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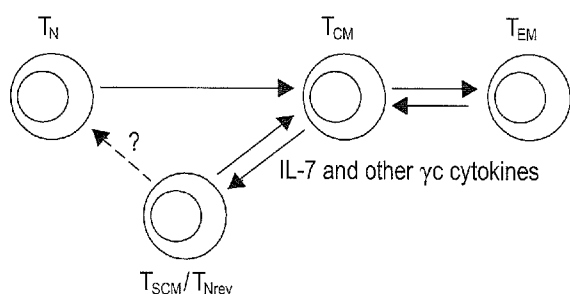


Figure 4E

(57) Abstract: Provided is a method of sorting T cells based on expression of CD117. The T cells may comprise a nucleotide sequence encoding a TCR and/or a CAR. Prior to sorting, the cells may have undergone reversion to a naïve phenotype. Also provided is a method of generating T cells having a naïve phenotype which comprises culturing central memory and/or effector memory T cells in a culture medium comprising a cytokine. Cell populations and uses of the T cells in therapy are also described.

Methods of sorting and culturing T Cells

Field of the invention

The present invention relates to methods for sorting T cells, as well as methods for generating precursor memory T cells, including memory T-stem cells (T_{scm}). The invention also relates to methods for generating naïve T cells.

Background of the invention

The development of T-cell memory is an intrinsic characteristic of the adaptive immune system and is essential for long-term health and survival. A progressive developmental model of T cell differentiation has been proposed within the human lineage which presumes a one way irreversible differentiation pathway. Specifically, initial antigen stimulation is proposed to trigger differentiation from naïve (T_N) to central memory (T_{CM}), effector memory (T_{EM}), and effector (T_{Eff}) cells. Along this pathway, $CD8^+$ T cells acquire increasing effector function whilst progressively losing survival and proliferative capacity.

Memory T-stem cells (T_{scm}), recently identified within human peripheral blood (PB), are proposed to constitute the precursor memory T cell pool. Although T_{scm} display many phenotypic features in common with naïve T cells and show capacity for self-renewal, they exhibit functional features of antigen-experienced cells and differentiate into memory and effector cell subsets upon re-encounter with the antigen. These cells are believed to represent an intermediate stage between T_N and T_{CM} , and therefore represent the key subset in memory T-cell generation. T_{scm} are relatively rare, accounting for 2-3% of all $CD3^+$ T-lymphocytes in PB. It is currently uncertain how such cells are formed and which mechanisms determine their expansion and differentiation.

Adoptive T cell therapy has been shown to be an effective treatment for a range of infectious and malignant diseases. The transfer of antigen-specific T cells is particularly advantageous, since cells can be transduced with a specific TCR (T cell receptor) or CAR (chimeric antigen receptor) in order to target cells having particular biomarkers. The quality of the transferred T cells is an important factor in the efficacy of therapy, especially in relation to the survival and persistence of T cells and their proliferative capacity *in vivo* after transfer. Highly differentiated T-cells can be less effective in immunotherapy, since they can become senescent soon after infusion and die. Thus, the pluripotent yet antigen-experienced features of T_{scm} are potentially

advantageous in immunotherapy. However, the low frequency of T_{SCM} in blood means that T_{SCM} are difficult to isolate and engineer, for example, by TCR or CAR transduction, in sufficient numbers for therapy. Due to their low frequency, the isolation of T_{SCM} from blood can also be an unpleasant and/or painful experience for the patient.

5 The present invention has been devised with these issues in mind.

Summary of the invention

10 According to a first aspect of the invention, there is provided a method of generating T memory stem cells (Tscm), the method comprising culturing central memory or effector memory T cells in a culture medium comprising one or more cytokines selected from the group consisting of IL-2, IL-4, IL-7 and IL-15.

15 It will be appreciated that any of the statements below may be applicable to the first, second, third, fourth, fifth, sixth, seventh, eighth, ninth aspects, or any of the other aspects of the invention, as appropriate.

As used herein, 'Tscm' refers to cells having a CD45RA⁺,CCR7⁺,CD95⁺ phenotype.

20 The Tscm may additionally express CD117.

The Tscm may additionally express CD122, CD127, integrin β 7 and/or CXCR3.

25 In some embodiments the Tscm may additionally express CD62L, CD31, CD27 and/or CD25.

Methods of identifying markers present on and/or in T cells will be known to the skilled person. For example, markers may be identified by antibody labelling methods such as flow cytometry or ELISA.

30 The central memory or effector memory T cells may be CD8⁺. In some embodiments, the method comprises culturing CD8⁺ central memory T cells.

The central memory or effector memory T cells may be CD4⁺. Thus, in some embodiments, the method comprises culturing CD4⁺ central memory T cells. The CD4⁺ central memory T cells may be cultured separately to CD8⁺ central memory T cells, or may be cultured with CD8⁺ central memory T cells.

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In some embodiments, the method may generate at least $5 \times 10^4/\text{ml}$, $1 \times 10^5/\text{ml}$, $3 \times 10^5/\text{ml}$, $4 \times 10^5/\text{ml}$, $5 \times 10^5/\text{ml}$, at least $6 \times 10^5/\text{ml}$, at least $7 \times 10^5/\text{ml}$, at least $8 \times 10^5/\text{ml}$, at least $9 \times 10^5/\text{ml}$, at least $1 \times 10^6/\text{ml}$, at least $1.5 \times 10^6/\text{ml}$, at least $2 \times 10^6/\text{ml}$, at least $3 \times 10^6/\text{ml}$, at least $4 \times 10^6/\text{ml}$, at least $5 \times 10^6/\text{ml}$, or at least $6 \times 10^6/\text{ml}$ Tscm.

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In some embodiments, the culture medium comprises IL-7.

In some embodiments, the culture medium comprises only one of: IL-2, IL-4, IL-7 and IL-15. It will be appreciated that other cytokines may or may not be present. Optionally, the cytokine is IL-7.

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In some embodiments, the concentration of IL-7 in the culture medium is from 10 to 50 ng/ml, from 15 to 40 ng/ml or from 20 to 30 ng/ml, e.g. 25 ng/ml.

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In some embodiments, the concentration of IL-2 in the culture medium is from 10 to 100 U/ml, from 20 to 80 U/ml, from 30 to 70 U/ml or from 40 to 60 U/ml, e.g. 50 U/ml.

In some embodiments, the concentration of IL-4 in the culture medium is from 10 to 50 ng/ml, from 15 to 40 ng/ml or from 20 to 30 ng/ml, e.g. 25 ng/ml.

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In some embodiments, the concentration of IL-15 in the culture medium is from 10 to 100 ng/ml, from 20 to 80 ng/ml, from 30 to 70 ng/ml or from 40 to 60 ng/ml, e.g. 50 ng/ml.

