Figure 4). The total number of cells across the different DASbox conditions and controls was reduced at day 16 compared to day 9 as the cells were reseeded at day 9 in all cases.

Lastly, the overall reactive CD8⁺ T cells population was quantified across the different DASbox conditions and the GMF and GSD controls. As shown in Figure 5, the overall reactive CD8⁺ T cells population was observably increased in the constant (high) agitation condition compared with both the GSD and GMF controls. The overall reactive CD8⁺ T cells population for the two intermittent (high) agitation conditions was equivalent to the GMF and GSD controls. Slight decreases were observed between the two low intermittent agitation conditions and the GMF and GSD controls.

Therefore, these results demonstrate that the DASbox conditions with intermittent agitation using high RPM results in overall reactive CD8⁺ T cells populations that are at least equivalent to those obtained using the current gold-standard (GMF) method and the GSD control. Further, the DASbox conditions with continuous agitation at high RPM resulted in a significant increase in the overall reactive CD8⁺ T cells population compared with both the current gold-standard (GMF) method and the GSD control.

Example 2 – Dynamic suspension co-culture produces reactive CD4⁺ yields at least equivalent to those produced by static co-culture

The same co-culture reactions investigated in Example 1 were further analysed with respect to the production of reactive CD4⁺.

In particular, the number of CD4⁺ T cells was quantified, and the overall expansion of CD4⁺ reactive T cells was calculated as follows:

$$1. \frac{D9 \text{ Reactive } CD4+\text{Harvest Population}}{D0 \text{ Reactive } CD4+\text{Seed Population}} \times \frac{D16 \text{ Reactive } CD4+\text{Harvest Population}}{D9 \text{ reactive } CD4+\text{Seed Population}} = \text{Overall Fold Expansion}$$

2.
$$\text{Overall Fold Expansion} \times \text{D0 reactive CD4}^+ \text{ Seed Population} = \text{Total reactive CD4 population}$$

(where D0 = reactive CD4⁺ cell count on day 0; D9 = reactive CD4⁺ cell count on day 9; D16 = reactive CD4⁺ cell count on day 16)

These values were then compared with the GMF/GSD controls. From a commercial perspective, it is desirable for the dynamic co-culture/DASbox conditions to support reactive CD4⁺/CD8⁺ expansion in-line with or better than the GMF and/or GSD controls. This potentially allows commercially viable numbers of cells to be produced, whilst providing the other advantages associated with dynamic suspension culture (e.g. continuous monitoring, real-time parameter adjustment, reduced costs, etc.).

As shown in Figure 6, the CD4⁺ cells produced by all the DASbox conditions apart from the DB3 and DB4 (intermittent agitation, low RPM) were at or above the base line required reactivity, and were not statistically different in reactivity compared with the GMF/GSD controls either at day 9 or day 16.

As shown in Figure 7, at day 9, the total number of CD4⁺ T cells was observably increased in the continuous (high) agitation condition, and the two intermittent (high) agitation conditions were equivalent to the GSD and GMF controls. Slight decreases were observed between the two low intermittent agitation conditions and the GSD and GMF controls.

At day 16, the total number of CD4⁺ T cells was increased in all DASbox conditions compared with the total number of CD4⁺ T cells produced in the GSD and GMF controls, with the greatest increase for the continuous agitation (also shown in Figure 7). The total number of cells across the different DASbox conditions and controls was reduced at day 16 compared to day 9 as the cells were reseeded at day 9 in all cases.

The number of reactive CD4⁺ T cells was quantified across the different DASbox conditions and the GMF and GSD controls. As shown in Figure 8, at day 9, the number of reactive CD4⁺ T cells was equivalent to the GMF and GSD controls for the constant (high) agitation condition, as well as the two intermittent (high) agitation conditions. Slight decreases were observed between the two low intermittent agitation conditions and the GSD and GMF controls.

At day 16, the total of reactive CD4⁺ T cells was increased compared with the number of reactive CD4⁺ T cells produced in the GSD or GFM controls (also shown in Figure 8). The total number of cells across the different DASbox conditions and controls was reduced at day 16 compared to day 9 as the cells were reseeded at day 9 in all cases.

Lastly, the overall reactive CD4⁺ T cells population was quantified across the different DASbox conditions and the GMF and GSD controls. As shown in Figure 9, the overall reactive

CD4⁺ T cells population was increased in across all DASbox conditions compared with both the GSD and GMF controls. The overall reactive CD4⁺ T cells population in the constant (high) agitation condition was significantly increase compared with both the GMF and GSD controls.

