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(54) Title: DIAGNOSTIC AND THERAPEUTIC USES OF AN *IN VITRO* SYSTEM OF AUTOLOGOUS LYMPHOCYTES CULTURE

(57) Abstract: An *in vitro* system of autologous lymphocytes culture, a method for the identification and classification of individuals suffering from a lymphopenia-related disease, and the use of the regulatory T cells obtained by a method of the present invention for treating autoimmune diseases, inflammatory diseases, allergic or asthmatic condition, graft versus host disease or for preventing graft rejection.

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Diagnostic and therapeutic uses of an *in vitro* system of autologous lymphocytes culture

FIELD OF THE INVENTION

The present invention relates to the field of immunology and medicine, particularly refers to an *in vitro* system of autologous lymphocytes culture that allows studying the homeostatic proliferation mechanisms of human naïve CD4 T-cells. The *in vitro* system of the present invention can be used in a method for the identification and classification of individuals suffering from a lymphopenia-related disease. Also refers to the regulatory T cells obtained in the present invention and their use in cell therapy for treating autoimmune diseases, inflammatory diseases, allergic or asthmatic condition, graft versus host disease or for preventing graft rejection.

BACKGROUND OF THE INVENTION

The size and composition of human peripheral T-cell pool is homeostatically kept constant throughout the life. However, immune insults as several viral infections, autoimmune disorders, chemotherapy against cancer and other drugs can lead to a lymphopenic status. The restoration of the T-cell pool after such aggressions is not uniform. While lymphodepletion of memory T-cell numbers is usually reverted early, the restoration of naïve CD4 and CD8 T-cell pools are slower and delayed. Importantly, the size and functional restoration of the naïve T-cell pool is of vital importance in order to recover the immune homeostasis.

Several mechanisms are involved in the reconstitution of naïve T-cell pool. On one hand, thymus is a lymphoid organ that supports the maturation of hematopoietic precursors leading to generation of new T-cells and ensuring the preservation of TCR variability. On the other hand, the homeostatic proliferation (HP) process involves a peripheral clonal expansion of T-cells in response to homeostatic stimuli like self- or commensal-antigens and cytokines. Indeed, it is known that different types of homeostatic proliferation exist. The fast or spontaneous HP is defined by a rapid cellular division of naïve T-cells mediated by the low avidity TCR-contact (antigen-driven proliferation) that generate memory-like T-cells. Otherwise, the slow HP is defined by a slower cellular division of naïve T-cells that do not result in a change of phenotype and carried out by homeostatic cytokines like IL7.

Nevertheless, while both thymopoiesis and homeostatic proliferations are essential to full reconstitution of T-cell pool, the homeostatic proliferation has an important role in early stages of immune reconstitution. For this reason, the comprehension of homeostatic proliferation process in human lymphopenic disorders has a pivotal importance.

In order to understand such process in different lymphopenic disorders, several *in vivo* models using different species of animals and chimeras, and several *in vitro* models using generated antigen-presenting cells or anti-CD3/CD28 stimulation, have been developed. However, self- and commensal antigens, that are well-known triggers of the HP, show a large variability among different subjects and scenarios and such natural composition is not considered in these experimental approximations.

Although the therapeutic potential of regulatory T cells was envisioned decades ago, clinical implementation of their potent immune regulatory activity by in vivo administration of agents has proven challenging.

Therefore, there is a need for a new method for regulatory T cell therapy that is more efficient for treating a patient in need thereof.

SUMMARY OF THE INVENTION

In a first aspect, the present invention relates to the use of antigen-presenting cells (APCs) for studying the homeostatic proliferation mechanisms in humans. Preferably, for studying the homeostatic proliferation mechanisms in a specific individual, the antigen-presenting cells are autologous antigen-presenting cells (aAPCs).

A **second aspect** of the present invention relates to a method to evaluate the potential homeostatic proliferation of T-cells of a human subject using APCs, preferably using their autologous APCs (aAPCs)defined in the first aspecto, hereinafter first method of the invention, comprising:

a) Isolation of two cellular subsets and control of purity from a human biological sample, wherein:

   I. Cellular subset 1 comprising APCs, preferably aAPCs, and

   II. Cellular subset 2 comprising naïve CD4 T-cells,

b) Irradiate the APCs, preferably aAPCs,of the cellular subset 1

c) Dye the naïve CD4 T-cells preferably with carboxyfluorescein succinimidyl ester (CFSE) for cell proliferation tracking

d) Cryopreservation of several aliquots of irradiated APCs, preferably aAPCs, of the step (b)

e) Cultivate the naïve CD4 T-cells from cellular subset 2 of step (c) in a plate and add the APCs, preferably aAPCs, of the cellular subset 1 and rIL7,
f) Perform the basal analysis (day 0) in an aliquot of the cell culture of step (e),
g) Refresh supplemented medium and thawed irradiated APCs, preferably aAPCs, from the aliquots of step (d) and IL7, preferably rIL7, for re-stimulate the culture each 5 days of culture
h) Culture for a total of 10-15 days, preferably 10 days.

A **third aspect** of the present invention relates to the *in vitro* model for studying homeostatic proliferation process, hereinafter *in vitro* model of the invention, comprising the two cellular subsets as described in the first method of the invention.

A **fourth aspect** of the present invention relates to a method for obtaining data that can be used for the characterization and classification of human subject suffering from a lymphopenia-related disease, hereinafter second method of the invention, comprising the steps according to the first method of the invention, and also comprising:

i) quantitation of the T-cells that undergo homeostatic proliferations: slow (with partial CFSE dilution and maintenance of the naïve phenotype) and fast homeostatic proliferation (with full CFSE dilution and acquisition of memory phenotype)

In a preferred embodiment the second method of the invention also comprises:

ii) the characterization and, optionally quantification, of different cellular markers in both types of proliferated cells including at least those related with differentiation and maturation (CD45RA,CD27,CCR7), activation (HLA-DR,CD25), survival (BCL-2), apoptosis-prone (CD95) or homing (β7-integrin).

A **fifth aspect** of the present invention relates to a method to **characterize** and classify a human subject suffering from lymphopenia-related disease, hereinafter third method of the invention, comprising the steps according to the first and/or the second method of the invention, and further comprising:

iii) assigning the subjects from steps (i-ii) preferably attending to frequencies of slow and fast homeostatic proliferation and to the ratio between these types of homeostatic proliferation.

A **sixth aspect** of the invention relates to a specific/autologous generated regulatory T cell during the HP process, hereinafter regulatory T cell of the invention, obtainable by the steps as described in the first method of the invention, and furthermore comprising the cell sorting of such cells.

Specifically, cellular markers like CD25, CD45RA and the levels of CFSE could be used to get a cellular fraction enriched in such regulatory T-cells, although other cellular marker associated to these cells like CTLA-4, FoxP3, CD39 or CD141 could be also used

A **seven aspect** of the invention relates to a composition, hereinafter composition of the invention, comprising a regulatory T cell or regulatory T cell population of the invention.

An **eight aspect** of the invention relates to the specific/autologous regulatory T cell of the invention, and/or the regulatory T cell population of the invention, for use in therapy.

A **ninth aspect** of the present invention relates to the specific/autologous regulatory T cell of the invention, and/or the regulatory T cell population of the invention, for use in the treatment, amelioration or prevention of autoimmune and inflammatory events associated with lymphopenia-related diseases.

A tenth **eleventh aspect** of the invention relates to use of the *in vitro* model of the invention for evaluating a pharmacological and/or chemical product.

The pharmacological and/or chemical product derived from this approach potentially impacting in the homeostatic proliferation process, and are useful for in the treatment, amelioration or prevention of autoimmune and inflammatory events associated with lymphopenia-related diseases.

DESCRIPTION OF THE FIGURES

Figure 1: Schematic representation of purity controls of cellular subsets isolations.

Figure 2: Schematic diagram of the gating strategy.

Figure 3: T-cell viability in different culture conditions. **a)** naïve CD4 T-cells stimulated with aAPCs (1/1 ratio aAPC/Naïve); **b)** naïve CD4 T-cells stimulated with rIL7 (10 ng/ml); **c)** naïve CD4 T-cells stimulated with both, aAPCs (1/1 ratio aAPC/Naïve) and rIL7 (10 ng/ml); **d)** naïve CD4 T-cells without stimuli. Data are represented as median value and standard deviation (SD). Data are representative of at least 3 independent experiments

Figure 4: Effect of different homeostatic stimuli on proliferation of human naïve CD4 T-cells and representative plots. a) naïve CD4 T-cells stimulated with aAPC (1/1 ratio aAPC/Naïve); **b)** naïve CD4 T-cells stimulated with rIL7 (10 ng/ml); **c)** naïve CD4 T-cells stimulated with aAPC (1/1 ratio aAPC/Naïve) and rIL7 (10 ng/ml). Plots (right panels) are representative of the 10^{th}, 22^{th} and 10^{th} days of culture, respectively. Bars data on left panels represent median value and standard deviation (SD). Fast homeostatic proliferation, slow homeostatic proliferation and non-proliferating T-cells are highlighted in right panels using red, green and blue boxes and lines, respectively.

Figure 5: Effect of different aAPC/Naïve ratios and rIL7 concentrations on both types of homeostatic proliferation. a) effect of different aAPC/Naïve ratios on homeostatic proliferation process (with 2.5 ng/ml rIL7); **b)** effect of different concentrations of rIL7 (0-10 ng/ml) on homeostatic proliferation process (with 1/1 ratio aAPC/Naïve). Bars data are represented as median value Data are representative of at least 3 independent experiments.

Figure 6: Immunophenotyping of the homeostatic proliferation process. a) cycling-marker (Ki67) upregulation during homeostatic proliferation; **b)** rIL7-related upregulation of MFI BCL2 during homeostatic proliferation. Data are represented as median value and standard deviation (SD). Fast homeostatic proliferation, slow homeostatic proliferation and non-proliferating T-cells are highlighted using red, green and blue dots, respectively.

Figure 7: CD4 T-cell subsets generation during homeostatic proliferation. Data are represented as median value; **a)** CD4 T-cell subsets generated by slow homeostatic proliferation; **b)** CD4 T-cell subsets generated by fast homeostatic proliferation, **c)** CCR7 expression in memory cells generated by fast homeostatic proliferation, **d)** CD95 expression in in memory cells generated by fast homeostatic proliferation. Data are representative of 3 independent experiments.

Figure 8: OX40 and α4-integrin upregulation during the homeostatic proliferation (HP) of human naïve CD4 T-cells. Purified naïve CD4 T-cells were culture in presence of rIL7 (10

ng/ml) and irradiated autologous APCs (1/1 ratio). At baseline and after 10 days, the expression of the different cellular markers was evaluated by flow cytometry. Cytometry plot of: **A)** OX40, **B)** α4-integrin upregulation during HP. Plots are representative of at least 3 independent experiments.

Figure 9: **HLA-DR, CD25, β7-integrin and α4-integrin upregulation during the homeostatic proliferation (HP) of human naïve CD4 T-cells**. Purified naïve CD4 T-cells were culture in presence of rIL7 (10 ng/ml) and irradiated autologous APCs (1/1 ratio). At baseline and after 10 days, the expression of the different cellular markers was evaluated by flow cytometry. Cytometry plot and data of: **A)** HLA-DR, **B)** CD25 and **C)** β7-integrin in the different cellular fractions after HP. Data are expressed as mean value and standard deviation (SD).

Figure 10: Human homeostatic proliferation generates regulatory T cell (Treg); a) representative gating plot of FoxP3 upregulation during homeostatic proliferation; **b)** representative gating plot of generated total, resting and activated Treg; **c)** representative histograms of TGF-β production by the different Treg subsets; **d)** negative correlation between the frequencies of generated activated Tregs and the size of fast homeostatic proliferation; **e)** total Treg generation in different APCs/Naïves CD4 T-cell ratios; **f)** total Treg generation in the different cellular subsets. Data are expressed as mean value and standard deviation (SD).

Figure 11: Generated regulatory T-cells show *in vitro* suppressive capacity; Sorted Treg subsets were seeded with autologous PBMCs in different Tconv/Treg ratios and then stimulated. The suppression capacity of the in vitro generated regulatory T-cells was assayed evaluating the expression of activation markers (CD69 and CD154) on conventional $CD25^-$ CD4 T-cells after stimulation. a) expression of CD154 on $CD25^-$ CD4 T-cells; b) expression of CD69 on $CD25^-$ CD4 T-cells. Results are representative of 5-6 independent experiments.

Figure 12: Scale-up of coculture system; we seeded increased numbers of naïve CD4 T-cells and aAPCs with a fixed 1:1 aAPC/Naïve ratio in the presence of 2.5 ng/ml rIL7. a) frequency of generated $CD25^{high}FoxP3^{high}$ Tregs; b) frequency of generated resting Tregs ($CD45RA^{pos}FoxP3^{pos}$); c) frequency of generated activated Tregs ($CD45RA^{neg}FoxP3^{high}$); d) Absolute numbers of generated $CD25^{high}FoxP3^{high}$ Tregs; e) absolute numbers of generated resting Tregs ($CD45RA^{pos}FoxP3^{pos}$); e) absolute numbers of generated activated Tregs ($CD45RA^{neg}FoxP3^{high}$). Graphs are representative of one experiment.

Figure 13: Frequency of Viable T-cells in the different culture conditions from the scale-up. Frequencies of viable T-cells were determined after counting absolute viable T-cell numbers and total T-cell numbers.

DETAILED DESCRIPTION OF THE INVENTION

The examples of the present invention show a new *in vitro* model based on a cellular culture of human lymphocytes exposed to natural and autologous homeostatic stimuli, which allows simultaneously studying both types of homeostatic proliferation (fast and slow HP) in response to homeostatic stimuli.

The *in vitro* method allows to reproduce simultaneously both types of homeostatic proliferation process of human naïve CD4 T-cells, as well as to reproduce several *in vivo* observed phenomena related to such homeostatic proliferation. Hence, the *in vitro* model of the present invention can be a useful tool to explore specific features of human homeostatic proliferation process in different lymphopenic human conditions and diseases.

Then, in **one aspect**, the present invention relates to the use of antigen-presenting cells (APCs), preferably autologous antigen-presenting cells (aAPCs), for studying the homeostatic proliferation mechanisms in humans.

The antigen-presenting cells (APCs) are cells that display antigens soaked with major histocompatibility complexes (MHCs) on their surfaces; this process is known as antigen presentation. These cells process antigens and present them to T-cells. T cells may recognize these complexes using their T cell receptors (TCRs). Preferably these APCs are of autologous origin and are isolated from each subject.