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The culture medium may be an aqueous solution which, in addition to the cytokine(s), comprises one or more amino acids, vitamins, minerals, salts and/or buffers. A suitable culture medium for use in the present invention is RPMI. Other suitable culture media will be known to the skilled person, for example, PBS. The culture medium may contain serum, for example foetal calf serum (FCS) or human serum. In some embodiments, the culture medium specifically excludes the use of FCS or human serum.

In some embodiments, the method comprises culturing the central memory or effector memory T cells in the culture medium for a period of from 4 to 25 days. In some embodiments, the method comprises culturing the central memory or effector memory T cells in the culture medium for a period of from 4 to 19 days, or for a period of from 9 to 24 days. In some embodiments, the method comprises culturing the central memory or effector memory T cells in the culture medium for a period of from 13 to 19 days. In some embodiments, the method comprises culturing the central memory or effector memory T cells in the culture medium for 16 days.

In some embodiments, the proportion of the T cell population constituted by Tscm cells after culture (i.e. incubation) with the one or more cytokines is at least 20%, 30%, 40%, 50%, 60%, 70%, at least 75%, at least 80, at least 85% or at least 90%.

The culture medium may be replaced at least once during the culture period. In some embodiments, the culture medium is replaced at least once, at least twice or at least three times per week. Various methods for the replacement of T cell culture medium will be known to those skilled in the art. A method for medium replacement may, for example, include the removal of a proportion e.g. a quarter, a third, a half, $\frac{2}{3}$ or $\frac{3}{4}$ of the medium from the culture, followed by an equivalent volume of replacement medium. Alternatively, all of the medium may be replaced by medium. In some circumstances the culture may be centrifuged before medium removal.

The central memory or effector memory T cells may themselves be generated by activation of naïve T cells. Thus, in some embodiments the method further comprises the step of activating naïve T cells to generate the central memory and/or effector memory T cells.

As used herein, "activation" will be understood to mean the initiation of at least a proportion of the downstream signaling pathway of a TCR of a T cell. Activation may be defined as the clonal expansion of T cells, the upregulation of activation markers on the cell surface, the differentiation of T cells into later stage cells (e.g. effector cells), the induction of cytotoxicity, the induction of cytokine secretion, and/or the induction of apoptosis.

Activation of naïve T cells may be carried out by culturing the naïve T cells in the presence of any suitable activating agent. It will be understood that an activating agent is an agent which can initiate at least a proportion of the downstream signaling pathway of a TCR.

- 5 In some embodiments the activating agent is a mitogen. The skilled person will understand “mitogen” to be a substance which triggers cell division.

In some embodiments the activating agent is a protein or peptide which is the ligand of the T cell's TCR (i.e. the TCR can recognize and bind to the protein or peptide), or equivalent to the
10 TCR, for example CAR. Such proteins or peptides may be considered to be specific mitogens.

In some embodiments, the protein or peptide may be presented (i.e. expressed on the surface) by a dendritic cell (DC) or other suitable antigen presenting cell (APC). The skilled person will be aware of suitable methods for the presentation of the protein or peptide on a DC or other
15 suitable APC, for example, the method of antigen-pulsing. In some embodiments the protein or peptide may be bound to a major histocompatibility complex (MHC) molecule.

In some embodiments, the activating agent is a non-specific mitogen. Non-specific mitogens include anti-CD3 and/or anti-CD28 antibodies, anti-CD3 and/or anti-CD28 antibody-coated
20 beads, phytohaemagglutinin (PHA), concanavalin A (ConA), phorbol myristate acetate (PMA) and/or ionomycin, 4.1BB and *Staphylococcus* enterotoxin B (SEB). The skilled person may obtain anti-CD3 and/or anti-CD28 beads from a number of different suppliers, including, but not limited to Dynabeads T Activator CD3/CD28 beads by Life Technologies. Alternatively, a person skilled in the art may generate their own anti-CD3 and/or anti-CD28 beads, using methods
25 known to those in the art.

The naïve T cells may be cultured in the presence of a suitable activating agent for a period of from 3 to 10 days, from 4 to 9 days, from 5 to 8 days or from 6 to 7 days in order to activate the
30 T cells.

In some embodiments, the T cells are considered activated when the proportion of naïve T cells in the cell population is less than 25%, less than 20%, less than 15% or less than 10%.

In some embodiments the method comprises:

(a) activating naïve T cells by culturing the naïve T cells in the presence of an activating agent, thereby generating central and/or effector memory T cells;

(b) culturing the central and/or effector memory T cells generated in (a) in a culture medium comprising one or more cytokines selected from the group consisting of IL-2, IL-4, IL-7 and IL-15;

wherein optionally, the culture medium of step (b) may be added to the same vessel as step (a); and

wherein optionally, the culture medium is added when the proportion of naïve T cells in the cell population is less than 20%.

It will be appreciated that step (b) may be carried out immediately after step (a). In some embodiments, there may be a period of time of at least 1 hour, at least 5 hours, at least 12 hours, at least 16 hours, at least 1 day, at least 2 days, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 2 weeks or at least 3 weeks between step (a) and step (b), wherein the central memory and/or effector T cells generated in step (a) may be stored. It will be understood that "stored" may refer to T cells being frozen, for example at -20°C, -80°C or in liquid nitrogen and/or being maintained in culture.

As used herein, 'naïve' refers to T cells having a CD45RA⁺, CCR7⁺, CD95⁻ phenotype. Naïve cells may additionally express CD62L. The naïve T cells may be obtained from cord blood (CB) or adult lymphocytes. In some embodiments the naïve T cells may be obtained by immunomagnetic separation from adult lymphocytes. In some embodiments the naïve T cells may be obtained by fluorescence activated cell sorting from adult lymphocytes. Other methods to isolate naïve T cells from CB or adult lymphocytes will be known to those skilled in the art. In some embodiments CB mononuclear cells may be considered to be naïve.

In contrast, central memory T cells have the phenotype CD45RA⁻, CCR7⁺, while effector memory T cells have the phenotype CD45RA⁻, CCR7⁻. Thus, these markers may be used to distinguish between central memory and effector memory T cells, and reverted Tscm and naïve T cells.

In the context of the present invention naïve T cells may be virgin naïve T cells (i.e. cells which are not activated and have not previously been activated, for example, by exposure to antigen), or they may be activated T cells which have been reverted to a naïve-like phenotype, for example antigen-experienced T cells with a naïve-like phenotype.

Naïve T cells may be distinguished from Tscm by their lack of expression of CD95 and/or CXCR3, both of which are expressed by Tscm. In some embodiments, naïve T cells may be distinguished from Tscm by their lack of expression of CD117.