5 Therefore, these results demonstrate that the DASbox conditions with agitation (continuous or intermittent) using high RPM, and particularly continuous agitation at high RPM results in overall reactive CD4⁺ T cells populations that are at least equivalent to those obtained using the current gold-standard (GMF) method and the GSD control. Further, the DASbox conditions with continuous agitation resulted in a significant increase in the overall
10 reactive CD4⁺ T cells population.

Example 3 – Comparison of a dynamic suspension co-culture with a hybrid control

By way of further validation of the dynamic co-culture protocols investigated in Examples 1 and 2, these dynamic suspension protocols were compared with a third set of
15 control conditions. This third control was a “hybrid control”, in which static culture in a G-Rex100M was set up for the first 48 hours and then the cells were re-seeded into a DASbox vessel for co-culture in a dynamic suspension thereafter (Figure 10).

These further experiments demonstrated comparable results for those observed in Examples 1 and 2 with the GMF and GSD controls (data not shown). Again, this supports the
20 conclusion that the dynamic co-culture protocols tested support commercially desirable reactive CD4⁺/CD8⁺ expansion.

Example 4 – Patient cNeT generation in dynamic system

Prior to initiation of the patient co-culture in dynamic system, autologous patient-derived
25 intermediate products, TILs and mDCs, and associated patient specific-peptides were generated as described:

TIL Generation

4 x 4 mm tumour pieces were weighed and transferred to the appropriately sized, closed
30 system G-Rex vessels. TexMACs media containing 22.5 U/mL IL-21, 6000 U/mL IL2, IL-15, 5% platelet lysate and 1% antibiotic/antimitotic agent was added to the G-Rex vessels. Vessels were then incubated at 37°C for 15-17 days.

At the end of 15-17 days the expansion vessels were removed from the incubator and
35 TIL/CD3⁺ cells counted using flow cytometry. TILs were then harvested into cryopreservation media in cryopreservation bags and frozen using a controlled rate freezer before transfer to liquid nitrogen for long-term storage.

Peptide Generation

Prior to initiation of the co-culture, Achilles' proprietary PELEUS™ platform was used to generate patient clonal neoantigens (from the sequenced blood and tumour) with the following steps:

- 5 (i) identify patient-specific somatic mutations (including single nucleotide variants (SNVs), multiple nucleotide variants (MNVs) and insertions/deletions (indels)) by comparing DNA sequence data from the germline (blood) sample and the matched tumour samples to each other and to a reference genome;
- 10 (ii) identify a set of mutations that are likely to be clonal in view of the sequence data from the patient using a Bayesian approach (see e.g. *McGranahan et al.*, Science Vol 135:6280, p. 1463-1469; Roth et al., Nat Methods. 2014 April ; 11(4): 396–398); and
- (iii) design a set of peptides comprising the set of somatic mutations identified as likely to be clonal.

15 The resulting set of candidate antigenic peptides was manufactured using standard peptide synthesis methods. These were pooled with commercially available tumour associated antigens to create patient specific peptide pools. The pool of peptides may be a masterpool of long peptides (LMP) and/or a masterpool of short peptides.

Patient co-culture in dynamic system

20 The co-culture was initiated by bringing together patient dendritic cells, pulsed with the pre-prepared patient peptide pools and TILs as described herein. The dynamic agitation conditions outlined in Figure 11 were used and were compared to a GSD/G-Rex control, as previously described for the healthy donor model (Figures 1 and 10). As before, cells were harvested at day 9 (D9_H) and reseeded (D9_S), without APCs. In this experiment, an AMBR®250 vessel obtained from Sartorius, was used as an alternative bioreactor to the DASbox bioreactor used in the above examples. As such, agitation conditions used in the DASbox were transferred to the AMBR®250 using P/V as described above.

Analysis Methods

Flow cytometry

35 Flow cytometry was used to phenotypically count T cells at intervals (Days 0,2,5,7,9,12,14&16) to monitor the expansion process. Since the patient dynamic co-culture experiment was performed at a different site to that used previously for healthy donor cells, and with somewhat different peptides and flow cytometer with different configuration, in this

example the complete CD3+ T cell population was used as a performance metric, as shown in Figures 12a and 12b, rather than CD4+ and CD8+ cells. Since CD4+ and CD8+ cells combine to comprise the total CD3+ population, these performance metrics are considered equivalent. Figures 12a and 12b show that there is steady cell growth of CD3+ cells in the AMBR (dynamic) condition, particularly for the polyclonal, also known as the second expansion phase. This demonstrates that the results obtained with the HD model are applicable to the patient derived material.