The antigens that present these cells could be self-antigens or exogenous-antigens. Moreover, a fraction of such exogenous antigens are derived from commensal organisms (commensal antigens) and are tolerated by immune system. An important source of these commensal antigens is the microbioma of each individual. Both self- and commensal antigens have been reported as triggers of fast HP. The present invention, instead of other types of stimulation like anti-CD3/CD28 and in vitro generated APCs, use autologous APCs that potentially expose the self- and commensal-antigens present in each subject. This is important because:

- the relative frequencies in the different host APCs subsets varies,

- the functionality of the cells is variable (activated, not activated, more or less mature, etc ...) which affect to their presentation capacity,

- the type of antigens that are presented by these cells, which are highly variable between subjects.

The APCs is understood to comprise cells that display antigens soaked with major histocompatibility complexes (MHCs) on their surfaces. Methods for the identification of cells that display antigens soaked with major histocompatibility complexes (MHCs) on their surfaces are well known in the state of the art, for example, but without limitation, by cytometry.

In a preferred embodiment of this aspect of the invention, the APCs fraction comprises all B cells, dendritic cells (DC), myeloid derived suppressor cells (MDSC), plasmacytoid dendritic cells (pDCs), monocites and any other potential antigen-presenting cells speciffically present in each individual (donor for the experiment).

METHOD OF IDENTIFY THE HOMEOSTATIC PROLIFERATION

A **second aspect** of the present invention relates to a method to evaluate the homeostatic proliferation of T-cells of human subjects using their autologous APCs (aAPCs) as previously described , hereinafter first method of the invention, comprising:

a) Isolation of two cellular subsets and control of purity from a human biological sample, wherein:

III. Cellular subset 1 comprising aAPCs, and

IV. Cellular subset 2 comprising naïve CD4 T-cells,

b) Irradiate the aAPCs of the cellular subset 1,
c) Dye the naïve CD4 T-cells with a fluorescent marker (preferably with CFSE) for cell proliferation tracking,
d) Cryopreservation of several aliquots of irradiated aAPCs of the step (b),
e) Cultivate the naïve CD4 T-cells from cellular subset 2 of step (c) in a plate and add the aAPCs of the cellular subset 1 and rIL7,
f) Perform the basal analysis (day 0) in an aliquot of the cell culture of step (e),
g) Refresh supplemented medium and thawed irradiated aAPCs from the aliquots of step (d) and rIL7 for re-stimulate the culture each 5 days of culture, and
h) Culture for a total of 10-15 days, preferably 10 days.

In a preferred embodiment of the present aspect of the invention, the aAPCs are obtained after depleting T-cells (CD3+) and NK cells (CD56+).

In another preferred embodiment of the present aspect of the invention, the naïve CD4 T-cells are obtained after an enrichment of CD4 T-cells and a subsequent depletion of CD45RO+ cells.

In another preferred embodiment of the present aspect of the invention, the aAPCs are irradiated in a γ-irradiator at dosis of 15 Gy to 35 Gy, preferably 20 Gy to 30 Gy, and more preferably at 25 Gy (25J/kg).

In another preferred embodiment of the present aspect of the invention, the naïve CD4 T-cells are stained with carboxyfluorescein succinimidyl ester (CFSE).

In another preferred embodiment of the present aspect of the invention, the concentration of naïve CD4 T-cells in the culture medium is about $1x10^6$ cells/mL.

In another preferred embodiment of the third aspect of the invention, the irradiated aAPCs and the naïve CD4 T-cells in culture ranges in a ratio from 1:1 to 1:40, more preferably is about 1:1.

A person skill in the art, changing the ratios, could modulate the production of regulatory T cells, for example, using aAPCs and naïve CD4 T-cells ratios from 2:1 to 10:1 (or more) for increasing the production of regulatory T cell.

This ratio could be varied in order to modify homeostatic conditions in the cell culture.

In another preferred embodiment of the third aspect of the invention, the concentration of rIL7 ranges between 0 to 10 ng/ml, and more preferably is about 2.5 ng/ml.

A **third aspect** of the present invention relates to the *in vitro* model for studying homeostatic proliferation, hereinafter *in vitro* model of the invention, comprising the two cellular subsets as described in the first method of the invention.

As described in this memory, an "*in vitro* model for studying homeostatic proliferation" means an *in vitro* representation of this particular phenomenon making this process easier to understand and to get conclusions.

METHOD OF CLASSIFICATION OF INDIVIDUALS SUFFERING FROM A LYMPHOPENIA-RELATED DISEASE OR A DISEASE ASSOCIATED WITH LYMPHOPENIA

A **fourth aspect** of the present invention relates to a method for obtaining data that can be used for the characterization and classification of human subjects suffering from a lymphopenia-related disease, hereinafter second method of the invention, comprising the steps according to the first method of the invention, and also comprising:

i) quantitation of the T-cells that undergo homeostatic proliferations: slow (with partial CFSE dilution and maintenance of the naïve phenotype) and fast homeostatic proliferation (with full CFSE dilution and acquisition of memory phenotype).

In a preferred embodiment the second method of the invention also comprises:

ii) the characterization of different cellular markers in both types of proliferated cells including, at least, those related with differentiation and maturation (CD45RA,CD27,CCR7), activation (HLA-DR,CD25), survival (BCL-2),apoptosis-prone (CD95) or homing (β7-integrin).

Then, the cellular markers of step ii) are related with differentiation, maturation, activation, survival or death or homing, or combinations thereof, and are known in the state of the art.

In the present invention a "lymphopenia-related disease" means a human disorder characterized or showing an abnormally low level of peripheral lymphocytes in blood.

Asthma, an allergic disease, transplant rejection and an autoimmune disease have one fundamental principle in common, they all are triggered by an imbalanced immune system which reacts hyperactive against a specific exogenic and/or endogenic challenge and therewith contributes significantly to the disease status.

It is generally accepted that such aberrations of the immune system have a common pathophysiological mechanism triggered by hyper-responsive effector T cells playing a central role in immune reactivity. Effector T cell-directed immunomodulation therefore is the key to successful treatment of asthma, an autoimmune condition, prevention of graft vs. host disease (GVHD) and prevention of graft rejection.

T lymphocytes, designated as regulatory T cells control immune responses by suppressing the effector function of $CD4^+$ T cells and $CD8^+$ T cells.

Then, in another preferred embodiment the lymphopenia-related disease is selected from the group consisting on: autoimmune diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis or insulin-dependent diabetes mellitus, inflammatory diseases like Crohn's disease, allergic or asthmatic condition, graft versus host disease or graft rejection, or combinations thereof.

In the present invention a regulatory T cell (Treg) means a CD4 T-cell subset which has a suppressive function over other immune cells preventing the excessive cellular activation and the occurrence of inflammation and autoimmune disorders.

In a more preferred embodiment the lymphopenia-related disease could be caused by the Human Immunodeficiency Virus (HIV) infection, which has been associated with several autoimmune and inflammatory disorders. In another preferred embodiment the lymphopenia-related disease is selected from potential rejection of a solid organ transplant or potential graft versus host disease.

A **fifth aspect** of the present invention relates to a method to **characterize** and classify a human subject suffering from lymphopenia-related disease, hereinafter third method of the invention, comprising the steps according to the first and/or the second method of the invention, and further comprising:

iii) assigning the subjects from steps (i-ii) preferably attending to frequencies of slow and fast homeostatic proliferation and to ratio between these types of homeostatic proliferation.

In a preferred embodiment the lymphopenia-related disease is selected from the group consisting on: autoimmune diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis or insulin-dependent diabetes mellitus, inflammatory diseases like Crohn's disease, allergic or asthmatic condition, graft versus host disease or graft rejection, or combinations thereof.

In a more preferred embodiment the lymphopenia-related disease is the Human Immunodeficiency Virus (HIV) infection.

In another preferred embodiment the lymphopenia-related disease is selected from potential rejection of a solid organ transplant or potential graft versus host disease.

CELL, CELL POPULATION AND COMPOSITION OF THE INVENTION

A **sixth aspect** of the invention relates to a specific/autologous generated regulatory T cell during the HP process, hereinafter regulatory T cell of the invention, obtainable by the steps as described in the first method of the invention, and optionally comprising the cell sorting of such cells. Preferably, cellular markers like CD25, CD45RA and the levels of CFSE could be used to get a cellular fraction enriched in such regulatory T-cells, although other marker associated to these cells like CTLA-4, FoxP3, CD39 or CD141 could be also used

A **seventh aspect** of the invention relates to a specific/autologous regulatory T cell population, hereinafter regulatory T cell population of the invention, obtainable by the steps as described in the first method of the invention, and optionally comprising cell sorting.

The regulatory T cell population of the invention comprises at least one regulatory T cell of the invention. More preferably, the regulatory T cell population is substantially pure.

Preferably, said population of the invention is a cell population in which 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 96%, at least about 97%, at least about 98%, or at least about 99%, of the cells are regulatory T cell of the invention. In other words, in some embodiments 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 96%, at least about 97%, at least about 98%, or at least about 99%, of the cells in the population are regulatory T cells of the invention. More preferably the regulatory T cell and/or the regulatory T cell population of the invention are isolated.

The term "isolated" indicates that the regulatory T cell or regulatory T cell population to which it refers is not within its natural environment. The regulatory T cell or regulatory T cell population has been substantially separated from surrounding tissue. In some embodiments, the cell or cell population is substantially separated from surrounding tissue if the sample contains at least about 75%, in some embodiments at least about 85%, in some embodiments at least about 90%, and in some embodiments at least about 95% regulatory T cells. In other words, the sample is substantially separated from the surrounding tissue if the sample contains less than about 25%, in some embodiments less than about 15%, and in some embodiments less than about 5% of materials other than the regulatory T cells. Such percentage values refer to percentage by weight or by cell number. The term encompasses cells which have been removed from the organism from which they originated, and exist in culture. The term also encompasses cells which have been removed from the organism from which they originated, and subsequently re-inserted into an organism. The organism which contains the re-inserted cells may be a different organism, i.e. a different individual of the same species, or even still more preferable the same individual or organism from which the cells were removed.

An **eight aspect** of the invention relates to a composition, hereinafter composition of the invention, comprising a regulatory T cell or regulatory T cell population of the invention.

In a preferred embodiment, the composition of the invention may comprise 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 96%, at least about 97%, at least about 98%, or at least about 99%, of the

regulatory T cells of the invention, either calculated by number, or by weight or by volume of the composition.

In another preferred embodiment, the composition of the invention also comprises a pharmaceutically acceptable vehicle and/or a pharmaceutically acceptable excipient. In another preferred embodiment, the composition of the invention is a pharmaceutical composition.

As used herein, the term "active ingredient", "active substance", "pharmaceutically active substance", "active ingredient" or "pharmaceutically active ingredient" means any component which potentially provides a pharmacological activity or another different effect in diagnosing, curing, mitigating, treating, or preventing a disease, or which affects the structure or function of the human body or body of other animals. Examples of active ingredients of biological origin include growth factors, hormones, and cytokines. A variety of therapeutic agents is known in the art and may be identified by their effects. Certain therapeutic agents are capable of regulating cell proliferation and differentiation. Examples include chemotherapeutic nucleotides, drugs, hormones, non-specific (non-antibody) proteins, oligonucleotides (e.g., antisense oligonucleotides that bind to a target nucleic acid sequence (e.g., mRNA sequence)), peptides, and peptidomimetics.

The pharmaceutical compositions of the present invention can be used in a treatment method in an isolated manner or together with other pharmaceutical compounds.

The term "pharmaceutically acceptable excipient" as used here refers to the fact that it must be approved by a regulatory agency of the federal government or a national government or one listed in the United States Pharmacopoeia or the European Pharmacopoeia, or some other pharmacopoeia generally recognized for use in animals and in humans. The term "vehicle" relates to a diluent, excipient, carrier or adjuvant with which the regulatory T cells of the invention, as well as the cells of the cell population of the invention, must be administered; obviously, said vehicle must be compatible with the cells. Illustrative, non-limiting examples of said vehicle include any physiologically compatible vehicle, for example isotonic solutions (e.g. sterile saline solution (0.9% NaCl), phosphate -buffered saline solution (PBS), Ringer-lactate solution, etc.), optionally supplemented with serum, preferably with autologous serum; culture media (e.g. DMEM, RPMI, McCoy, etc.); or, preferably, a solid, semisolid, gelatinous or viscous support medium, such as collagen, collagen-glycosamine-glycan, fibrin, polyvinyl chloride, poly-amino acids, such as polylysine, or polyornithine, hydrogels, agarose, dextran sulphate silicone. Moreover, if desired, the support medium can, in special embodiments, contain growth factors or other agents. If the support is solid, semisolid, or gelatinous, the cells can be introduced in a liquid phase of the vehicle that is treated subsequently so that it is converted into a more solid

phase. In some embodiments of the invention in which the vehicle has a solid structure, said vehicle can be configured according to the form of the lesion.

The pharmaceutical composition of the invention can, if desired, also contain, when necessary, additives for increasing and/or controlling the desired therapeutic effect of the cells, e.g. buffering agents, surface-active agents, preservatives, etc. The pharmaceutically acceptable carrier may comprise a cell culture medium which supports the cells' viability. The medium will generally be serum-free in order to avoid provoking an immune response in the recipient. The carrier will generally be buffered and/or pyrogen free. Also, for stabilizing the cellular suspension, it is possible to add chelating agents of metals. The stability of the cells in the liquid medium of the pharmaceutical composition of the invention can be improved by adding additional substances, such as, for example, aspartic acid, glutamic acid, etc. Said pharmaceutically acceptable substances that can be used in the pharmaceutical composition of the invention are generally known by a person skilled in the art and are normally used in the production of cellular compositions. Examples of suitable pharmaceutical vehicles are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Additional information on said vehicles can be found in any manual of pharmaceutical technology (that is, galenical pharmacy).

The pharmaceutical composition of the invention will be administered in a suitable pharmaceutical form of administration. For this, the pharmaceutical composition of the invention will be formulated according to the chosen form of administration. The formulation will be adapted to the method of administration. In a special embodiment, the pharmaceutical composition is prepared in a liquid, solid or semisolid dosage form, e.g. in the form of suspension, in order to be administered by implanting, injection or infusion to the subject needing treatment. Illustrative, non- limiting examples include formulation of the pharmaceutical composition of the invention in a sterile suspension with a pharmaceutically acceptable excipient, e.g. an isotonic solution, for example, phosphate-buffered saline solution (PBS), or any other suitable, pharmaceutically acceptable vehicle, for administration to a subject parenterally, although other routes of administration can also be used.