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Surprisingly, it has been found that, in culture, in the presence of one or more cytokines selected from the group consisting of IL-2, IL-4, IL-7 and IL-15, the Tscm continue to revert back to a phenotype resembling naïve T cells. Thus, in some embodiments, the method of the first aspect of the invention further comprises continuing to culture the Tscm so as to produce naïve T cells.

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In some embodiments, the method comprises continuing to culture the Tscm so as to produce naïve T cells for at least 3, 5 or 7 days. In some embodiments, the method comprises continuing to culture the Tscm so as to produce naïve T cells for no more than 35, 33 or 31 days. In some

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embodiments the method comprises continuing to culture the Tscm for a further 7 days to 28 days, or 14 to 21 days.

Surprisingly, the inventors have found that Tscm can undergo more than one cycle of phenotypic reversion. "Phenotypic reversion" refers to activation of T cells, followed by culture

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(i.e. incubation) in the presence of at least one cytokine, for example IL-7, to generate Tscm or revertant naïve T cells. In some embodiments, the Tscm undergo at least 1, at least 2, at least 3 or at least 4 cycles of phenotypic reversions.

Without wishing to be bound by theory, the inventors believe that multiple cycles of phenotypic reversion will be beneficial in an *in vivo* setting, for example, in immunotherapy or for the long term study of animal models, since the multiple cycles enable the long-term survival of the T cells in a "latent" state as Tscm while still retaining the ability to reactivate upon encountering the antigen(s) to which the T cell is specific. Furthermore, multiple cycles of phenotypic reversion may be beneficial for the *in vitro* study of any T cell, particularly rare T cells, or small numbers of T cells, since this will enable researchers to study activated cells and then "revert" the cells to a naïve-like phenotype to facilitate continued and long-term research.

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According to a second aspect of the invention, there is provided a method of generating naïve T cells, the method comprising culturing Tscm in a culture medium comprising one or more cytokines selected from the group consisting of IL-2, IL-4, IL-7 and IL-15.

- 5 In some embodiments, the method comprises culturing the Tscm for a period of from 7 days to 28 days, or 14 to 21 days.

In some embodiments, the cells may be cultured until at least 50%, at least 60%, at least 70%, at least 80 or at least 90% of the cells obtain a CD95⁺ phenotype.

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In some embodiments, the method additionally comprises transducing the T cells with a nucleic acid sequence comprising a sequence which encodes a T cell receptor (TCR) or a chimeric antigen receptor (CAR). Other receptors suitable for the transduction of T cells will be known to those skilled in the art, for example, Natural Killer cell receptors such as CD16, CD56, NK1.1 or
15 NK1.2, or any suitable combination of receptors. Transduction may be carried out simultaneously with activation. In some embodiments transduction is carried out before or after activation. The method may comprise transducing naïve T cells. Alternatively, or in addition, the method may comprise transducing Tscm cells.

- 20 The skilled person will be aware that transduction is a process by which genetic material, for example DNA or siRNA, is artificially introduced into the cell. Transduction may be carried out using a virus, for example, a lentivirus or a retrovirus. Transduction of T cells with a nucleic acid comprising a sequence encoding a TCR or CAR gene can be carried out using standard techniques known to those skilled in the art, and as described, for example, by Frumento, G
25 (Cord blood T cells retain early differentiation phenotype suitable for immunotherapy after TCR gene transfer to confer EBV specificity. *Am J Transplant* **13**, 45-55 (2013)). For example, the sequence encoding the TCR may be isolated from a naturally occurring chromosome of a T cell and incorporated into a suitable vector.

- 30 In some embodiments, the method additionally comprises reactivation of the T cells. As would be understood by the skilled person, the reactivation of T cells may be required in order to induce cell division to enable the transduction of previously activated T cells.

It will be appreciated that cells can be reactivated using the same or different activating agent to the activating agent used in the initial or prior activation step.

In some embodiments, T cells may be activated, followed by simultaneous reactivation and transduction. In some embodiments, T cells may be activated, after which T cells may be re-activated, after which T cells may be transduced. In some embodiments, T cells may be activated, after which T cells may be transduced, following which T cells may be re-activated.

The insertion of a TCR or CAR gene into the T cell population enables the antigen specificity of the central memory or effector memory T cells, and thus the Tscm and naïve T cells generated therefrom, to be programmed. The methods of the invention therefore enable the generation of reverted Tscm or naïve T cells having specificity for a particular antigen as well as the properties of self-renewal, proliferation and differentiation typically associated with early-stage cells. This combination of features makes the cells particularly useful for therapy, since they can be used to target cells having particular biomarkers. The properties of self-renewal, proliferation and/or differentiation also make the cells particularly advantageous for therapy, since they can be produced in large numbers.

Specific TCRs and/or CARs which are useful for therapy will be known to the skilled person.

Example TCR and/or CARs may include any of (but are not limited to), an Epstein-Barr virus (EBV) antigen-specific TCR or CAR (i.e. the TCR/CAR can recognise and bind to the EBV antigen), a TCR and/or CAR specific for an antigen associated with melanoma, a TCR and/or CAR specific for an antigen associated with synovial sarcoma, a TCR and/or CAR specific for an antigen associated with testicular cancer and a CD19-specific TCR or CAR. An example EBV antigen is the SSCSSCPLSK (SSC) peptide of the LMP2 protein of Epstein Barr virus.

In some embodiments the TCR is not encoded by the T cell's innate genome.

The EBV-antigen specific TCR may be useful for the therapy of EBV-associated tumours, for example nasopharyngeal carcinoma, NK T cell lymphoma and post-transplant lymphoproliferative disease (PTLD).

The CD19-specific CAR may be useful for the therapy of B cell malignancies, for example B-CLL Acute Lymphoblastic Leukaemia and chronic lymphocytic leukaemia (CLL).

It will be understood that the TCRs, CARs and therapies exemplified above are in no way limiting to the scope of the invention and that the selection of a particular TCR or CAR suitable for use in therapy against a particular disease or disorder will be within the realms of a person skilled in the art.

In some embodiments, there is provided a method of generating T memory stem cells (Tscm), the method comprising culturing central memory and/or effector memory CD8⁺T cells in a culture medium comprising IL-7,

wherein optionally, the concentration of IL-7 in the culture medium is from 20 to 30 ng/ml; and optionally, the method comprises culturing the central memory and/or effector memory T cells in the culture medium for from 4 to 25 days.

In some embodiments, there is provided a method of generating T memory stem cells (Tscm), the method comprising activating naïve T cells to generate central memory and/or effector memory CD8⁺T cells; and

culturing central memory and/or effector memory CD8⁺T cells in a culture medium comprising IL-7,

wherein optionally, the naïve T cells are obtained from cord blood or adult lymphocytes; and

optionally, the concentration of IL-7 in the culture medium is from 20 to 30 ng/ml; and optionally, the method comprises culturing the central memory and/or effector memory T cells in the culture medium for from 4 to 25 days.