Intracellular Cytokine Staining (ICS)

The Intracellular staining (ICS) assay is the test method used to identify cells producing cytokines and other cytotoxic molecules in response to peptide stimulation using flow cytometry. Overnight the cells are treated with Golgi inhibitors which leads to intracellular retention of cytokines. Simultaneously cells are stimulated with peptide pools specific to the antigens generated during the co-culture process. This causes build-up of cytokines inside stimulated cells.

The cells are then fixed, permeabilized and fluorochrome-conjugated, antigen-specific antibodies are incubated with the peptide-stimulated cells. This enables fluorescence detection of phenotypes (CD3+, CD4+, CD8+) which are producing cytokines of interest. Cells are acquired using a flow cytometer to identify antigens present on the surface and the presence of intracellular cytokines. Cells producing cytokines of interest (IFN γ and TNF α) are referred to as 'Reactive' cells and their frequency is expressed as a % of the whole population. Reactivities for CD8+ cells are shown in Figures 14a and 14b.

Figure 14a and 14b show that a higher percentage reactivity is observed for CD8+ cells from days 0-9 in the AMBR (dynamic) condition compared with the static G-Rex/GSD (static) control. This demonstrates that patient reactive T cells can be grown successfully during the co-culture phase under dynamic conditions, performing even better than the G-Rex/GSD control.

CLAIMS

1. A method for producing a population of immune cells, wherein said method comprises co-culture of isolated immune cells with antigen presenting cells (APCs) in a dynamic suspension.
2. The method of claim 1, wherein the dynamic suspension results from agitation of the co-culture.
3. The method of claim 2, wherein the agitation is:
- (a) continuous; and/or
- (b) intermittent.
4. The method of claim 3, wherein the intermittent agitation of the co-culture comprises agitation for between about 5 minutes to about 30 minutes every 1 to 5 hours, wherein optionally:
- (a) the agitation is for between about 5 minutes to about 15 minutes, preferably about 10 minutes, every hour; or
- (b) the agitation is for between about 5 minutes to about 15 minutes, preferably about 10 minutes, every 3 hours.
5. The method of any one of claims 2 to 4, wherein:
- (a) a first period of the co-culture is subjected to intermittent agitation, wherein said first period is optionally between about 0 to about 2 days, preferably between about 1 to about 2 days; and
- (b) said first period of co-culture is followed by a second period of co-culture which is subjected to constant agitation, wherein said second period is optionally between about 2 to about 21 days, preferably between about 2 to about 10 days.
6. The method of any one of claims 2 to 5, wherein said agitation is:
- (a) mechanical agitation;
- (b) rocking motion agitation;

- (c) vertical wheel agitation; or
- (d) pneumatic agitation;

wherein preferably the isolated immune cells and APCs are co-cultured under
5 mechanical agitation and more preferably wherein a co-culture is carried out in a stirred tank
bioreactor.

7. The method of any one of claims 2 to 6, wherein the agitation is mechanical agitation
at:
- 10 (a) an RPM of between about 20 RPM to about 80 RPM, preferably between about 20
RPM to about 75 RPM, more preferably between about 25 RPM to about 70 RPM;
or
(b) an RPM of between about 70 RPM to about 150 RPM, preferably between about
15 70 RPM to about 100 RPM.
8. The method of any one of the preceding claims, wherein the immune cells are T cells,
optionally tumour infiltrating lymphocytes (TILs).
- 20 9. The method of claim 8, wherein the T cells are:
- (a) CD8⁺ T cells;
(b) CD4⁺ T cells, optionally Th1, Th2, Th17, Tfh and/or Th9 cells;
(c) NKT cells, optionally invariant NKT cells; and/or
25 (d) regulatory T cell (Treg) cells.
10. The method of any one of the preceding claims, wherein the APCs are:
- (a) dendritic cells (DCs);
30 (b) B cells; and/or
(c) macrophages.
11. The method of any one of the preceding claims, wherein the antigen is a tumour
antigen, preferably a neoantigen, more preferably a clonal neoantigen.
- 35