The administration of the pharmaceutical composition of the invention to the subject who needs it will be carried out using conventional means. In a particular embodiment, said pharmaceutical composition of the invention can be administered to the subject parenterally using suitable devices such as syringes, catheters, trocars, cannulas, etc. In all cases, the pharmaceutical composition of the invention will be administered using equipment, apparatus and devices suitable for the administration of cellular compositions and known by a person skilled in the art. In another embodiment, direct administration of the pharmaceutical composition of the invention

to the site that is intended to benefit may be advantageous. In this method, direct administration of the pharmaceutical composition of the invention to the desired organ or tissue can be achieved by direct administration (e.g. by injection, etc.) on the external surface of the affected organ or tissue by inserting a suitable device, e.g. a suitable cannula, by infusion (including reverse flow mechanisms) or by other means described in this patent or known in the art.

The pharmaceutical composition of the invention can be stored until the moment of its application by the conventional methods known by a person skilled in the art. For short-term storage (less than 6 hours), the pharmaceutical composition of the invention can be stored at or below room temperature in a sealed container, supplemented or not with a nutrient solution. Medium-term storage (less than 48 hours) is preferably carried out at 2-8 °C, and the pharmaceutical composition of the invention includes, in addition, an iso-osmotic, buffered solution. Longer-term storage is preferably carried out by means of suitable cryopreservation and storage in conditions that promote the retention of cellular function.

In a concrete embodiment, the pharmaceutical composition of the invention can be used in combination therapy. Said additional medicinal products can form part of the same pharmaceutical composition or can, alternatively, be supplied in the form of a separate composition for simultaneous or successive (sequential in time) administration relative to the administration of the pharmaceutical composition of the invention.

MEDICAL USES OF THE INVENTION

A **ninth aspect** of the invention relates to the specific/autologous generated regulatory T cell of the invention, the regulatory T cell population of the invention, and/or the composition of the invention, for use in therapy.

Differently to other approaches which expand regulatory T cells from other preexisting mature regulatory T cell, the present invention is able to generate specific/autologous regulatory T cell from non-differentiated naïve CD4 T cells through a homeostatic process, which constitutes a important advantage in comparison to others.

A **tenth aspect** of the present invention relates to the specific/autologous generated regulatory T cell of the invention, the regulatory T cell population of the invention, and/or the composition of the invention, for use in the treatment, amelioration or prevention of a lymphopenia-related disease.

As used in the present invention, a lymphopenia-related disease is a human disorder characterized or showing an abnormally low level of peripheral lymphocytes in blood.

In a preferred embodiment the lymphopenia-related disease is selected from the group consisting on: autoimmune diseases, inflammatory diseases, allergic or asthmatic condition, graft versus host disease or for preventing graft rejection, or combinations thereof.

In a more preferred embodiment the lymphopenia-related disease is the Human Immunodeficiency Virus (HIV) infection. Human Immunodeficiency Virus (HIV) infection is characterized by a progressive loss of CD4 T-cells that hampers the function of the immune system. As a result, those afflicted with the syndrome are susceptible to a variety of opportunistic infections. The etiologic agent is a cytopathic retrovirus designated the human immunodeficiency virus (HIV). One of the major targets of the HIV in humans is T helper cells (CD4+ cells). The infection of T helper cells by HIV results in a profound dysregulation of the immune system including both depleted numbers and impaired functioning of T lymphocytes.

The specific regulatory T cell population according to claim that could be obtained using APCs and the naïve CD4 T-cells from the donor of the solid organ for transplantation, or using APCs and the naïve CD4 T-cells from the recipient of the solid organ for transplantation.

In another more preferred embodiment the lymphopenia-related disease is the inflammatory bowel disease.

In another preferred embodiment the lymphopenia-related disease is selected from potential rejection of a solid organ transplant or potential graft versus host disease.

The specific regulatory T cell population could be obtained using APCs and the naïve CD4 T-cells from the donor of the solid organ for transplantation, or using APCs and the naïve CD4 T-cells from the recipient of the solid organ for transplantation.

A preferred embodiment of this aspect of the invention refers to the regulatory T cell or a regulatory T cell population of the invention for use in the treatment, amelioration or prevention of transplant rejection, wherein the regulatory T cells are obtained from the host.

A preferred embodiment of this aspect of the invention refers to the regulatory T cell or a regulatory T cell population of the invention for use in the treatment, amelioration or prevention of graft versus host disease, wherein the regulatory T cells are obtained from the donor.

As shown in the examples and in the figures 12 and13, the production of regulatory T cells can be scaled, increasing their production by changing the parameters.

Along the description and claims, the word "comprises" and variants thereof do not intend to exclude other technical features, supplements, components or steps. For persons skilled in the art, other objects, advantages and features of the invention will be understood in part from the

description and in part from the practice of the invention. The following examples and drawings are provided by way of illustration and they are not meant to limit the present invention.

SCREENING METHODS

The *in vitro* model of the invention is useful for developing and testing of drugs or any therapeutic intervention in the global scenario of lymphopenia-related diseases, and more particularly, for HIV infection, trying to regulate the homeostatic proliferation process, since some immune alterations found in such scenario are congruent with an exacerbation of such process. For instance, in HIV-infected subjects known as immunodiscordants to antiretroviral treatment, who show a chronic and severe lymphopenic state despite therapeutic control of viremia.

Therefore, an **eleventh aspect** of the invention relates to use of the *in vitro* model of the invention for evaluating a pharmacological and/or chemical product.

Along the description and claims, the word "comprises" and variants thereof do not intend to exclude other technical features, supplements, components or steps. For persons skilled in the art, other objects, advantages and features of the invention will be understood in part from the description and in part from the practice of the invention. The following examples and drawings are provided by way of illustration and they are not meant to limit the present invention.

EXAMPLES OF THE INVENTION

The following specific examples provided in this patent document serve to illustrate the nature of the present invention. These examples are included only for illustrative purposes and must not be interpreted as limiting to the invention which is claimed herein. Therefore, the examples described below illustrate the invention without limiting the field of application thereof.

Samples and Culture design

Buffy coat from HIV-1 and hepatitis C virus (HCV)-seronegative blood bank donors were obtained from the *Centro Regional de Transfusión Sanguínea de Sevilla-Huelva y Banco de Tejidos* (Seville, Spain). Using these samples, we developed an *in vitro* approximation based on a cellular culture in order to study the homeostatic proliferation of human naïve CD4 T-cells in response to different homeostatic stimuli: recombinant interleukin 7 (rIL7) and autologous antigen-presenting cells (aAPCs). All experimental procedures with human blood were approved by local Ethical Committee for human research and were done according to the European Union guidelines and the Declaration of Helsinki.

Cellular subsets isolation

Briefly, peripheral blood mononuclear cells (PBMCs) were firstly obtained from *buffy coats* using a *ficoll* gradient centrifugation (2500 rpm, 30 min), and then were washed and placed in cold supplemented medium (RPMI 1640 supplemented with 10% fetal bovine serum, 1.7 mM glutamine, 100 µl/ml streptomicine and 100 U/ml peniciline). Magnetic negative isolation (StemCell, Vancouver, Canada) was used according to manufacturer's instructions to isolate cellular fractions from previously obtained PBMCs. Specifically, aAPCs were obtained after depleting T-cells (CD3+) and NK cells (CD56+) (human CD3 and CD56 positive selection kits), whereas naïve CD4 T-cells were obtaining after an enrichment of CD4 T-cells and a subsequent depletion of CD45RO+ cells (human NAÏVE CD4+ T-cell enrichment kit). Treg-depleted fraction was obtained by depletion of CD25+ cells (human CD25 positive selection kit) in the naïve T-cell fraction. A purity control was always performed after each isolation procedure by flow cytometry and only cellular fractions with a purity ≥95% were used in experiments. A representative example is shown in **Figure 1.**

CFSE staining

Isolated naïve CD4 T-cells were stained with carboxyfluorescein succinimidyl ester (CFSE) (Sigma-Aldrich, Missouri, USA) to determine lymphocyte proliferation. Briefly, up to $1x10^7$ cells/mL of naïve CD4 T-cells were stained in PBS (Phosphate-buffered saline) with an excess of CFSE (2.5 µM) during 8 minutes at 37°C. Finally, cells were washed and placed in cold supplemented medium.

Autologous antigen-presenting cells (aAPCs) irradiation

In order to avoid the response of antigen-presenting cells (APCs) to homeostatic stimuli and their potential interference on T-cell proliferation, autologous APCs were irradiated in a γ-irradiator (IBL437C, Cis bioInternational, France) at dosis of 25 Gy (25J/kg). Several aliquots of irradiated aAPCs were frozen in order to re-stimulate culture with fresh aAPCs each 5 days of cell culture. The aAPCs aliquots were frozen in fetal bovine serum with 10% v/v of Dimethil Sulfoxide (DMSO).

Culture conditions

In all culture conditions, 200.000 naïve CD4 T-cells per well were seeded in a 96-well plate (Thermo Fissher, Massachusetts, USA) with supplemented medium at a concentration of $1x10^6$ cells/mL. Then, the homeostatic stimuli (autologous APCs and rIL7) were provided. Specifically, 200.000 irradiated aAPCs were added, stablishing a 1:1 ratio of aAPCs/naïve CD4 T-cells (aAPC/Naïve ratio), although other aAPC/Naïve ratios were also tested, varying the amount of

aAPC fraction. On the other hand, rIL7 was used at a concentration between 1-10 ng/ml, whereas the condition without rIL7 only included the rIL7-carrier solution (PBS with 2% fetal bovine serum and 1 mM EDTA). All these components were placed at the 96-well plate and an aliquot was therefore used to perform the basal analyses (day 0). The culture was then placed in a culture incubator at 37°C and 5% CO_2 atmosphere. After each 5 days of culture, the half of medium volume was refreshed with new supplemented medium and thawed irradiated aAPCs and rIL7. Thus, cells were recovered from independent wells at day 5, 10 and 15 of culture, and cellular viability was determined using Trypan Blue (Sigma-Aldrich, Missouri, USA). To determine the intracellular production of TGF-β, 10 ng/ml of brefeldin (Biolegend, USA) was added 12 hours before the end of culture.

Immunophenotyping

Immunophenotyping was carried out using multiparametric flow cytometry in the general condition of culture. Briefly, cells were incubated at 4°C with surface antibodies for 20 minutes, followed by fixation and permeabilization according to the manufacturer's instructions (Foxp3/Transcription Factor Staining Buffer, Ebioscience), and subsequently incubated with intracellular antibodies for 30 minutes. Viable cells were identified using LIVE/DEAD fixable Aqua Blue Dead Cell Stain (Life Technologies, USA). Extracellular and intracellular antibodies were: anti-CD3, anti-CD27, anti-CD197/CCR7, anti-CD25, anti-HLADR, anti-FoxP3, anti-CD45RA, anti-TGF-β, anti-Ki67, anti-BCL2, anti-CD134/OX40, anti-CD95, anti-β7-integrin (BD Biosciences, USA); anti-CD49d/α4-integrin (Biolegend, USA). Fluorescence Minus One (FMO) and isotype controls were included when necessary. A schematic diagram of the gating strategy used is shown in **Figure 2**. CD4 T-cell subsets were defined as follows: naïve (T_N; $CD45RA^+CD27^+$), central memory (T_{CM}; $CD45RA^-CD27^+CCR7^+$), transitional memory (T_{TM}; $CD45RA^-CD27^+CCR7^-$), effector memory (T_{EM}; $CD45RA^-CD27^-$) and terminally differentiated (T_{EMRA}; $CD45RA^+CD27^-$). Fast homeostatic proliferation was defined as viable CD4 T-cells which fully diluted CFSE and acquired memory phenotype ($CD45RA^-$), while slow homeostatic proliferation was defined as viable $CD45RA^+$ CD4 T-cells which partially diluted CFSE, without acquiring memory phenotype. Treg subsets were defined as follows: total Treg ($CD25^{high}FoxP3^{high}$), resting Treg ($CD45RA^+FoxP3^+$) and activated Treg ($CD45RA^-FoxP3^{high}$). Flow cytometry was performed on a LSR Fortessa (BD, USA). Analysis was performed using FlowJo version 9.2 (Tree Star) and data is expressed as mean values and standard deviation (σ) of the frequencies and MFI (Mean Fluorescence Intensity). Graphs were generated using Prism version 5.0 (GraphPad Software, USA).

Cell sorting of Regulatory T-cells

We sorted generated Tregs to determine their suppression capacity. After 10 days of culture, cells were washed and stained at 4°C with surface antibodies for 20 minutes. Antibodies used were: anti-CD3, anti-CD25, anti-CD45RA, anti-CD4 (BD Biosciences, USA). Treg subsets were defined and sorted as follows: resting Treg ($CD45RA^{+}CD25^{+}$) and activated Treg ($CD45RA^{-}CD25^{high}$). Sorted was performed in a Facs Aria Fusion (BD Bioscience, USA).

Regulatory T-cell suppression assay *in vitro*

The suppression capacity of the *in vitro* generated regulatory T-cells was assayed following the manufacturer instructions of the Regularoty T-Cell Functon Kit (BD bioscience, USA). Briefly, frozen autologous PBMCs previously isolated from Buffy coat were thawed and resting overnight in a culture incubator at 37°C and 5% CO_2 atmosphere. Then, PBMCs were stimulated with Dynabeads Human T-Activator CD3/CD28 (Life Technologies, USA), and seeded at 1:2 and 1:4 sorted Treg/PBMCs and placed in a culture incubator at 37°C and 5% CO_2 atmosphere. After 7 hours, cells were collected, washed and stained with anti-CD3, anti-CD4, anti-CD45RA, anti-CD25, anti-CD154, anti-CD69 (BD Bioscience, USA). An unstimulated condition was always used as control for each experiment. Flow cytometry was performed on a LSR Fortessa (BD, USA). Analysis was performed according to the manufacturer's instructions and graphs were generated using Prism version 5.0 (GraphPad Software, USA).