In some embodiments, there is provided a method of generating T memory stem cells (Tscm), the method comprising culturing central memory and/or effector memory CD8⁺ T cells in a culture medium comprising IL-7,

wherein optionally, the concentration of IL-7 in the culture medium is from 20 to 30 ng/ml; and

(a) optionally, the method comprises culturing the central memory and/or effector memory T cells in the culture medium for from 4 to 25 days; and

(b) optionally, the method further comprises continuing to culture the Tscm so as to produce naïve T cells.

In some embodiments, there is provided a method of generating T memory stem cells (Tscm), the method comprising:

culturing central memory and/or effector memory CD8⁺ T cells in a culture medium comprising IL-7; and;

wherein optionally, the concentration of IL-7 in the culture medium is from 20 to 30 ng/ml; and

(a) optionally activating naïve T cells to generate the central memory and/or effector memory T cells; and

(b) optionally transducing the T cells with a nucleic acid encoding a T cell receptor (TCR) or a chimeric antigen receptor (CAR), wherein step (b) is carried out prior to, or after or simultaneously with step (a);

(c) optionally culturing the central memory and/or effector memory T cells in the culture medium for from 4 to 25 days to obtain Tscm; and

(d) optionally continuing to culture the Tscm so as to provide naïve T cells.

In some embodiments, there is provided a method of generating T memory stem cells (Tscm), the method comprising culturing central memory and/or effector memory CD4⁺ T cells in a culture medium comprising IL-7,

wherein optionally, the concentration of IL-7 in the culture medium is from 20 to 30 ng/ml; and

(a) optionally, the method comprises culturing the central memory and/or effector memory T cells in the culture medium for from 4 to 25 days; and

(b) optionally, the method further comprises continuing to culture the Tscm so as to produce naïve T cells.

In some embodiments the method further comprises identifying and/or isolating T cells which express CD117. For example, a subset of CD117⁺ cells may be selected from a mixed population of T cells. The skilled person will be aware of methods for isolating cells, for example, the method of Fluorescence Activated Cell Sorting (FACS).

Expression of CD117 may be used for identifying, sorting or isolating cells before or after transducing T cells with a nucleic acid encoding a T cell receptor (TCR) or a chimeric antigen receptor (CAR). In some embodiments, CD117⁺ cells are isolated prior to administration to a patient.

According to a third aspect of the invention, there is provided a population of Tscm, wherein the population comprises at least 5×10^4 /ml Tscm.

In some embodiments, there is provided at least $5 \times 10^4/\text{ml}$, $1 \times 10^5/\text{ml}$, $3 \times 10^5/\text{ml}$, $4 \times 10^5/\text{ml}$, $5 \times 10^5/\text{ml}$, at least $6 \times 10^5/\text{ml}$, at least $7 \times 10^5/\text{ml}$, at least $8 \times 10^5/\text{ml}$, at least $9 \times 10^5/\text{ml}$, at least $1 \times 10^6/\text{ml}$, at least $1.5 \times 10^6/\text{ml}$, at least $2 \times 10^6/\text{ml}$, at least $3 \times 10^6/\text{ml}$ Tscm, at least $4 \times 10^6/\text{ml}$, at least $5 \times 10^6/\text{ml}$, or at least $6 \times 10^6/\text{ml}$ Tscm.

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In some embodiments, the population of Tscm is obtainable by the method of the first aspect of the invention.

10 In some embodiments, the Tscm comprise a nucleotide sequence encoding a TCR/CAR. The TCR/CAR may be encoded by a transduced nucleotide sequence. Advantageously, this provides Tscm programmed with specificity to a desired antigen. This specificity may find particular use in therapy.

15 According to a fourth aspect of the invention, there is provided a population of naïve T cells obtainable by the method of the second aspect of the invention, wherein the population comprises at least $5 \times 10^4/\text{ml}$ naïve T cells.

20 In some embodiments, there is provided at least $5 \times 10^4/\text{ml}$, $1 \times 10^5/\text{ml}$, $3 \times 10^5/\text{ml}$, $4 \times 10^5/\text{ml}$, $5 \times 10^5/\text{ml}$, at least $6 \times 10^5/\text{ml}$, at least $7 \times 10^5/\text{ml}$, at least $8 \times 10^5/\text{ml}$, at least $9 \times 10^5/\text{ml}$, at least $1 \times 10^6/\text{ml}$, at least $1.5 \times 10^6/\text{ml}$, at least $2 \times 10^6/\text{ml}$, at least $3 \times 10^6/\text{ml}$ naïve T cells, at least $4 \times 10^6/\text{ml}$, at least $5 \times 10^6/\text{ml}$, or at least $6 \times 10^6/\text{ml}$ naïve T cells.

In some embodiments, the naïve T cells comprise a nucleotide sequence encoding a TCR/CAR. The TCR/CAR may be encoded by a transduced nucleotide sequence.

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In addition to the characteristic markers identified above, the Tscm and naïve T cells provided by the methods of the invention are characterized by their self-renewal ability, their potential to differentiate and their high proliferative capacity. All of these properties are important for effective immunotherapy.

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The efficacy of immunotherapy using T cells depends on the quality of the cells and their stage of differentiation. Generally, the more differentiated the cells, the less efficacious they are. The methods of the invention advantageously enable the reversion of T cells to an early stage. Such early-stage Tscm or naïve T cells are more effective for immunotherapy than later stage T

cells since they are not exhausted. Tscm or naïve cells also have a greater ability to persist *in vivo* than later stage T cells. Thus, both the Tscm and naïve T cells described herein find use in therapy.

- 5 According to a fifth aspect of the invention, there is provided a Tscm cell which comprises a nucleotide sequence encoding a TCR/CAR. The Tscm cell may express the TCR/CAR on the cell surface. The Tscm cell may additionally express CD117.

The Tscm cell may additionally express CD122, CD127, integrin $\beta 7$ and/or CXCR3.

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In some embodiments, the TCR/CAR encoded by the nucleotide sequence may be overexpressed by the Tscm cell. By “overexpressed” the skilled person will understand that the TCR/CAR is expressed at an increased level in comparison to normal expression (i.e. of an endogenous TCR). For example, expression of the nucleotide sequence may be increased by a

15 factor of at least 2x, 3x, 4x, 5x, 10x, 15x, 20x, 50x or 100x.

The Tscm cell may overexpress at least one co-stimulatory molecule, for example CD28, CD3 or 4.1BB.

- 20 The nucleotide sequence may be transgenic. By transgenic, it will be understood that the sequence encoding the TCR/CAR has been artificially introduced into the cell, for example, by transduction. A nucleotide sequence encoding a TCR may be engineered or modified. It will be appreciated that a CAR is an artificial T cell receptor and so is always engineered. For example, the nucleotide sequence may be modified so that the TCR is a high affinity/avidity variant of an
- 25 endogenous TCR. For example, the nucleotide sequence encoding the TCR may be modified so that the TCR has a sulphide bond between the alpha and beta chains.