12. The method of any one of the preceding claims, wherein the immune cells are T cells, preferably TIL, and the APCs are DCs.
13. The method of any one of the preceding claims, wherein one or more of the following parameters is monitored and maintained at a desired set point using a feedback mechanism:
- (a) pH;
- (b) dissolved oxygen (DO);
- (c) temperature;
- (d) gas mix of O₂, N₂, CO₂ and/or compressed air; and/or
- (e) nutrient and/or metabolite concentrations;
- wherein optionally said one or more parameter is monitored and maintained using a control loop mechanism.
14. The method of any one of the preceding claims, wherein fresh medium is introduced into the co-culture:
- (a) at least once, twice, three times, four times, five times, six times or more during the method; and/or
- (b) every 2 to 3 days during the method.
15. The method of any one of the preceding claims, wherein the number of immune cells and/or APCs is determined:
- (a) at least once, twice, three times, four times, five times, six times or more during the method; and/or
- (b) every 2 to 3 days during the method;
- wherein optionally the number of immune cells and/or APCs is determined using flow cytometry.
16. The method of any one of the preceding claims, wherein at the start of the co-culture the immune cells are seeded:
- (a) at about 1×10^6 immune cells/mL; and/or

(b) in a ratio of about 10:1 immune cells:APCs.

17. The method of any one of the preceding claims, which further comprises a step of non-specifically expanding the immune cells before co-culturing with the APCs, wherein optionally:

(a) the immune cells are T cells; and/or

(b) the step of non-specifically expanding the immune cells comprises culturing the immune cells with one or more of IL-2, IL-21, IL-15, anti-CD3 antibodies, anti-CD28 antibodies and anti-CD2 antibodies, preferably all of IL-2, IL-21, IL-15, anti-CD3 antibodies, anti-CD28 antibodies and anti-CD2 antibodies.

18. The method of any one of the preceding claims, wherein the co-cultured cells are harvested after a total duration of co-culture of between about 2 days to about 21 days, preferably of between about 2 days to about 10 days, and the immune cells are used to re-seed the culture vessel, wherein optionally:
the immune cells are reseeded at a density of about 1×10^6 immune cells/mL in the absence of APCs.

19. The method of any one of the preceding claims, wherein following co-culture, the immune cells are cultured in the absence of the APCs for a further period of time, wherein optionally:

(a) in said further period of time the immune cells are cultured in a dynamic suspension; and/or

(b) said further period is optionally between about 2 to about 21 days, preferably between about 2 to about 10 days.

20. The method of claim 19, wherein the dynamic suspension results from agitation of the immune cells.

21. The method of claim 20, wherein the agitation is:

(a) continuous; and/or

(b) intermittent.

22. The method of claim 21, wherein the intermittent agitation of the immune cells comprises agitation for between about 5 minutes to about 30 minutes every 1 to 5 hours, wherein optionally:
- 5 (a) the agitation is for between about 5 minutes to about 15 minutes, preferably about 10 minutes, every hour; or
- (b) the agitation is for between about 5 minutes to about 15 minutes, preferably about 10 minutes, every 3 hours.
- 10 23. The method of any one of claims 20 to 22, wherein the agitation is:
- (a) mechanical agitation;
- (b) rocking motion agitation;
- (c) vertical wheel agitation; or
- 15 (d) pneumatic agitation;
- wherein preferably the isolated immune cells and APCs are co-cultured under mechanical agitation and more preferably wherein a co-culture is carried out in a stirred tank bioreactor.
- 20 24. The method of any one of claims 20 to 23, wherein the agitation of the immune cells is mechanical agitation at:
- (a) an RPM of between about 20 RPM to about 80 RPM, preferably between about 20 RPM to about 75 RPM, more preferably between about 25 RPM to about 70 RPM;
- 25 or
- (b) an RPM of between about 70 RPM to about 200 RPM, preferably between about 70 RPM to about 150 RPM or between about 70 RPM to about 100 RPM.
25. The method of any one of the preceding claims, said method comprising:
- 30 (a) (i) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 25 RPM followed by no agitation for about 1 hour for a first period of about 24 hours;
- (ii) co-culturing the isolated immune cells and APCs with intermittent
- 35 agitation for about 10 minutes at an RPM of about 50 RPM followed by no agitation for about 1 hour for a second period of about 24 hours;

(iii) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 70 RPM for a third period of about 1 week;

optionally

(iv) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and

(v) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a fourth period of about 1 week;

or

(b) (i) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 25 RPM followed by no agitation for about 3 hours for a first period of about 24 hours;