Scale-up of the coculture system

In order to obtain higher numbers of regulatory T-cells, we aimed to scale-up the culture. For this purpose, we seeded increased numbers of naïve CD4 T-cells and aAPCs keeping the fixed 1:1 aAPC/Naïve ratio in the presence of 2.5 ng/ml rIL7. Specifically, we seeded $2x10^4$ cells each type in a 96-well plate, $4x10^4$ cells each type in a 48-well plate, $1.125x10^5$ cells each type in a 24-well plate and $2.25x10^5$ cells each type in a 12-well plate. The cultures were then placed in a culture incubator at 37°C and 5% CO_2 atmosphere. After each 5 days of culture, the half of medium volume was refreshed with new supplemented medium and thawed irradiated aAPCs and rIL7. After 10 days, cells were collected, washed and placed in cold supplemented medium. Finally, we performed an immunecharacterization of generated regulatory T-cells as previously described on a LSR Fortessa (BD, USA). Absolute cell numbers were calculated using the Absolute Perfect-Count Microbeads (Cytognos, Spain) according to the manufacturer's instructions. Results are shown in frequencies and absolute cell numbers. Graphs were generated using Prism version 5.0 (GraphPad Software, USA).

Results

Effects of homeostatic stimuli on cellular viability and proliferation of human naïve CD4 T-cells

We first explored the effects of each stimulus on cellular viability and proliferation over time. For this purpose, we firstly tried only one stimulus per each condition (aAPCs or rIL7). T-cells maintained a good viability in all culture conditions except when they were cultivated without any stimuli (**Figure 3**). When naïve CD4 T-cells were only stimulated with aAPCs (1/1 aAPC/Naïve ratio), we found that cellular proliferation at 5^{th} day was negligible (**Figure 4.a left**), though at 10^{th} and 15^{th} day we observed that fast HP was occurring (**Figure 4.a left**) and generating memory cells ($CD45RA^-$) (**Figure 4.a right**). On the other hand, when naïve CD4 T-cells were stimulated only with rIL7 (10 ng/ml), we did not find proliferation at 10^{th} neither 15^{th} day of culture (**Figure 4.b left**) but it was scarcely observed after 22 days of culture (**Figure 4.b left-right**). Eventually, a third condition including both stimuli was performed. In this condition, naïve CD4 T-cells underwent fast and slow HP at 10^{th} and 15^{th} day of culture (**Figure 4.c left**), generating both memory T-cells and naïve T-cells (**Figure 4.c right**). Interestingly, no enhancing effect of rIL7 was observed over fast HP regarding the time point in that such process occurred. In contrast, when naïve CD4 T-cells were stimulated with both stimuli (aAPCs and rIL7), the generation of slow H.P was significantly anticipated.

Effect of aAPC/Naïve ratio and rIL7 concentrations on both types of homeostatic proliferation

First, we performed the culture using different aAPC/Naïve ratios (1/1, 1/10, 1/20 and 1/30 ratios). When aAPC/Naïve ratio was decreased, a global reduction of total homeostatic proliferation was observed, mainly due to the reduction of fast HP whereas slow H.P seemed even to increase (**Figure 5.a**). As it can also be observed, when aAPC/Naïve ratio decreased, the presence of rIL7 significantly enhanced fast homeostatic proliferation in comparison to condition without such cytokine (**Figure 5.a**).

In order to study the effect of rIL7 concentrations on homeostatic proliferation, we used a range of rIL7 (0-10 ng/ml) in our culture (1/1 aAPC/Naïve ratio). We observed that low rIL7 concentrations were sufficient to induce slow HP (**Figure 5.b**), but a dose-dependent effect on such HP was also observed at increasing rIL7 concentrations, though a *plateau* appeared at the highest rIL7 concentrations (5-10 ng/ml). In contrast, no effect of different rIL7 concentrations was observed over fast HP with 1/1 aAPC/Naïve ratio. .

Fast homeostatic proliferation of human naïve CD4 T-cells mainly generates central memory-like cells

To explore the usefulness of our *in vitro* system, we performed an immunophenotyping of cells undergoing HP. As expected, cells that diluted CFSE upregulated the cellular-cycling marker Ki67 while cells that did not diluted CFSE scarcely expressed such marker (**Figure 6.a**). As it can be observed, the upregulation of Ki67 was higher in fast HP than in slow HP in all studied culture conditions. Moreover, Ki67 expression was slightly increased in non-proliferating cells in the presence of rIL7. Likewise, we observed a rIL7 dose-dependent upregulation of the anti-apoptotic molecule Bcl2 expression in non-proliferating naïve CD4 T-cells and cells that underwent slow HP (**Figure 6.b**), though this effect seemed to be absent in cells undergoing fast HP.

Additionally, we explored the maturational profile of cells undergoing homeostatic proliferation in our *in vitro* system. Cells undergoing slow HP maintained their naïve phenotype ($CD45RA^+$ $CD27^+$) (**Figure 7.a**), whereas cells undergoing fast HP acquired mostly central and/or transitional memory phenotype ($CD45RA^-$ $CD27^+$) (**Figure 7.b**). We observed that most cells undergoing fast HP also expressed CCR7 (**Figure 7.c**), denoting that fast HP of human naïve CD4 T-cells mainly generates central memory T-cells. Moreover, as expected, generated memory T-cells strongly acquired a constitutive expression of FAS-L receptor (CD95) (**Figure 7.d**), and also upregulated TNFR-related molecule OX40 (CD134) during the process of fast HP (**Figure 8.a**).

Cellular activation is increased during the homeostatic proliferation process

We also explored whether the HP was associated with an increase in the expression of cellular activation markers (HLA-DR and CD25). On one hand, we observed that cells undergoing fast HP strongly upregulated HLA-DR expression, whereas no effect was observed in non-proliferating naïve T-cells and a negligible upregulation seemed to occur in cells undergoing slow HP (**Figure 9.a**). As it can be observed, this effect was independent of the rIL7 concentration. Regarding CD25 expression, we also found that cells undergoing fast HP strongly upregulated such activation marker in a rIL7-independent manner (**Figure 9.b**). In contrast with HLA-DR expression, we observed a rIL7-dependent upregulation of CD25 in non-proliferating cells and cells undergoing slow HP.

Homing molecules are upregulated during the homeostatic proliferation process

Next, we explored the expression of the β7-integrin in CD4 T-cells undergoing homeostatic proliferation. We found a strong upregulation of β7-integrin in cells undergoing fast HP but in a rIL7-independent manner (**Figure 9.c**). As it can be observed, cells that underwent slow HP as

well as non-proliferating cells in the presence of rIL7 also upregulated β7-integrin. We finally explored the expression of another integrin (CD49d) related to cellular homing during the process of homeostatic proliferation. Interestingly, we observed that CD49d (α4-integrin) was upregulated during both types of HP, but mainly during the fast HP (**Figure 8.b**).

Human homeostatic proliferation generates TGF-β-producing regulatory T-cells

Finally, because of HP has been pointed as a potential source of regulatory T-cells (Treg) in murine models, we aimed to explore the generation of Treg in our *in vitro* model. For this propose, we seeded Treg-depleted naïve CD4 T-cells, and after 10 days of culture, an upregulation of Foxp3 was found in cells that undergone HP, mainly during fast HP (**Figure 10.a**). Concretely, different types of Treg were generated during HP: total Treg ($CD25^{high}Foxp3^{high}$), resting Treg ($CD45RA^{+}Foxp3^{+}$) and activated Treg ($CD45RA^{-}Foxp3^{high}$) (**Figure 10.b**). Moreover, all these generated Treg were able to produce suppressive cytokines as TGF-β, showing the activated Treg ($CD45RA^{-}Foxp3^{high}$) the highest TGF-β production, whereas resting Treg ($CD45RA^{+}Foxp3^{+}$) showed the lowest (**Figure 10.c**). Intriguingly, we also found an interesting negative association between the frequency of activated Treg generated by HP, and the size of fast homeostatic proliferation (**Figure 10.d**). Such association was not present with other types of Treg (data not shown).

Next, trying to explore how the homeostatic stimuli can impact on Treg generation during HP, we analyzed the generation of Treg ($CD25^{high}Foxp3^{high}$) in different aAPC/Naïve ratios and rIL7 concentrations. We found that Treg generation during HP was highly dependent on cellular contact with aAPC (**Figure 10.e**), but no dependence on rIL7 concentrations was observed (**Figure 10.f**). Similar results were found with the other phenotypes of Treg.

Generated regulatory T-cells show *in vitro* suppressive capacity

Since FoxP3 is a nuclear transcription factor, it could not be used for cell sorting neither definition of Treg during sorting. Thus, we sorted the regulatory T-cell generated in the culture using the expression of CD25 (Miyara. Immunity. 2009). Thus, sorted Treg subsets were resting Treg ($CD45RA^{+}CD25^{+}$) and activated Treg ($CD45RA^{-}CD25^{high}$). Both Treg subsets, resting and activated Treg showed suppressive capacity (**Fig. 11 a-b**), which showed a dose-dependent effect depending on the Treg/Tconv ratio used in each condition.

Scale-up of coculture system

In order to obtain higher numbers of regulatory T-cells, we tried to scale-up the culture system. We analyzed the frequencies and number of generated regulatory T-cells according different phenotypes such Total Treg ($CD25^{high}FoxP3^{high}$), resting Treg ($CD45RA^{+}FoxP3^{+}$) and activated

Treg ($CD45RA^{-}FoxP3^{high}$). As it can be observed in the **Figure 12**, while frequencies of all these Treg subsets were mostly similar between the different size culture conditions (**Figure 12 a-c**), greater number of cells were found as the number of cells seeded increased (**Figure 12 d-f**). Importantly, no differences were found in viability of T-cells obtained between the different size culture conditions (**Figure 13**).

CLAIMS

1.- Use of antigen-presenting cells (APCs) for studying the homeostatic proliferation mechanisms in humans.

2.- The use according to claim 1, wherein APCs is understood to comprise cells that display antigens soaked with major histocompatibility complexes (MHCs) on their surfaces.

3.- The use of autologous antigen-presenting cells (aAPCs) according to anyone of claim 1-2, wherein the APCs fraction comprises all B cells, dendritic cells (DC), myeloid derived suppressor cells (MDSC), plasmacytoid dendritic cells (pDCs), monocites and any other potential antigen-presenting cells specifically present in each individual (donor for the experiment)

4.- A method to evaluate the homeostatic potential proliferation of T-cells of a human subject using autologous APCs (aAPCs), comprising:

i) Isolating two cellular subsets and control of purity from a human biological sample, wherein:

V. Cellular subset 1 comprising aAPCs, and

VI. Cellular subset 2 comprising naïve CD4 T-cells,

a) Irradiating the aAPCs of the cellular subset 1
b) Dyeing the naïve CD4 T-cells with a fluorescent marker preferable CFSE for cell proliferation tracking
c) Cryopreserving several aliquots of irradiated aAPCs of step (b)
d) Cultivating the naïve CD4 T-cells from cellular subset 2 of step (c) in a plate and adding the aAPCs of the cellular subset 1 and rIL7,
e) Performing the basal analysis (day 0) in an aliquot of the cell culture of step (e),
f) Refreshing the supplemented medium and thawed irradiated aAPCs from the aliquots of step (d) and rIL7 for re-stimulate the culture each 5 days of culture
g) Culturing for a total of 10-15 days, preferably 10 days.

5.- The method according to claim 4, wherein the aAPCs are obtained after depleting T-cells (CD3+) and NK cells (CD56+).

6.- The method according to anyone of claims 4-5, wherein the naïve CD4 T-cells are obtained after an enrichment of CD4 T-cells and a subsequent depletion of CD45RO+ cells.

7.- The method according to anyone of claims 4-6, wherein the aAPCs are irradiated in a γ-irradiator at dosis of 20 to 30 Gy, preferably at 25 Gy (25J/kg).

8.- The method according to anyone of claims 4-7, wherein the naïve CD4 T-cells are stained with carboxyfluorescein succinimidyl ester (CFSE).

9.- The method according to anyone of claims 4-8, wherein the concentration of naïve CD4 T-cells in the culture medium is about $1x10^6$ cells/mL.

10.- The method according to anyone of claims 4-9, wherein the irradiated aAPCs and the naïve CD4 T-cells in culture are in a ratio 1:1, though this ratio could be varied (up to 1:40) in order to modify homeostatic conditions in the cell culture.

11.- The method according to anyone of claims 4-10, wherein the concentration of rIL7 ranges between 0-10 ng/ml.

12.- An *in vitro* model for studying homeostatic proliferation process comprising the two cellular subsets as described in anyone of claims 4-11.

13.- A method for obtaining data that can be used for the characterization and classification of a human subject suffering from a lymphopenia-related disease comprising the steps according to anyone of claims 4-12, and also comprises:

i) quantitation of the T-cells that undergo homeostatic proliferations: slow (with partial marker (preferably CFSE) dilution and maintenance of the naïve phenotype) and fast homeostatic proliferation (with full marker (preferably CFSE) dilution and acquisition of memory phenotype), and/or optionally

ii) characterization of different cellular markers in both types of proliferated cells including at least those related with differentiation and maturation (CD45RA,CD27,CCR), activation (HLA-DR,CD25), survival (BCL-2),apoptosis-prone (CD95) or homing (β7-integrin).

14.- A method to characterize and classify a human subject suffering from lymphopenia-related disease comprising the steps according to claims 13, and further comprising:

iii) assigning the subjects from steps (i-ii) preferably attending to frequencies of slow and fast homeostatic proliferation and to ratio between these types of homeostatic proliferation.

15.- The method according to anyone of claims 13-14, wherein the lymphopenia-related disease is selected from the group consisting on: autoimmune diseases, inflammatory diseases, allergic or asthmatic condition, graft versus host disease or for preventing graft rejection, or combinations thereof.

16.- The method according to anyone of claims 13-15, wherein the lymphopenia-related disease is an autoimmune disease selected from the group consisting on: systemic lupus erythematosus (SLE), rheumatoid arthritis, insulin-dependent diabetes mellitus, or combinations thereof.

17.- The method according to anyone of claims 13-15, wherein the lymphopenia-related disease is an inflammatory disease, preferably the Crohn´s disease.

18.- The method according to anyone of claims 13-15, wherein the lymphopenia-related disease is the Human Immunodeficiency Virus (HIV) infection.

19.- The method according to anyone of claims 13-15, wherein the lymphopenia-related disease is selected from potential rejection of a solid organ transplant or potential graft versus host disease.

20.- A regulatory T cell obtainable by a method comprising the steps as described in anyone of claims 4-11 and furthermore comprising the cell sorting.

21.- A regulatory T cell population obtainable by a method comprising the steps as described in anyone of claims 4-11 and furthermore comprising cell sorting, comprising at least a regulatory T cell as described in claim 20.

22.- A composition comprising a regulatory T cell according to claim 20 or a regulatory T cell population according to claim 21.