The nucleotide sequence encoding the TCR/CAR may be operably linked to a suitable promoter. The promoter is preferably functional in T cells. The promoter may be a non-TCR

30 promoter which is at least T-cell specific. The promoter may be a CD marker promoter, for example, a CD2, CD3, CD4 or CD8 promoter.

In some embodiments the Tscm cell may additionally express CD62L, CD31, CD27 and/or CD25.

In some embodiments, the Tscm cell is obtainable by the method of the first aspect of the invention.

- 5 According to a sixth aspect of the invention, there is provided a naïve T cell which comprises a nucleotide sequence encoding a TCR/CAR. The naïve T cell may express a TCR/CAR.

In some embodiments, the naïve T cell is obtainable by the method of the second aspect of the invention.

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The naïve cell may additionally express CD62L.

- In some embodiments, the TCR/CAR encoded by the nucleotide sequence may be overexpressed by the naïve T cell. By “overexpressed” the skilled person will understand that
15 the TCR/CAR is expressed at an increased level in comparison to normal expression (i.e. of an endogenous TCR). For example, expression of the nucleotide sequence may be increased by a factor of at least 2x, 3x, 4x, 5x, 10x, 15x, 20x, 50x or 100x.

- The naïve T cell may overexpress at least one co-stimulatory molecule, for example CD28, CD3
20 or 4.1BB.

- The nucleotide sequence may be transgenic. By transgenic, it will be understood that the sequence encoding the TCR/CAR has been artificially introduced into the cell, for example, by transduction. A nucleotide sequence encoding a TCR may be engineered or modified. It will be
25 appreciated that a CAR is an artificial T cell receptor and so is always engineered. For example, the nucleotide sequence may be modified so that the TCR is a high affinity/avidity variant of an endogenous TCR. For example, the nucleotide sequence encoding the TCR may be modified so that the TCR has a sulphide bond between the alpha and beta chains.

- 30 The nucleotide sequence encoding the TCR/CAR may be operably linked to a suitable promoter. The promoter is preferably functional in T cells. The promoter may be a non-TCR promoter which is at least T-cell specific. The promoter may be a CD marker promoter, for example, a CD2, CD3, CD4 or CD8 promoter.

Naïve T cells may be distinguished from Tscm by their lack of expression of CD95, integrin $\beta 7$ and/or CXCR3, all of which are expressed by Tscm. In some embodiments, naïve T cells may be distinguished from Tscm by their lack of expression of CD117.

- 5 The invention thus allows the preparation of a Tscm or naïve T cell which expresses a particular TCR and/or CAR enabling specificity to a desired antigen.

According to a seventh aspect of the invention, there is provided Tscm or naïve T cells for use in immunotherapy or cancer therapy.

10

The Tscm may be obtained by a method in accordance with the first aspect of the invention.

The naïve T cells may be obtained by a method in accordance with the first or second aspect of the invention.

15

The Tscm or naïve T cells may be stored as a bank of cells for use in immunotherapy or cancer therapy when required.

20

The Tscm or naïve T cells may be used to treat patients. The patients may be mammalian, for example humans. The Tscm or naïve T cells may be autologous to the patient to which the therapy is directed. Alternatively, the Tscm or naïve T cells may be allogeneic to the patient to which the therapy is directed. Treatment may be carried out by administration of the Tscm or naïve T cells to the patient. Administration may be by an intravenous route.

25

According to an eighth aspect of the invention, there is provided a cell culture medium comprising IL-7.

The IL-7 may be present in the culture medium at a concentration of from 10 to 50 ng/ml, from 15 to 40 ng/ml or from 20 to 30 ng/ml, e.g. 25 ng/ml.

30

According to a ninth aspect of the invention, there is provided T cells having a CD117⁺ phenotype.

In some embodiments, the T cells may be CD8⁺. In some embodiments, the T cells may be CD4⁺.

The CD117⁺ T cells may comprise Tscm. In some embodiments, the CD117⁺ cells may additionally comprise Tcm. A CD117⁺ cell population comprising Tscm and, optionally, Tcm cells may be considered as early differentiation T cells. Such cells are highly proliferative and have long lived renewal capacity, and thus are particularly advantageous for immunotherapy.

According to a further aspect of the invention, there is provided a method of identifying Tscm cells, the method comprising analysing T cells for expression of CD95 and, optionally CD117, wherein T cells expressing CD95, and optionally CD117, are identified as Tscm cells.

The present inventors have identified CD117 as a useful marker which could be used for identifying and/or isolating early lineage (i.e. early differentiation) T cells, for example for T cell engineering. As a single marker, CD117 would permit the direct and positive enrichment of early lineage T cells.

Thus, according to a further aspect of the present invention there is provided a method of sorting T cells, the method comprising identifying and/or isolating T cells which express CD117.

Such T cells may be identified by analyzing T cells for expression of CD117. Methods of identifying markers present on and/or in T cells will be known to the skilled person. For example, markers may be identified by antibody labelling methods such as flow cytometry or ELISA. In some embodiments, the method comprises isolating T cells which express CD117.

For example, a subset of CD117⁺ cells may be isolated from a mixed population of T cells. The skilled person will be aware of methods for isolating cells, for example, the method of Fluorescence Activated Cell Sorting (FACS).

Expression of CD117 may be used for isolating cells before or after transducing the T cells with a nucleic acid encoding a T cell receptor (TCR) or a chimeric antigen receptor (CAR). In some embodiments, CD117⁺ cells are isolated prior to administration to a patient.

In some embodiments, the method of sorting T cells comprises isolating CD117⁺ T cells which have undergone reversion.

By “reversion”, it will be understood that the cells have been subjected to a process which causes them to revert (phenotypically) to an earlier state of differentiation.

- 5 In some embodiments, the T cells have undergone reversion in accordance with the method of the first or second aspects of the invention.

In some embodiments, the T cells comprise a nucleotide sequence encoding a TCR and/or CAR. The T cells may have been transduced with a TCR or a CAR, prior to reversion.

10

Thus, in some embodiments, the invention provides a method comprising:

- (a) optionally activating naïve T cells to generate central memory and/or effector memory T cells;
- (b) optionally transducing activated central memory and/or effector T cells with a nucleic acid encoding a T cell receptor (TCR) or a chimeric antigen receptor (CAR), wherein
15 step (b) is carried out after or simultaneously with step (a) (when present);
- (c) subjecting T cells to reversion, optionally by culturing activated (and optionally transduced) central memory and/or effector T cells in a culture medium comprising IL-7; and
- 20 (d) isolating T cells which express CD117.