(ii) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 50 RPM followed by no agitation for about 3 hours for a second period of about 24 hours;

(iii) culturing the isolated immune cells and APCs with constant agitation at an RPM of about 70 RPM for a third period of about 1 week;

optionally

(iv) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and

(v) co-culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a fourth period of about 1 week;

or

(c) (i) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 100 RPM followed by no agitation for about 1 hour for a first period of about 48 hours;

(ii) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 100 RPM for a second period of about 1 week;

optionally

(iii) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and

(iv) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a third period of about 1 week;

or

(d) (i) co-culturing the isolated immune cells and APCs with intermittent agitation for about 10 minutes at an RPM of about 100 RPM followed by no agitation for about 3 hours for a first period of about 48 hours;

5 (ii) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 100 RPM for a second period of about 1 week;

optionally

(iii) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and

10 (iv) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a third period of about 1 week;

or

(e) (i) co-culturing the isolated immune cells and APCs with constant agitation at an RPM of about 100 RPM for a first period of about 9 days;

15

optionally

(ii) re-seeding the immune cells, preferably to a density of about 1×10^6 cells/mL; and

(iii) culturing the isolated immune cells in the absence of APCs with constant agitation at an RPM of about 100 RPM for a second period of about 1 week.

20

26. The method of any one of the preceding claims, which is carried out using a stirred tank bioreactor, optionally with a marine impeller.

25

27. The method of any one of the preceding claims, wherein the yield of immune cells is at least equivalent to that produced by a corresponding method in which the isolated immune cells are co-cultured with APCs without agitation.

30 28. A method of producing a cell therapy product, comprising:

(a) producing a population of immune cells according to a method of any one of claims 1 to 27;

(b) optionally isolating and/or purifying the immune cell population; and

(c) formulating the immune cell population with a pharmaceutically acceptable carrier.

35

29. An immune cell population obtained or obtainable by the method according to any one of claim 1 to 27, an immune cell composition comprising said immune cell population or a cell therapy product obtained or obtainable by a method of claim 28, wherein preferably said population, composition or product comprises at least about 10×10^6 reactive immune cells, or at least about 0.2%-5%, 5%-10%, 10-20%, 20-30%, 30-40%, 40-50 %, 50-70% or 70-100% reactive immune cells.
30. The immune cell population, immune cell composition or cell therapy product of claim 29, wherein the immune cells are antigen-specific T cells.
31. An immune cell population or composition according to claim 29 or 30 for use in treating or preventing cancer in a subject, wherein preferably said cancer is bladder cancer, gastric, oesophageal, breast cancer, colorectal cancer, cervical cancer, ovarian cancer, endometrial cancer, kidney cancer (renal cell), lung cancer (small cell, non-small cell and mesothelioma), brain cancer (e.g. gliomas, astrocytomas, glioblastomas), melanoma, lymphoma, small bowel cancers (duodenal and jejunal), leukemia, pancreatic cancer, hepatobiliary tumours, germ cell cancers, prostate cancer, head and neck cancers, thyroid cancer or sarcomas, and wherein more preferably the subject is a human.
32. A system for the production of a population of immune cells, the system comprising:
- (a) a suspension bioreactor;
 - (b) isolated immune cells;
 - (c) APCs;
 - (d) a culture medium; and
 - (e) at least one sensor;
- wherein:
- the isolated immune cells are in co-culture with the APCs in a dynamic suspension; and
- optionally wherein the at least one sensor capable of monitoring one or more of the following parameters:
- (i) pH;

- (ii) dissolved oxygen (DO);
- (iii) temperature;
- (iv) gas mix of O₂, N₂, CO₂ and/or compressed air; and/or
- (v) nutrient and/or metabolite concentrations.

5

33. The system of claim 32, wherein the dynamic suspension results from agitation of the co-culture of isolated immune cells and APCs, and wherein optionally:

(a) said agitation is optionally selected from:

10

- (i) mechanical agitation;
- (ii) rocking motion agitation;
- (iii) vertical wheel agitation; or
- (iv) pneumatic agitation;

15

wherein preferably the isolated immune cells and APCs are co-cultured under mechanical agitation and more preferably wherein the agitated bioreactor is a stirred tank bioreactor; and/or

(b) said at least one sensor is comprised in a proportional–integral–derivative controller allowing for said one or more parameter to be monitored and maintained using a control loop mechanism.

20