23.- The composition according to claim 22, also comprising a pharmaceutically acceptable vehicle and/or a pharmaceutically acceptable excipient.

24.- The composition according to anyone of claims 21-22, wherein the composition of the invention is a pharmaceutical composition.

25.- The regulatory T cell according to claim 20, the regulatory T cell population according to claim 21, and/or the composition according to anyone of claims 22-24, for use in the treatment, amelioration or prevention of a lymphopenia-related disease.

26.- The regulatory T cell, the regulatory T cell population and/or the composition for use in the treatment, amelioration or prevention of a lymphopenia-related disease according to claim 25, wherein the lymphopenia-related disease is selected from the group consisting on: autoimmune diseases, inflammatory diseases, allergic or asthmatic condition, graft versus host disease or for preventing graft rejection, or combinations thereof.

27.- The regulatory T cell, the regulatory T cell population and/or the composition for use in the treatment, amelioration or prevention of a lymphopenia-related disease according to claim 25, wherein the lymphopenia-related disease is the Human Immunodeficiency Virus (HIV) infection.

28.- The regulatory T cell, the regulatory T cell population and/or the composition for use in the treatment, amelioration or prevention of a lymphopenia-related disease according to claim 25, wherein the lymphopenia-related disease is selected from potential rejection of a solid organ transplant or potential graft versus host disease.

29.- The regulatory T cell according to claim 20, the regulatory T cell population according to claim 21, and/or the composition according to anyone of claims 22-24, for use in the treatment, amelioration or prevention of transplant rejection, wherein the regulatory T cells are obtained from the host.

30.- The regulatory T cell according to claim 20, the regulatory T cell population according to claim 21, and/or the composition according to anyone of claims 22-24, for use in the treatment, amelioration or prevention of graft versus host disease, wherein the regulatory T cells are obtained from the donor.

31.- The regulatory T cell according to claim 20, the regulatory T cell population according to claim 21, and/or the composition according to anyone of claims 22-24, for use in the treatment, amelioration or prevention of the inflammatory bowel disease.

32.- Use of the *in vitro* model for studying homeostatic proliferation according to claim 12, for screening of a therapeutic agent useful for the treatment, amelioration or prevention of a lymphopenia-related disease.

33.- The use the *in vitro* model for studying homeostatic proliferation according to claim 32, wherein the lymphopenia-related disease is selected from the group consisting on: autoimmune diseases, inflammatory diseases, allergic or asthmatic condition, graft versus host disease or for preventing graft rejection, or combinations thereof.

Figures

Fig. 1

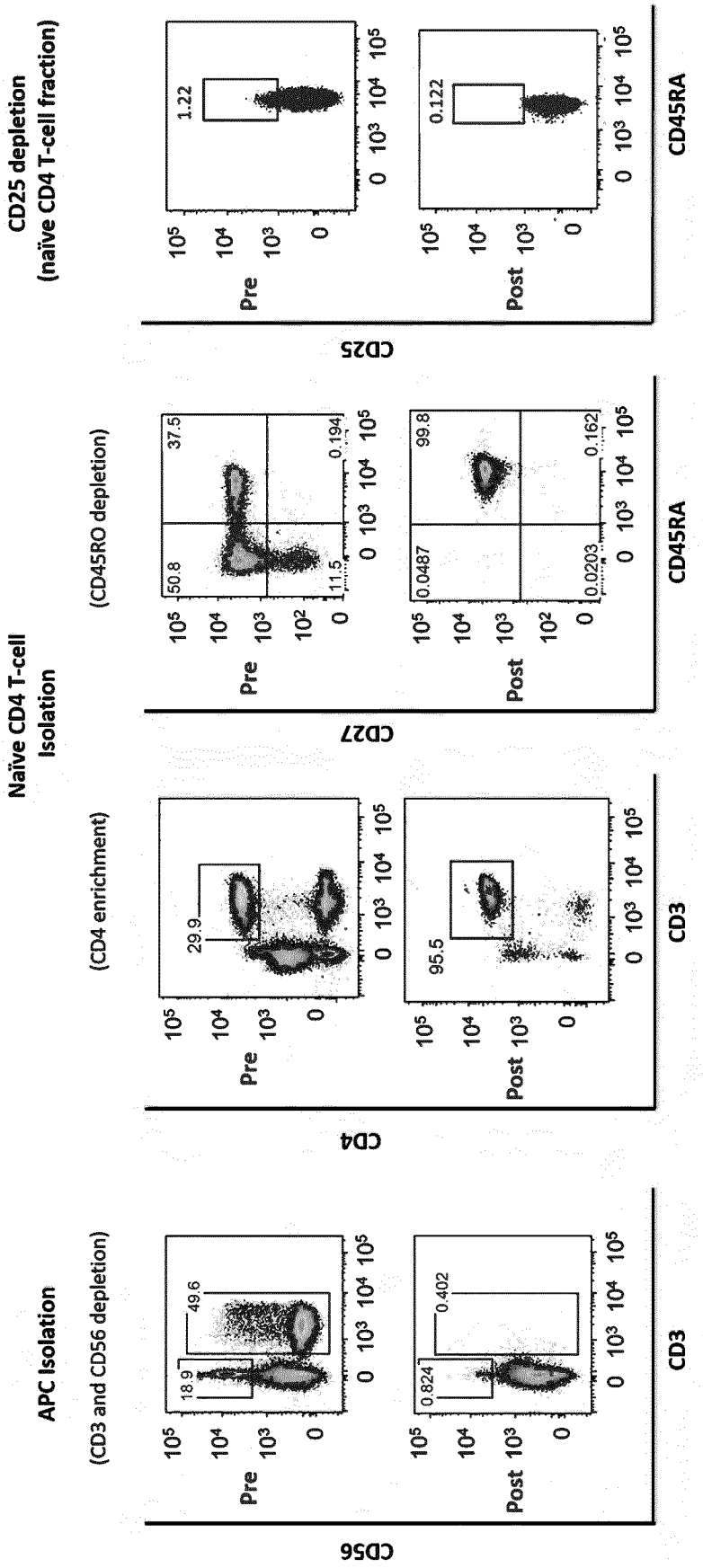


Fig. 2

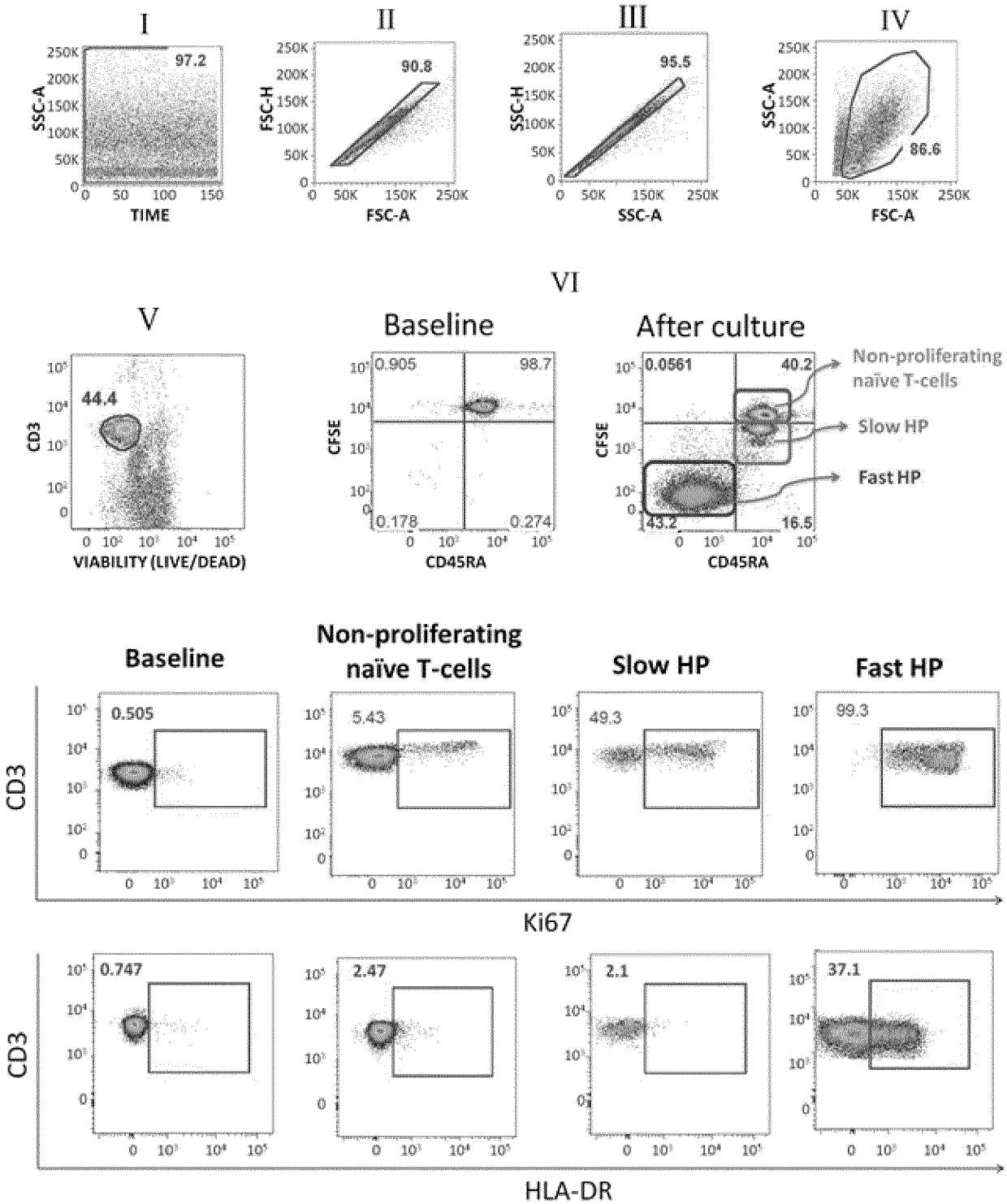
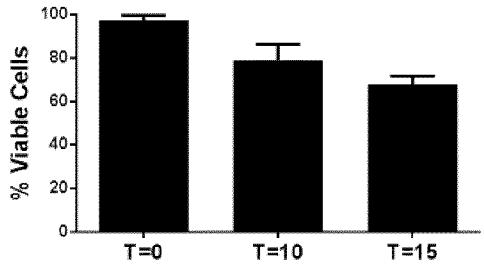
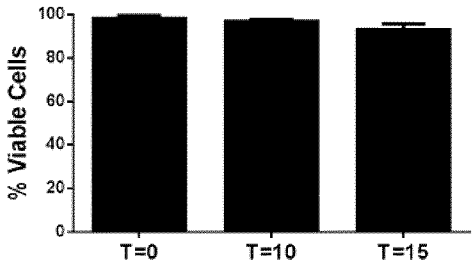

I
II
III
IV
V
VI
SSC-A
TIME
97.2
FSC-H
FSC-A
90.8
SSC-H
SSC-A
95.5
SSC-A
FSC-A
86.6
CD3
VIABILITY (LIVE/DEAD)
44.4
Baseline
After culture
CFSE
CD45RA
0.905
98.7
0.178
0.274
0.0561
40.2
43.2
16.5
Non-proliferating naïve T-cells
Slow HP
Fast HP
Baseline
Non-proliferating naïve T-cells
Slow HP
Fast HP
CD3
Ki67
0.505
5.43
49.3
99.3
HLA-DR
0.747
2.47
2.1
37.1

Fig. 3

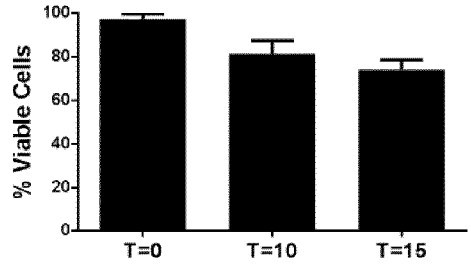
a) naïve CD4 T-cells + aAPCs



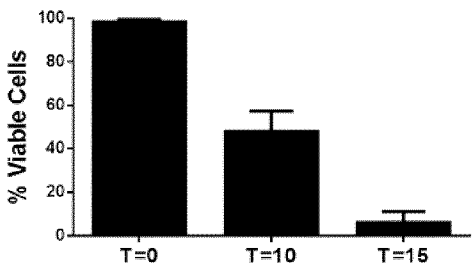
b) naïve CD4 T-cells + rIL7



c) naïve CD4 T-cells + aAPCs + rIL7



d) naïve CD4 T-cells without stimuli



INCORPORATED BY REFERENCE (RULE 20.6)

Fig. 3

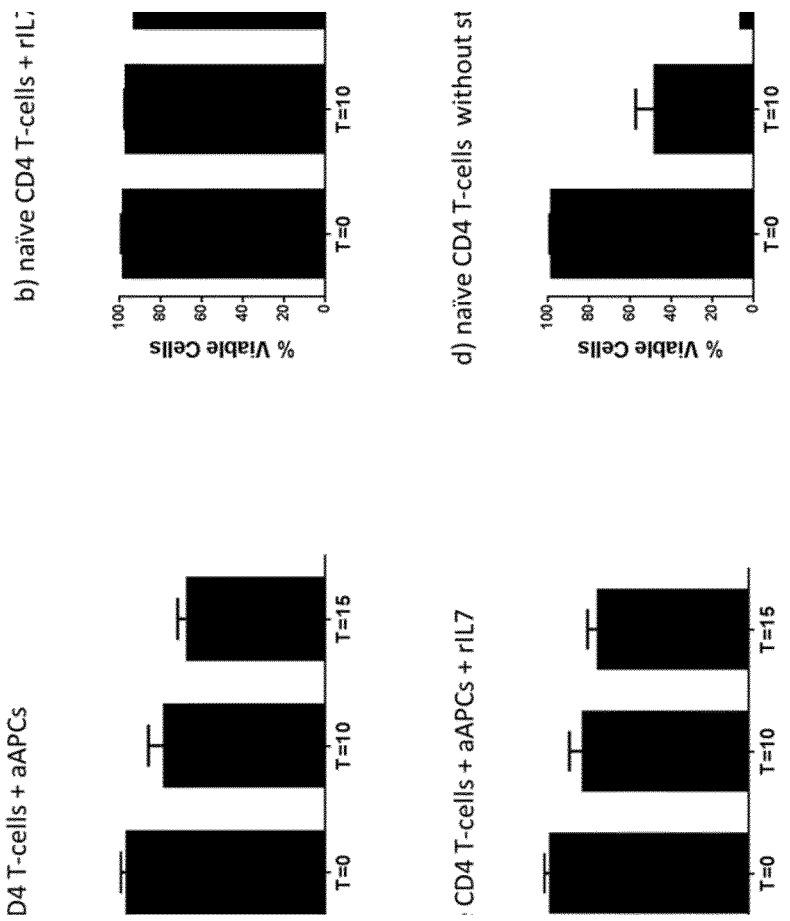

b) naïve CD4 T-cells + rIL7
% Viable Cells
100
80
60
40
20
0
T=0
T=10
d) naïve CD4 T-cells without st
% Viable Cells
100
80
60
40
20
0
T=0
T=10
D4 T-cells + aAPCs
T=0
T=10
T=15
CD4 T-cells + aAPCs + rIL7
T=0
T=10
T=15