In some embodiments step (d) is carried out between step (a) and step (b), between step (b) and step (c), before step (c) and/or after step (c). In some embodiments, step (d) is carried out after step (b). In some embodiments, step (d) is carried out after step (c).

25

The T cells subjected to reversion in step (c) may be recently activated (and optionally transduced) central memory and/or effector memory T cells. In other words, the cells are not steady state central memory and/or effector memory T cells.

- 30 Methods of identifying markers present on and/or in T cells will be known to the skilled person. For example, markers may be identified by antibody labelling methods such as flow cytometry or ELISA.

The method may further comprise isolating T cells having a CD95⁺, CD117⁺ phenotype. The skilled person will be aware of methods for isolating cells, for example, the method of Fluorescence Activated Cell Sorting (FACS).

5 Detailed description

Embodiments of the invention will now be described by way of example with reference to the accompanying figures, in which:

10 **Figure 1: IL-7 induced reversion of recently differentiated memory CD8⁺ T cells to a naïve-like phenotype.**

(a) Flow cytometric analysis of phenotypic changes in CD8⁺ T cells after activation and successive incubation with IL-7. CBMC were activated with anti-CD3 and the phenotype of CD8⁺ T cell was monitored over time. At day 5, when the percentage of naïve T cells dropped below 20%, IL7 was added to the cultures. Numbers indicate the percentage of cells in each quadrant. Single representative experiment out of 50. (b) Kinetics of phenotype reversion of CD8⁺ T cells from 50 different CB samples. The shaded area represents the timeframe when IL-7 was added for the first time. (c) CD8⁺ T cells proliferation after activation and after IL-7 administration. CBMC were stained with CFSE either before activation (left panels) or at day 14, during phenotype reversion (right panels). At the indicated time points, cell phenotype and CFSE content were assessed for T_N (light gray dots) and T_{CM} (dark gray dots) CD8⁺ cells. Dashed lines indicate basal content in CFSE. Single representative experiment out of three. (d) Flow cytometry evaluation of IL-7-dependent phenotype reversion in recently differentiated T_{CM} and T_{EM}. Enriched CD8⁺ T_N were stimulated and, when T_N percentage dropped below 20%, untouched T_{CM} and T_{EM} were isolated. The two cell subpopulations were then incubated with IL-7 and monitored for phenotype changes over time. The T_{CM} and T_{EM} shown here are from two different CB samples. Single representative experiment out of three shown for each subset.

Figure 2: The expression of CD27, CD45RO and CD62L is shown for the cells having undergone phenotype reversion in Figure 1a (right panel). Dashed lines represent isotype controls.

Figure 3: The expression of the indicated markers is shown for T_N, recently differentiated T_{CM} and early reverted T_{Nrev} CD8⁺ cells.

Figure 4: Comparative phenotype of CB CD8⁺ T_{Nrev}, T_{CM} and T_N cells, and dependence on culture conditions. (a) The expression of the markers that discriminate early T_{Nrev} from T_N was measured by flow cytometry. T_N (black histograms), recently differentiated T_{CM} (gray histograms) and early reverted T_{Nrev} (red histograms). Single representative experiment out of three. (b) Kinetics of CD25 and CD127 expression by reverted CD8⁺ T_{Nrev} cells in the presence of either IL-2 or IL-7. After phenotype reversion had occurred following activation, cells were either switched to IL-2 or maintained in IL-7. The absence of one cytokine led to increased expression of its cognate receptor. The mean fluorescence intensity (MFI) for CD25 and CD127 is shown. Data are represented as means $\pm$ 1SD of three samples. (c) Kinetics of the expression of discriminative markers for early reverted CD8⁺ T_{Nrev} cells. The MFI of CXCR3, integrin β 7 and CD95 expression was monitored at different time points during activation and reversion as in (b). Data are represented as means $\pm$ 1SD of three samples. (d) Summary of phenotypic changes for each sequential subset (T_N, T_{CM}, T_{SCM} and T_{Nrev}). (e) Proposed model of CD8⁺ T cell differentiation.

Figure 5: CD8⁺ T_{Nrev} cells have excellent differentiative and proliferative potential, and can acquire effector function upon secondary activation. (a) Flow cytometry analysis of activation-induced phenotype changes in CD8⁺ T_{Nrev} and T_N cells from the same CB sample. Cells were activated with PHA and phenotype assessed at different time points. Single representative experiment out of three. (b) Flow cytometry analysis of activation-induced proliferation in CD8⁺ T_{Nrev} and T_N cells. Cells from the samples shown in the panel above were stained with CFSE at day 0 and activated with PHA. The CFSE content in the two cell subsets is shown at the indicated time points. Dashed lines represent basal content of CFSE. Single representative experiment out of three. (c) Flow cytometry analysis of perforin and granzyme B expression in re-stimulated CD8⁺ T_{Nrev}. T_{Nrev} cells transduced with the SSC-TCR were re-stimulated twice with peptide-pulsed DCs, and their expression of perforin and granzyme B assessed. Black histograms indicate resting CD8⁺ T_{Nrev} cells, gray indicate re-stimulated cells. (d) Epitope-specific cytotoxicity assay of re-stimulated, transduced T_{Nrev} cells. The cells shown in the panel above were incubated in a standard ⁵¹Cr cytolytic assay with HLA A*1101-transduced T2 cells loaded with either 1 μ g/ml (diamonds), 10 ng/ml (squares) or 1 ng/ml of SSC peptide (triangles). The peptide solvent i.e. DMSO was used as control (crosses). The percentage of target cell killing at different E:T ratios is indicated.

Figure 6: Phenotype reversion can be induced by cytokines other than IL-7, and can be induced by activation with different stimuli (a) Viability of cells incubated with different cytokines.

Activated CBMC were incubated from day 4 with the indicated cytokine or medium, and CD8⁺ T cell viability was evaluated by flow cytometry using 7AAD uptake. Data are represented as means $\pm$ 1SD of three samples. (b) Kinetics of phenotype reversion of CD8⁺ T cells activated with different artificial stimuli. IL-7 was added on day 6, and CD8⁺ T cell phenotype was monitored throughout. Data are represented as means $\pm$ 1SD of three samples. (c) Kinetics of phenotype reversion of CD8⁺ T cells undergoing successive cycles of activation/IL-7 incubation. Newly generated CD8⁺ T_{Nrev} cells were stimulated twice with PHA and induced to revert twice with IL-7. (d) Flow cytometry analysis of phenotype changes of CD8⁺ T_{Nrev} upon activation with the cognate antigen. CBMC T lymphocytes were activated, retro-transduced with the SSC-TCR and induced to revert their phenotype with IL-7 (left panels). Afterward, cells were incubated with peptide-pulsed DCs to induce specific differentiation of transduced cells (central panels). IL-7 was then added driving the transduced cells (black dots), but not the non-transduced ones (gray dots) to revert their phenotype (right panels). Plots were gated on CD8⁺ T cells. In the upper panels the percentage of transduced and non-transduced CD8⁺ T cells is indicated.