Fig. 4

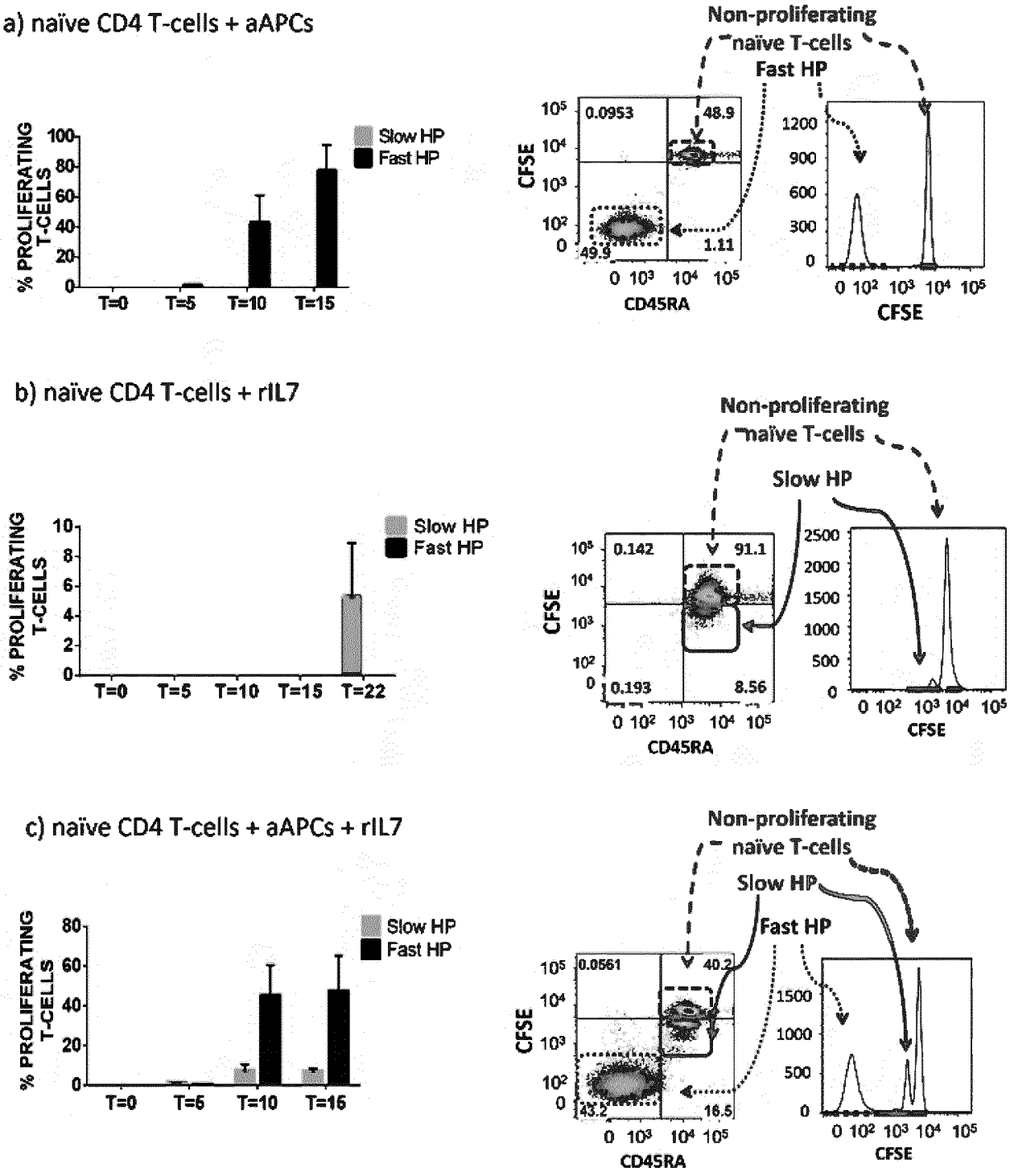

a) naïve CD4 T-cells + aAPCs
% PROLIFERATING T-CELLS
Slow HP
Fast HP
T=0
T=5
T=10
T=15
Non-proliferating naïve T-cells
Fast HP
CFSE
CD45RA
0.0953
48.9
49.9
1.11
b) naïve CD4 T-cells + rIL7
T=22
Slow HP
0.142
91.1
0.193
8.56
c) naïve CD4 T-cells + aAPCs + rIL7
0.0561
40.2
43.2
16.5

Fig.5

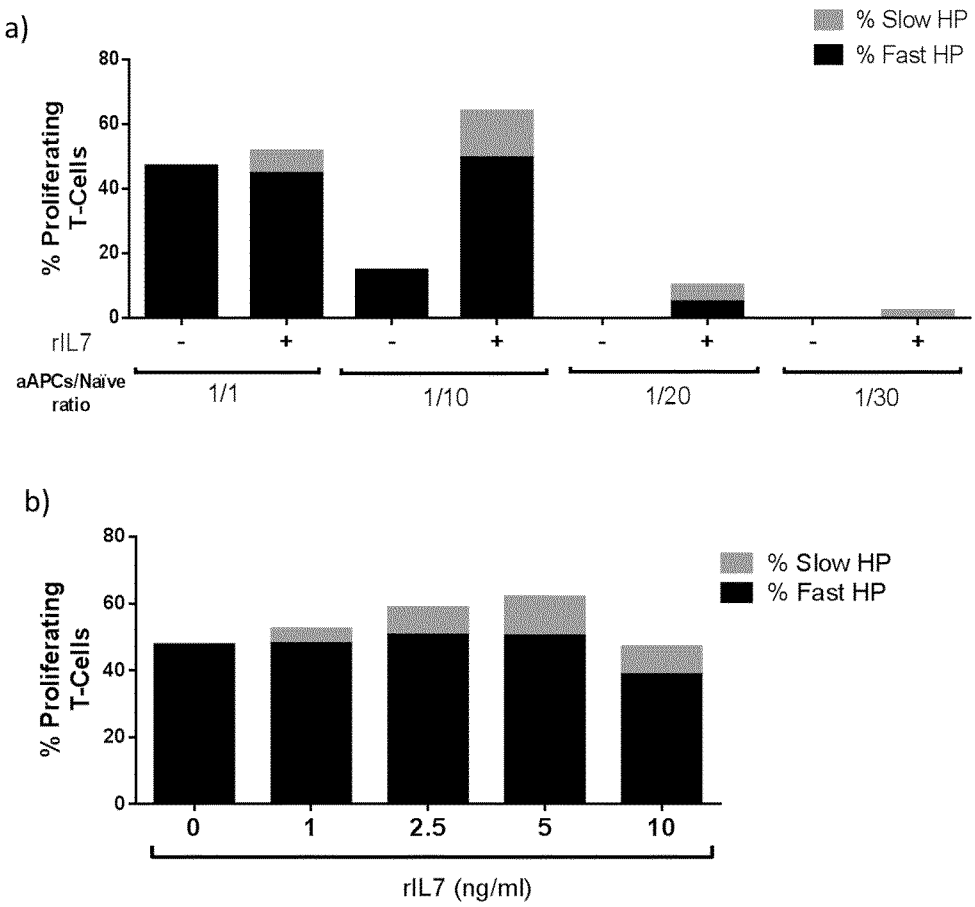

a)
% Slow HP
% Fast HP
% Proliferating T-Cells
80
60
40
20
0
rIL7
-
+
-
+
-
+
-
+
aAPCs/Naïve ratio
1/1
1/10
1/20
1/30
b)
% Slow HP
% Fast HP
% Proliferating T-Cells
80
60
40
20
0
0
1
2.5
5
10
rIL7 (ng/ml)

Fig.6

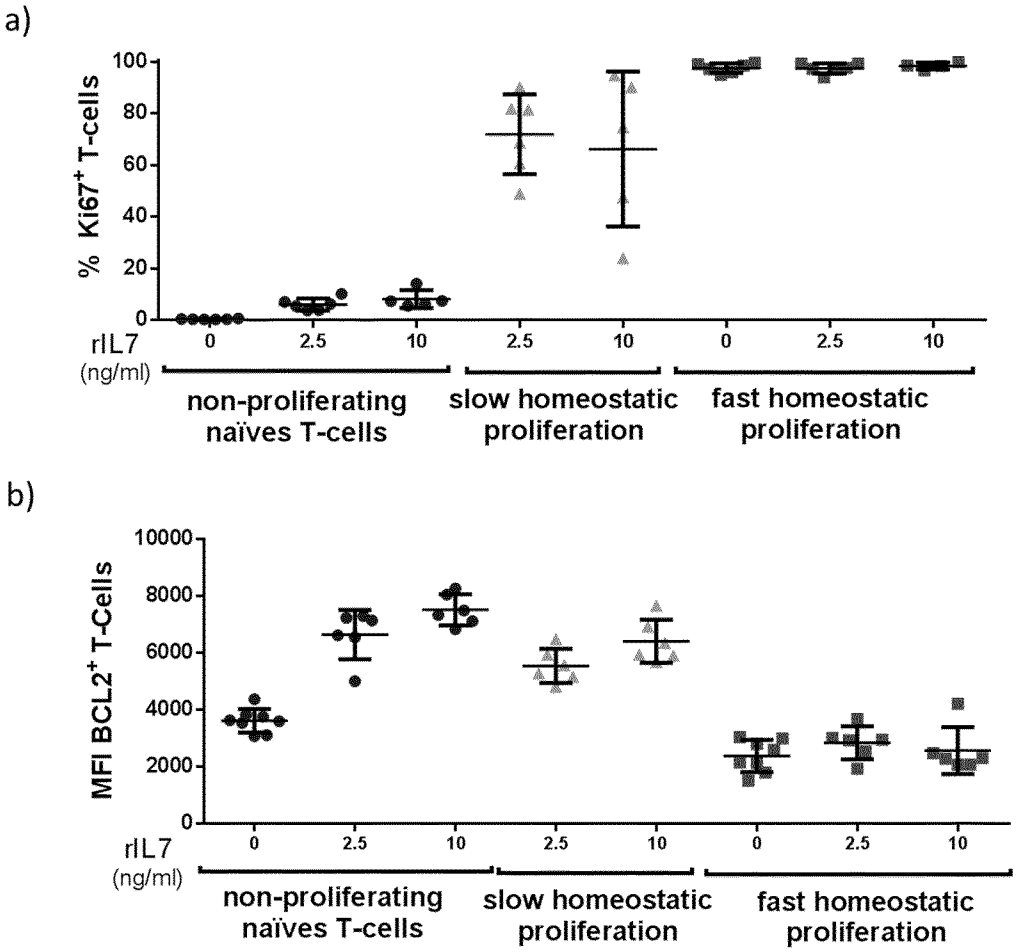

a)
% Ki67+ T-cells
100
80
60
40
20
0
rIL7
(ng/ml)
0
2.5
10
2.5
10
0
2.5
10
non-proliferating
naïves T-cells
slow homeostatic
proliferation
fast homeostatic
proliferation
b)
MFI BCL2+ T-Cells
10000
8000
6000
4000
2000
0
rIL7
(ng/ml)
0
2.5
10
2.5
10
0
2.5
10
non-proliferating
naïves T-cells
slow homeostatic
proliferation
fast homeostatic
proliferation

Fig.7

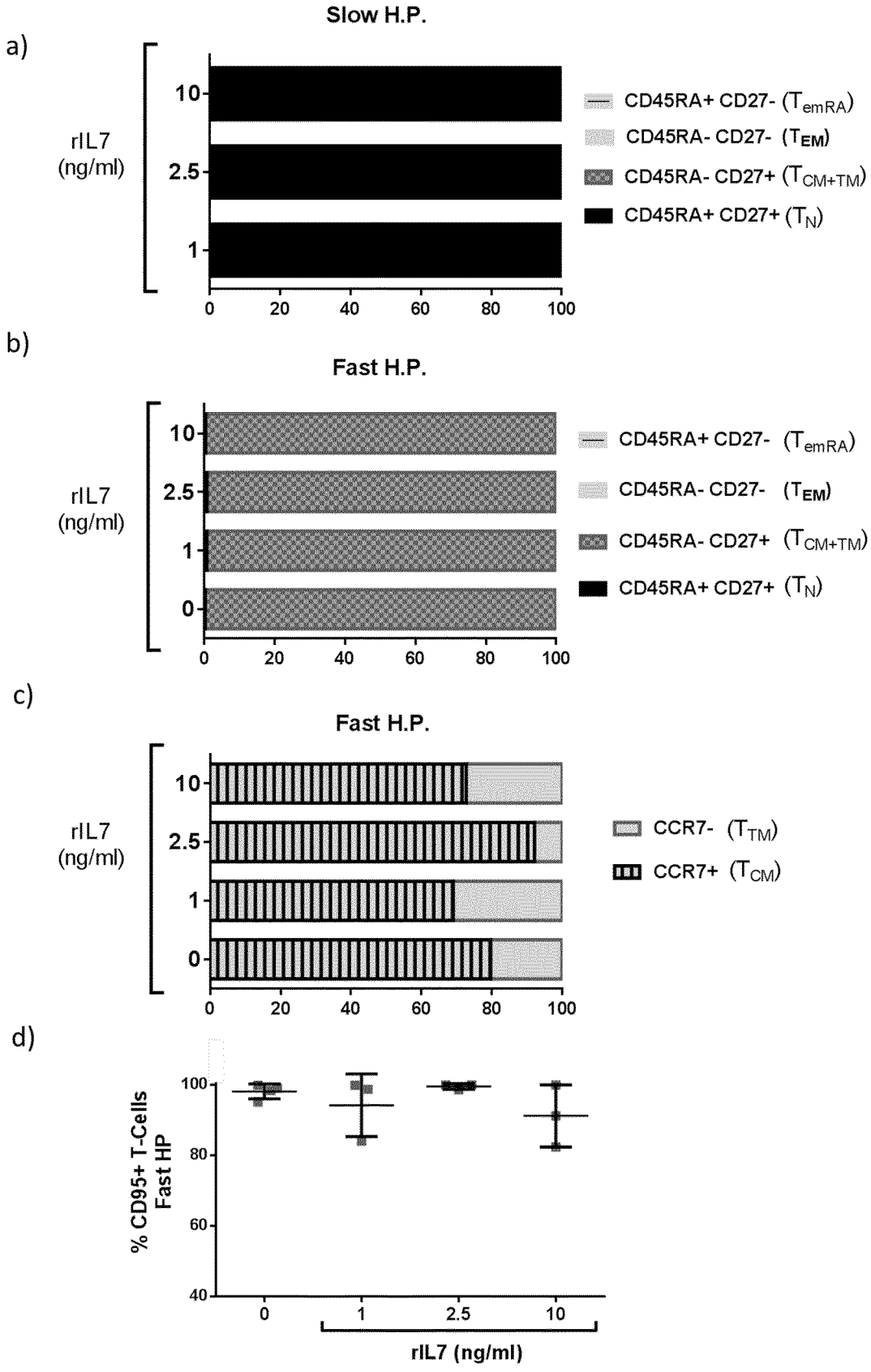

a)
Slow H.P.
rIL7 (ng/ml)
10
2.5
1
0
20
40
60
80
100
CD45RA+ CD27- (T_{emRA})
CD45RA- CD27- (T_{EM})
CD45RA- CD27+ (T_{CM+TM})
CD45RA+ CD27+ (T_N)
b)
Fast H.P.
rIL7 (ng/ml)
10
2.5
1
0
20
40
60
80
100
CD45RA+ CD27- (T_{emRA})
CD45RA- CD27- (T_{EM})
CD45RA- CD27+ (T_{CM+TM})
CD45RA+ CD27+ (T_N)
c)
Fast H.P.
rIL7 (ng/ml)
10
2.5
1
0
20
40
60
80
100
CCR7- (T_{TM})
CCR7+ (T_{CM})
d)
% CD95+ T-Cells Fast HP
100
80
60
40
0
1
2.5
10
rIL7 (ng/ml)

Fig.8

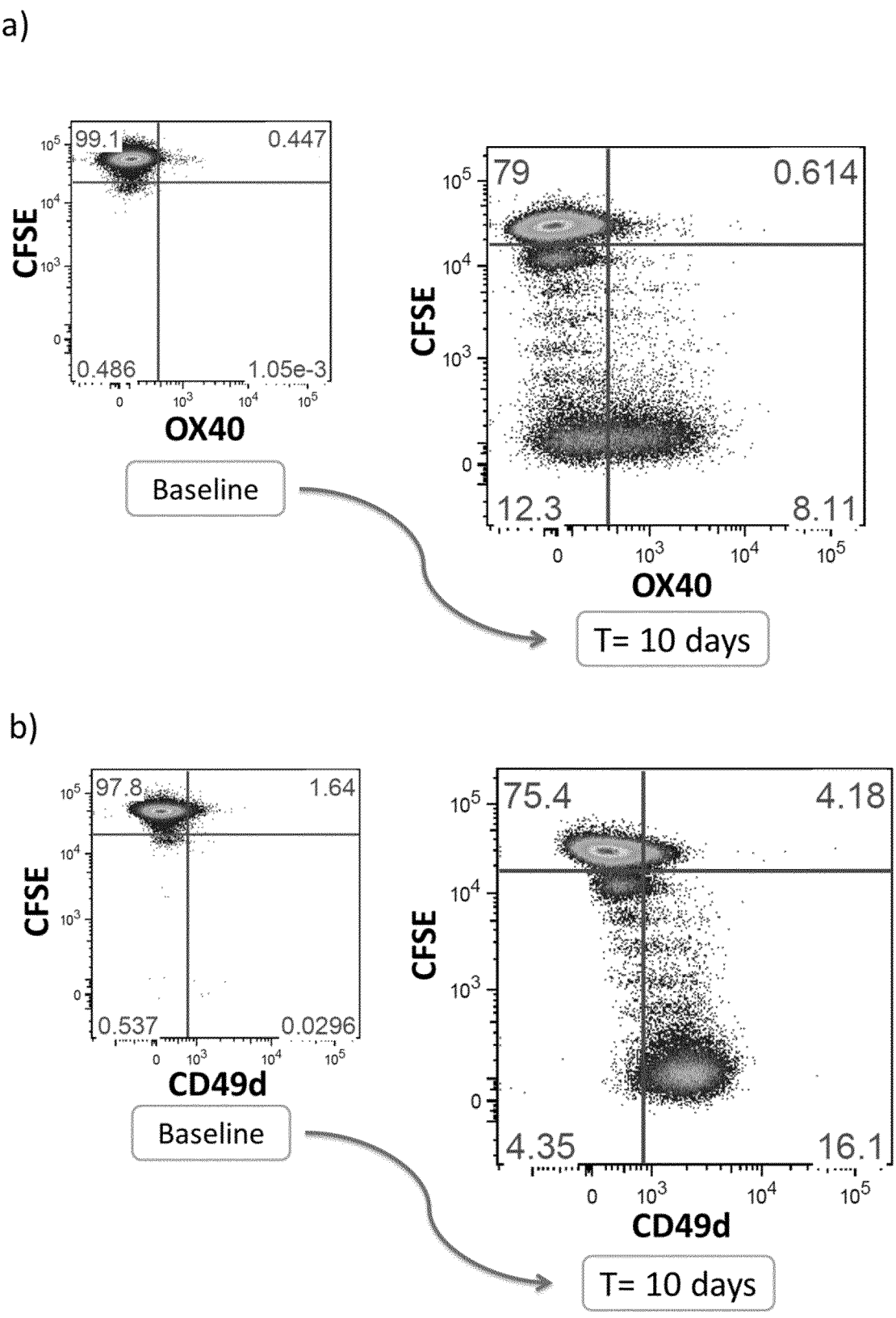

a)
99.1
0.447
0.486
1.05e-3
CFSE
OX40
Baseline
79
0.614
12.3
8.11
CFSE
OX40
T= 10 days
b)
97.8
1.64
0.537
0.0296
CFSE
CD49d
Baseline
75.4
4.18
4.35
16.1
CFSE
CD49d
T= 10 days

Fig.9

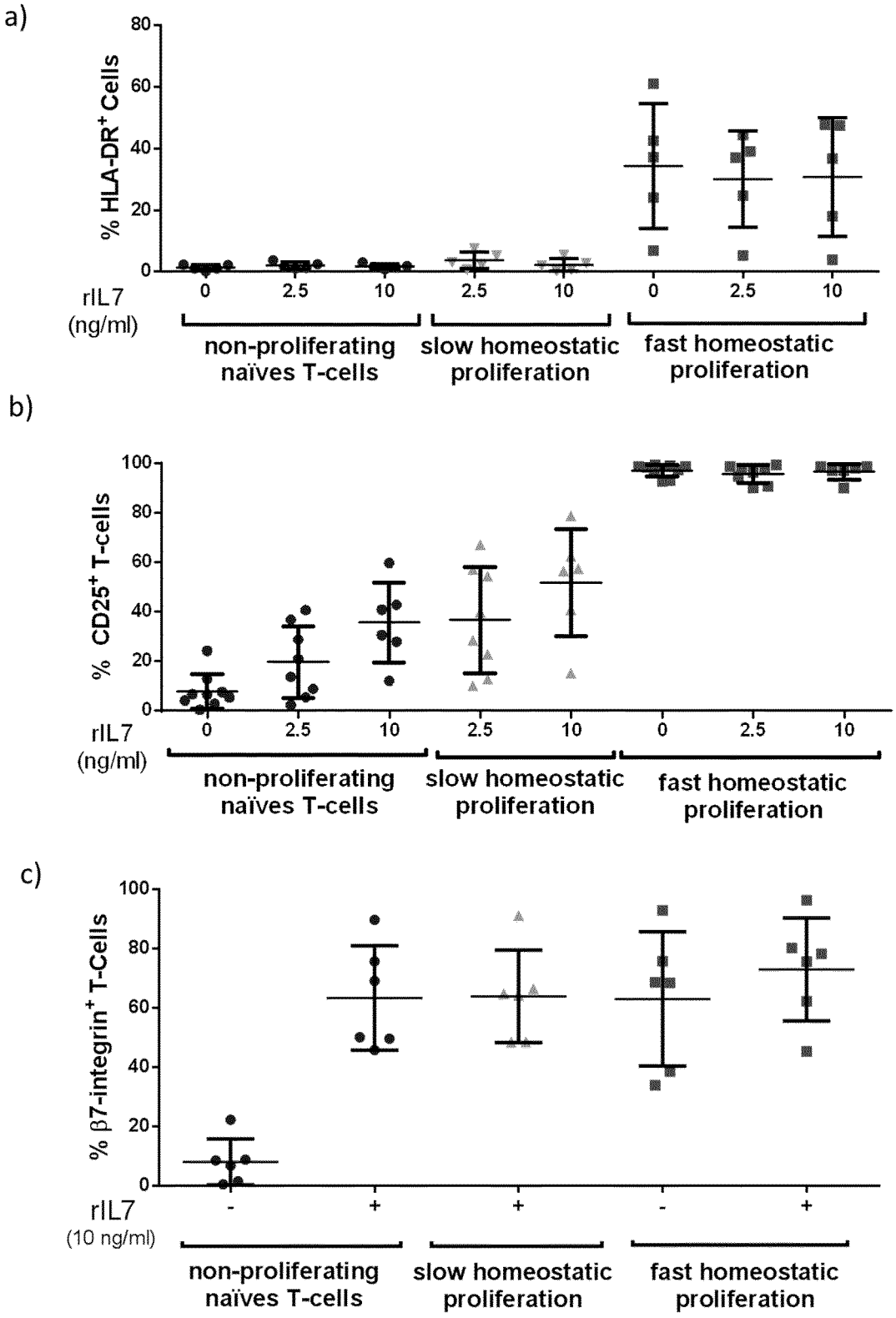

a)
% HLA-DR+ Cells
80
60
40
20
0
rIL7
(ng/ml)
0
2.5
10
2.5
10
0
2.5
10
non-proliferating naïves T-cells
slow homeostatic proliferation
fast homeostatic proliferation
b)
% CD25+ T-cells
100
80
60
40
20
0
rIL7
(ng/ml)
0
2.5
10
2.5
10
0
2.5
10
non-proliferating naïves T-cells
slow homeostatic proliferation
fast homeostatic proliferation
c)
% β7-integrin+ T-Cells
100
80
60
40
20
0
rIL7
(10 ng/ml)
-
+
+
-
+
non-proliferating naïves T-cells
slow homeostatic proliferation
fast homeostatic proliferation

Fig. 10

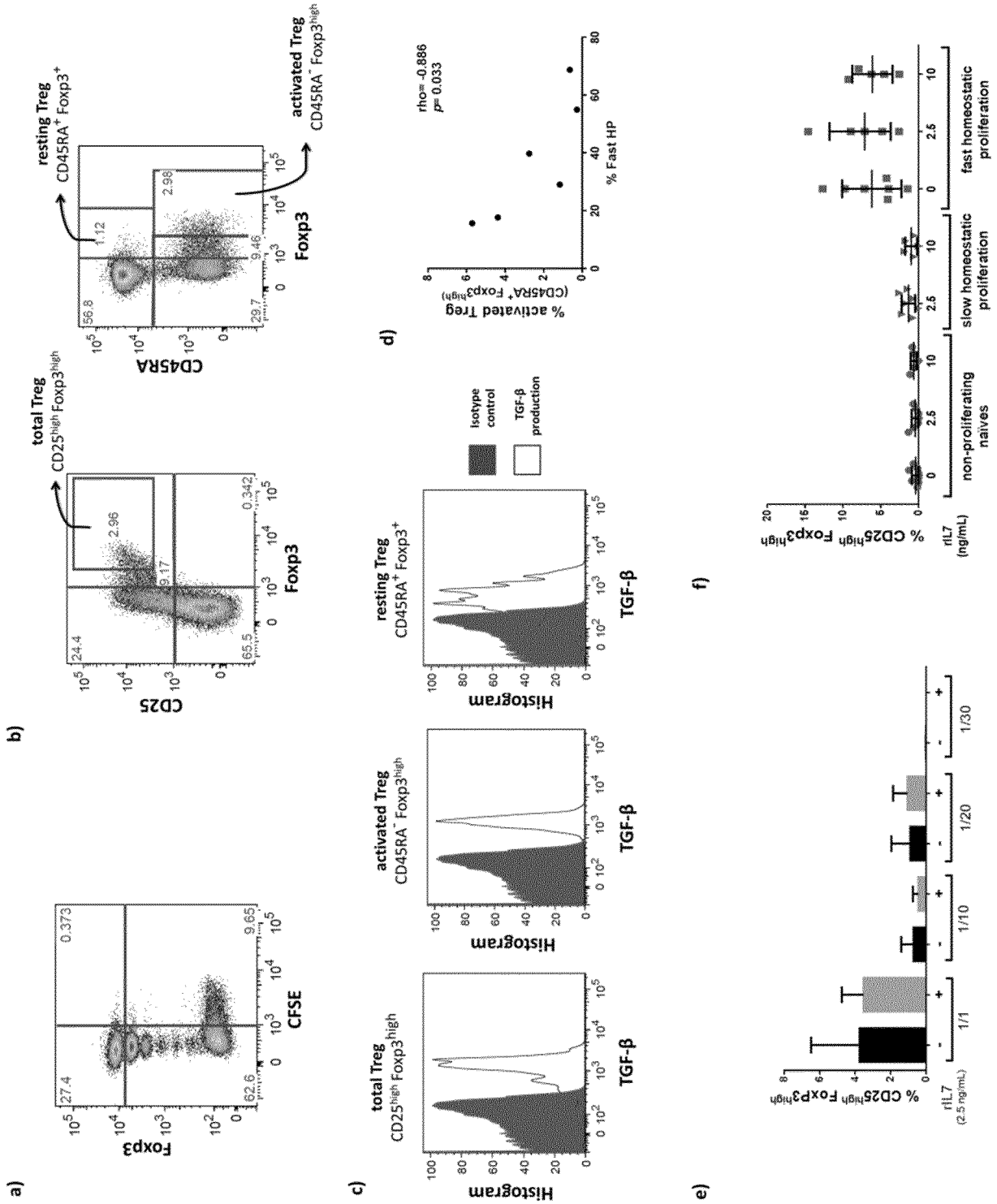

a)
Foxp3
CFSE
27.4
0.373
62.6
9.65
b)
total Treg
CD25high Foxp3high
CD25
Foxp3
24.4
2.96
9.17
65.5
0.342
resting Treg
CD45RA+ Foxp3+
activated Treg
CD45RA- Foxp3high
CD45RA
Foxp3
56.8
1.12
29.7
9.46
2.98
c)
total Treg
CD25high Foxp3high
activated Treg
CD45RA- Foxp3high
resting Treg
CD45RA+ Foxp3+
Histogram
TGF-β
Isotype control
TGF-β production
d)
rho= -0.886
p= 0.033
% activated Treg (CD45RA- Foxp3high)
% Fast HP
e)
% CD25high Foxp3high
rIL7 (2.5 ng/mL)
1/1
1/10
1/20
1/30
f)
% CD25high Foxp3high
rIL7 (ng/mL)
non-proliferating naïves
slow homeostatic proliferation
fast homeostatic proliferation