Figure 7: (a) CD117 expression was assessed by flow cytometry after activation and during phenotype reversion. Single representative experiment out of three. (b) As controls for CD117 normal PBMC and AML blast cells were used.

Figure 8: Reversion of activated memory CD4⁺ T cells to a naïve/T_{Nrev} phenotype. CD4⁺ T_N cells obtained from cord blood (CB) were activated with anti-CD3 and the phenotype was monitored over time. At day 5, when the percentage of T_N dropped below 20%, IL7 was added to the cultures. Numbers indicate the percentage of each cell type as calculated from flow cytometry.

Figure 9: Activated memory cells derived from adult CD8 T lymphocytes can also be induced to revert to a naïve-like (T_{Nrev}) phenotype. CD8⁺ T_N cells isolated from the peripheral blood of adult donors were activated with anti-CD3 and the phenotype was monitored over time. At day 5, when the percentage of T_N dropped below 20%, IL7 was added to the cultures. Numbers indicate the percentage of each cell type as calculated from flow cytometry.

Figure 10 Table showing the absolute number of CD8⁺ T cells in the different subsets at T_N nadir and T_{Nrev} plateau. Cells were enumerated using Trucount beads. ($\times 10^3$)

Figure 11 Table showing the effect of different cytokines on phenotype reversion. Cytokines were added on day 4, when the percentage of T_N dropped below 20%. The percentage of CD8⁺ T cells in each subset at T_N nadir and T_{Nrev} plateau is indicated.

Figure 12 Table listing the antibodies used for cell staining.

Figure 13 Plots showing expression of CD45RA and CD117 in cord blood-derived T cells cultured in anti-CD3, IL-2 and IL-7.

Figure 14 (a) Plots showing expression of CD117 of isolated adult naïve T cells cultured in anti-CD3, IL2 and IL-7; (b) Gating strategy on plot identifying differentiation subsets based on CCR7 and CD45RA expression.

EXAMPLE 1

A novel subset of memory T-stem cells (T_{SCM}) was recently identified within human peripheral blood (PB) which has been proposed to constitute the precursor memory T cell pool. Although T_{SCM} display many phenotypic features in common with naïve T cells and show capacity for self-renewal, they exhibit functional features of antigen-experienced cells and differentiate into memory and effector cell subsets upon re-encounter with the antigen. These cells are believed to represent an intermediate stage between T_N (naïve T cells) and T_{CM} (central memory T cells), and therefore the key subset in memory T-cell generation. T_{SCM} account for 2-3% of all $CD3^+$ T-lymphocytes in PB, and it is currently uncertain how such cells are formed and which mechanisms determine their expansion and differentiation. We explored the generation of T_{SCM} using lymphocytes from human cord blood which are unlikely to have encountered antigen and therefore have a very low frequency of T_{SCM} . We then investigated the functional phenotype of these T_{SCM} cells in comparison to naïve or memory T cells. In particular, we studied the proliferative capacity, effector function and marker expression of the cells. We term this population a T naïve-revertant subset (T_{Nrev}). This term encompasses T_{SCM} cells.

Materials and Methods

Cell separation and culture

Cord blood (CB) was obtained from CB collections unsuitable for transplant, provided by the NHS Cord Blood Bank, Colindale, UK. Peripheral blood (PB) (for the generation of DCs) was collected from platelet donors undergoing apheresis at the NHS Blood Donor Centre, Birmingham, UK. Informed consent was sought from each donor prior to sample collection. PB mononuclear cells (PBMC) and CB mononuclear cells (CBMC) were obtained by Ficoll separation.

In some instances, (i.e. to exclude the possibility of the selective death of memory cells, or the selective proliferation of naïve cells) CD8⁺ T_N cells were enriched by immunomagnetic separation, using the Naïve CD8⁺ T Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany), exactly following manufacturer's instructions. However, CBMC were generally
 5 activated following Ficoll separation without enrichment for T cells.

Untouched CD8⁺ T_{EM} cells were isolated after activation of enriched CD8⁺ T_N cells by removal of CCR7⁺ and CD45RA⁺ cells with anti CCR7/APC, anti CD45RA/APC and Anti-APC MicroBeads (all from Miltenyi). Untouched CD8⁺ T_{CM} cells were isolated from less differentiated samples by depletion of CD45RA⁺ cells with anti CD45RA/APC and Anti-APC MicroBeads.

10 Cells were cultured in RPMI 1640 plus 10% FCS (both from Sigma-Aldrich, St. Louis, MO).

TCR gene transduction and re-activation of transduced cells.

CBMC T cells were retrovirally transduced with an HLA A*1101-restricted TCR, specific for the SSCSSCPLSK (SSC) peptide of the LMP2 protein of Epstein Barr virus, as previously described¹. Also previously described is the generation of dendritic cells (DCs) from PBMCs, the loading
 15 of said DCs with the peptide, and the re-stimulation of transduced T cells with peptide-pulsed DCs.

Cell activation and treatment

Cells were activated, or re-activated, with either of the following stimuli:

Anti-CD3: cells were incubated in the presence of 66 ng/ml anti-CD3 antibody (OKT3), plus 300
 20 U/ml IL-2 (Miltenyi); cells were activated in this way throughout the work, unless otherwise stated.

Phytohaemagglutinin (PHA): cells were incubated in the presence of 1% PHA M form (Life Technologies), plus 50 U/ml IL-2;

IL-2 100 U/ml was added at day 2. The phenotype was checked every other day, and, unless
 25 otherwise stated, when the percentage of CCR7⁺/CD45RA⁺ CD8⁺ T cell dropped below 20% half of the medium was removed from cultures and replaced with fresh medium containing IL-7, (Miltenyi) at the final concentration of 25 ng/ml. The concentration was chosen on the basis of titration experiments. Thrice a week, half of the culture medium was removed and replaced with new medium plus cytokine(s).

Flow cytometry

The antibodies used for cell staining are listed in Figure 12. Gating strategy involved selection of single cells and use of a “dump channel” including either 7-aminoactinomycin D (7AAD, BD), and PerCP-conjugated CD14, CD16 and CD19, or Live/Dead Fixable Violet (Life Technologies,
 5 Thermo Fisher Scientific, Wilmington, DE, USA) and Pacific Blue-conjugated CD14, CD16 and CD19.

Proliferation was evaluated by staining cells for 2 min with 1 with 1 μ M carboxyfluorescein succinimidyl ester (CFSE). For intracellular staining, cells were fixed and permeabilized using the FIX&PERM kit (ADG, Kaumberg, Austria).