Fig. 11

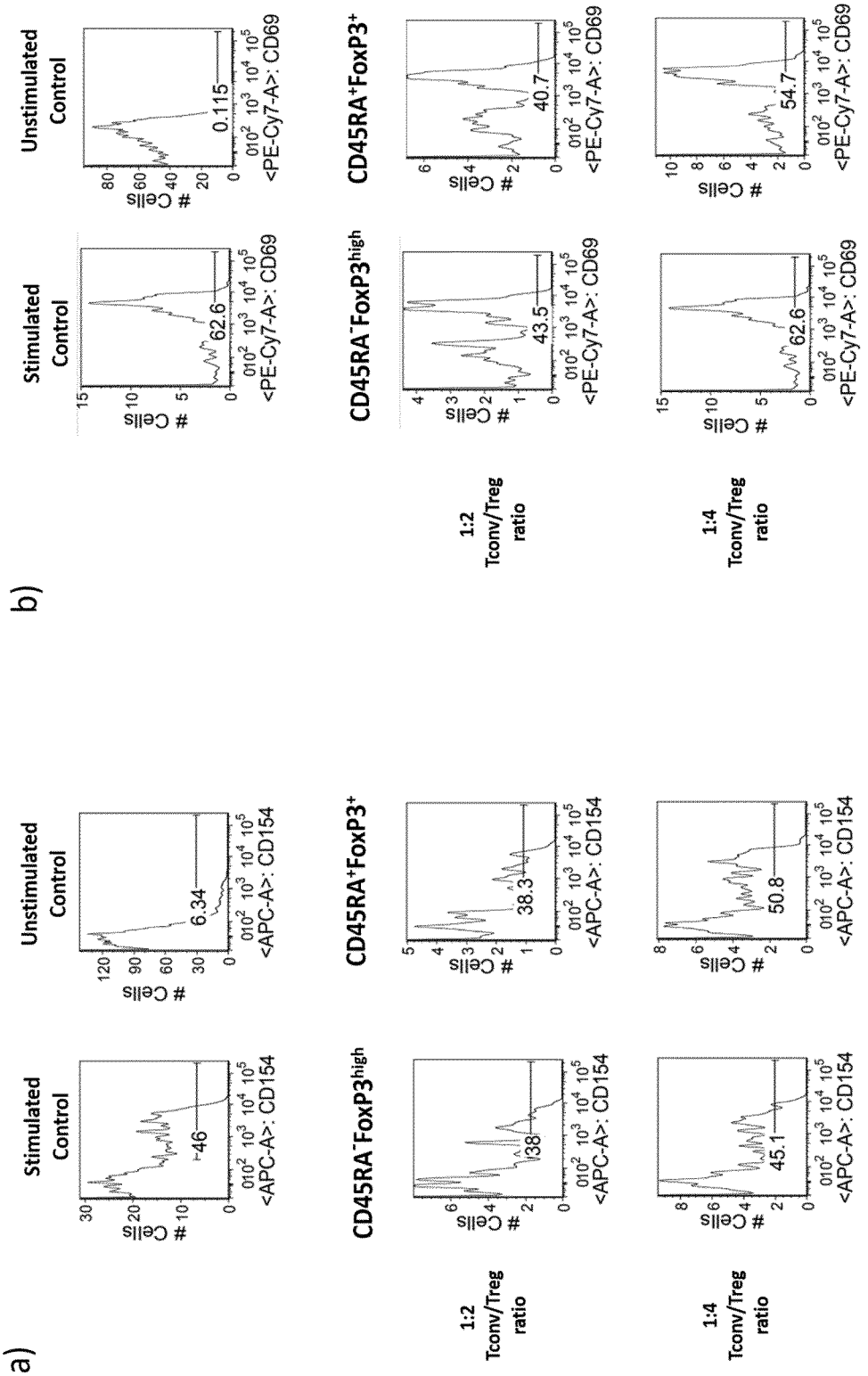

a)
Unstimulated Control
Stimulated Control
CD45RA⁺FoxP3⁺
CD45RA⁻FoxP3^high
1:2 Tconv/Treg ratio
1:4 Tconv/Treg ratio
Cells
<APC-A>: CD154
6.34
46
38.3
38
50.8
45.1
b)
<PE-Cy7-A>: CD69
0.115
62.6
40.7
43.5
54.7
62.6

Fig. 12

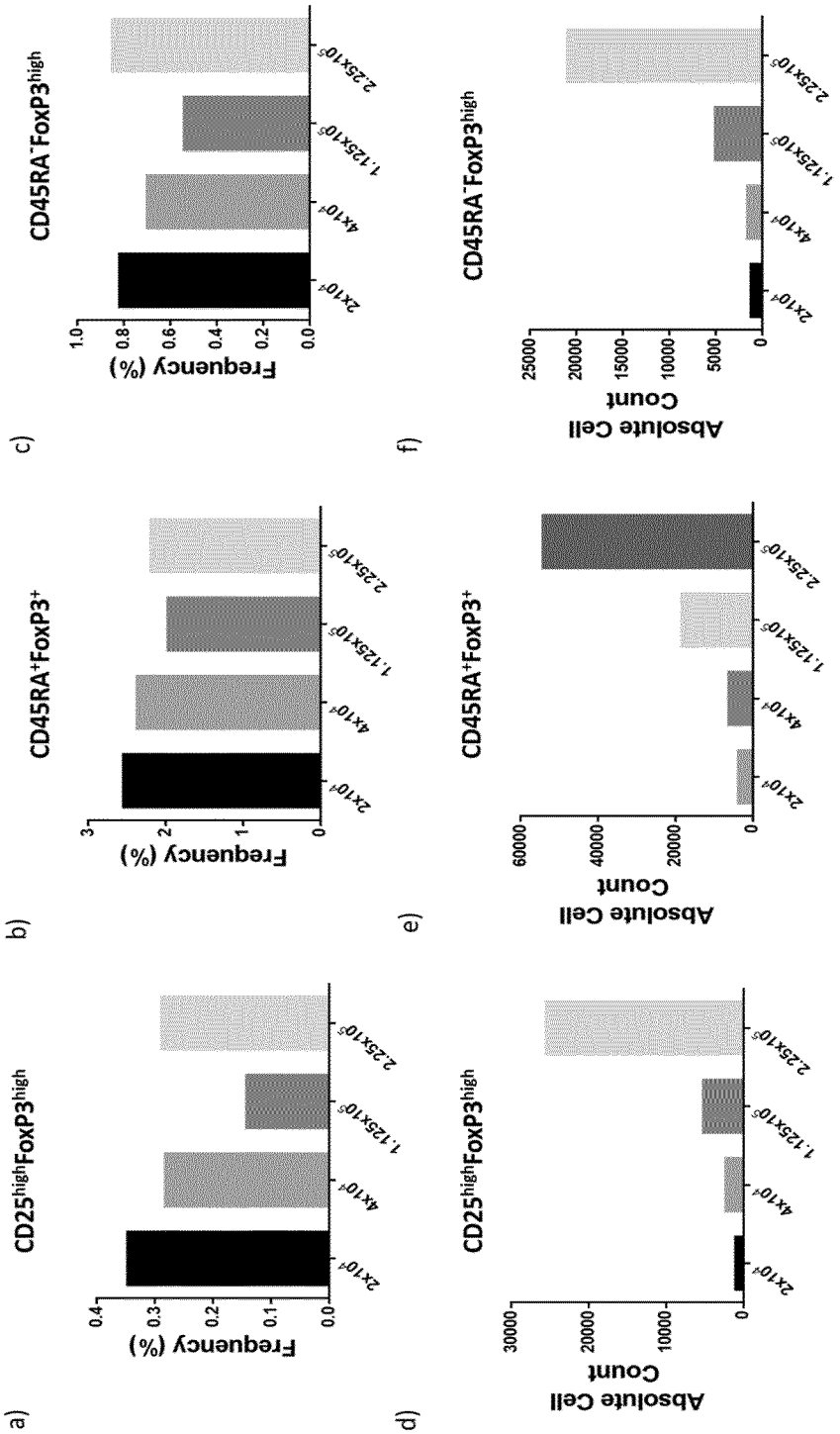
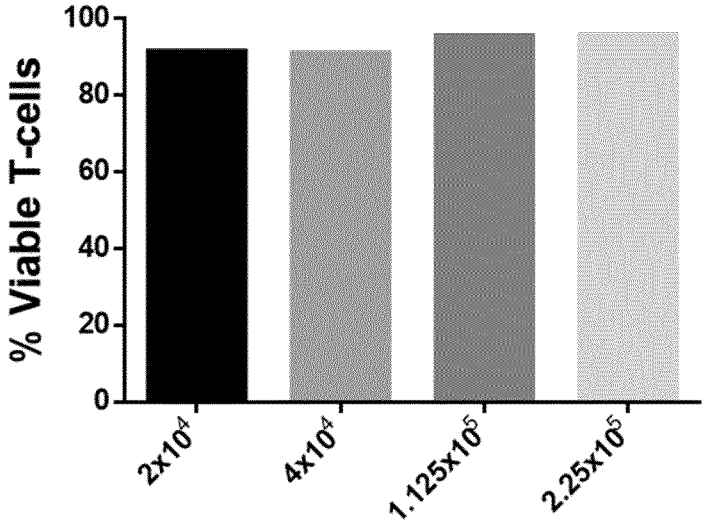

a)
CD25highFoxP3high
Frequency (%)
0.0
0.1
0.2
0.3
0.4
2x10⁴
4x10⁴
1.125x10⁵
2.25x10⁵
b)
CD45RA+FoxP3+
Frequency (%)
0
1
2
3
c)
CD45RA-FoxP3high
Frequency (%)
0.0
0.2
0.4
0.6
0.8
1.0
d)
CD25highFoxP3high
Absolute Cell Count
0
10000
20000
30000
e)
CD45RA+FoxP3+
Absolute Cell Count
0
20000
40000
60000
f)
CD45RA-FoxP3high
Absolute Cell Count
0
5000
10000
15000
20000
25000

Fig. 13



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/062256

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/17
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CIMBRO RAFFAELLO ET AL: "IL-7 induces expression and activation of integrin alpha 4 beta 7 promoting naive T-cell homing to the intestinal mucosa", BLOOD, vol. 120, no. 13, September 2012 (2012-09) , pages 2610-2619, XP002782994, ISSN: 0006-4971 see whole document and in particular the abstract.	12,20-31

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 2018	27/07/2018

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

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BEQ STEPHANIE ET AL: "IL-7 induces immunological improvement in SIV-infected rhesus macaques under antiviral therapy", JOURNAL OF IMMUNOLOGY, vol. 176, no. 2, January 2006 (2006-01), pages 914-922, XP002782995, ISSN: 0022-1767 see whole document and in particular the abstract.	12,20-31
X	DO JEONG-SU ET AL: "Both exogenous commensal and endogenous self antigens stimulate T cell proliferation under lymphopenic conditions", CELLULAR IMMUNOLOGY, vol. 272, no. 2, 2012, pages 117-123, XP002782996, ISSN: 0008-8749 see whole document and in particular the abstract.	12,20-31
X	A. GRUBER ET AL: "MHC Class I-Positive Dendritic Cells (DC) Control CD8 T Cell Homeostasis In Vivo: T Cell Lymphopenia as a Prerequisite for DC-Mediated Homeostatic Proliferation of Naive CD8 T Cells", THE JOURNAL OF IMMUNOLOGY, vol. 175, no. 1, 1 July 2005 (2005-07-01), pages 201-206, XP055492016, US ISSN: 0022-1767, DOI: 10.4049/jimmunol.175.1.201 see whole document and in particular the abstract.	1-3,12, 20-31
X	WO 2013/088114 A1 (CELL MEDICA LTD [GB]; ROONEY CLIONA M [US]; LEEN ANN M [US]; VERA JUAN) 20 June 2013 (2013-06-20) see whole document and in particular summary of the invention	1-3,12, 20-31
X	WO 2013/076268 A1 (INST NAT SANTE RECH MED [FR]) 30 May 2013 (2013-05-30) see whole document and in particular summary of the invention	1-3,12, 20-31
X	BOYMAN ONUR ET AL: "Homeostatic proliferation and survival of naive and memory T cells", EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 39, no. 8, August 2009 (2009-08), pages 2088-2094, XP002782997, ISSN: 0014-2980 the whole document	12,20-31

-/--

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	ROSADO-SÁNCHEZ ISAAC ET AL: "An in vitro system of autologous lymphocytes culture that allows the study of homeostatic proliferation mechanisms in human naive CD4 T-cells", LABORATORY INVESTIGATION, NATURE PUBLISHING GROUP, THE UNITED STATES AND CANADIAN ACADEMY OF PATHOLOGY, INC, vol. 98, no. 4, 18 January 2018 (2018-01-18), pages 500-511, XP036492981, ISSN: 0023-6837, DOI: 10.1038/S41374-017-0006-3 [retrieved on 2018-01-18] the whole document -----	1-33

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/062256

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013088114 A1	20-06-2013	AU 2012351347 A1	07-08-2014
		BR 112014014591 A2	13-06-2017
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		US 2015017723 A1	15-01-2015
		WO 2013088114 A1	20-06-2013
WO 2013076268 A1	30-05-2013	EP 2797623 A1	05-11-2014
		US 2015004182 A1	01-01-2015
		US 2017216417 A1	03-08-2017
		WO 2013076268 A1	30-05-2013