- 10 Transduced lymphocytes were identified using HLA A*1101:SSC peptide-specific pentamers and Tag/PE (Proimmune, Oxford, UK).

We performed flow cytometry acquisition on either a violet laser FacsCanto II, or a Fortessa (BD)

Cytotoxicity assay

- 15 The cytotoxicity of transduced T cells was assessed in a standard ^{51}Cr release assay as previously described.¹ Briefly, HLA A*1101-transduced T2 cells were loaded with different concentrations of SSC peptide, then used as targets at 2500 cells/well in a 5 hrs test.

Results

- 20 **IL-7 induces CD8⁺ memory T cells to revert to a naïve-like phenotype.**

In initial experiments cord blood-derived mononuclear cells (CBMCs) were cultured with anti-CD3 and IL-2 and the activation of CD8⁺ T cells was monitored by phenotypic analysis of CD45RA and CCR7 expression. These markers are used conventionally to categorize T-cells into naïve (CD45RA⁺/CCR7⁺, T_N), central memory (CD45RA⁻/CCR7⁺, T_{CM}), effector memory
 25 (CD45RA⁻/CCR7⁻, T_{EM}) and effector (CD45RA⁺/CCR7⁻, T_{Eff}) subsets. As expected, culture with these mitogens induced an expansion of T_{CM} and T_{EM} subsets with a concurrent reduction in the T_N population (Fig. 1a,b). CD45RA⁺/CCR7⁻ T_{Eff} were not generated in this model and so were not considered further.

We next examined a potential role for cytokines to modulate the differentiation of T-cells after activation. IL-7 was added to the culture medium when the proportion of T_N $CD8^+$ T cells dropped below 20%; this timing differed between different samples. IL-7 was added on day 5 after activation in Figure 1a. A reduction in the proportion of T_N $CD8^+$ T cells to below 20% occurred between days 4-9 after activation (mean 10.4 ± 6.23 , $n=50$) in Figure 1B. IL-7 was therefore added between days 4-9 after activation to the samples shown in Figure 1B (shaded area indicates the interval of time when IL-7 was added for the first time). After addition of IL-7 the percentage of T_N $CD8^+$ cells continued to diminish and reached a nadir of $8.39\% \pm 6.40$, after a further 3 days (Fig 1b). However, during further incubation with IL-7 the great majority of $CD8^+$ cells started to re-express CD45RA and reverted back to a phenotype resembling T_N cells, characterized by co-expression of CD45RA, CCR7, CD62L and CD27 and lack of expression of CD45RO (Fig. 1a and Fig. 2). This reacquisition of a naïve phenotype by $CD8^+$ T memory cells reached a plateau at 13-28 days after initial activation (mean 19.8 days, ± 4.66), and at that time typically represented over 70% of the $CD8^+$ T cell population (mean $71.1\% \pm 11.68$, range 44.7%- 95.3%). As such this value was only slightly below the mean of 86.6 % of $CD8^+$ T_N cells which was present at day 0 (± 5.84 ; range 74.3-97.7).

To demonstrate that the apparent phenotypic reversion of activated T cells was not due to selective death or proliferation of individual T cell subsets, we went on to enumerate the cells within each cell subset, monitored their proliferation, and then tracked the phenotypic changes of immunomagnetically-purified $CD8^+$ T cell subsets. Specifically, cell numbers were counted during incubation with IL-7 and despite changes in the proportion of naïve and memory subsets, the total number of all cells before and after reversion remained largely unchanged (Figure 10). Therefore selective death of memory cells could not explain the apparent re-population of the T_N compartment. To explore the possibility that $CD8^+$ T_N cells had selectively proliferated and retained their phenotype, we used CFSE-staining to track proliferation within T cell subsets. Although there was initial proliferation of both T_{CM} and, to a lesser extent, T_N $CD8^+$ T cells for 3 days after activation, no cell division was found after the addition of IL-7, during phenotypic reversion (Figure 1c).

To further confirm that there is true reversion of memory T cells to a naïve-like phenotype, recently differentiated T_{CM} and T_{EM} $CD8^+$ T cells, previously activated with anti-CD3 and IL-2, were then purified by magnetic selection and incubated with IL-7. Phenotype reversion was again demonstrated for over 80% of T_{CM} and T_{EM} cells, with the latter showing slightly delayed kinetics (Fig. 1d). The observation that acquisition of the naïve-like T memory cell phenotype

was obtained within a purified population of memory CD8⁺ T cells confirms that this is a definitive modulation of cellular phenotype and is not due to selective survival of T_N cells. The data also show that IL-7 can revert isolated CD8⁺ T cells without other cell types present. Henceforth the reverted naïve-like memory T cells will be referred to as T_{Nrev}.

5 During reversion the phenotype of CD8⁺ T_{Nrev} cells progresses through a profile resembling T_{SCM} cells before acquiring a fully naïve phenotype

We next decided to investigate the membrane profile of CD8⁺ T memory cells during the process of reversion and to compare this to the profile of CD8⁺ T_{CM}, T_{SCM}, and T_N cells.

27 membrane-bound proteins were selected, in order to identify distinct markers that could be used for discriminating CD8⁺ T_{Nrev} from T_N or T_{CM} subsets. T_{Nrev} were isolated and analysed between around 12-18 days post-activation. Most of the selected markers were unsuitable for discriminating between T_N and T_{Nrev} due to overlapping expression. Nevertheless, in some cases there were detectable small differences in mean fluorescence intensity (MFI) between the two such as for CD120b, CD122 or CD127. (Figure 3). On the other hand, integrin β7 and CXCR3 expression levels of T_{Nrev} were quite distinct from T_N but closer to and overlapping with recently differentiated T_{CM} (Figure 4a). Most discriminating were the expression of CD95 and CD25 by T_{Nrev}, lying intermediate between T_N and recently differentiated T_{CM}.

Indeed, the profile of T_{Nrev} was very similar to that of circulating T_{SCM}, with the two subsets sharing the phenotype CD45RA⁺/CCR7⁺/CD95⁺/CD122⁺/CXCR3⁺. Unlike T_{SCM}, T_{Nrev} were CD127^{low}/CD25^{high}. However, it is known that the expression of the receptors for IL-7 and IL-2 by T-cells is downregulated by their respective cytokine. T_{Nrev} that had just attained phenotype reversion with IL-7 (early T_{Nrev}), and thereafter deprived of IL-7 and maintained in low dose IL-2 (IL-7 was added at day 6 post activation, then at day 18 cells were switched to IL-2) converged to a phenotype even closer to T_{SCM}, with quick and sharp decrease in the expression of CD25 and progressive increase in the expression of CD127 (Figure 4b).

At this stage (i.e. T_{Nrev} deprived of IL-7 and maintained in low dose IL-2) only the expression of CD95, CXCR3 and integrin β7 differentiated T_{Nrev} and T_{SCM} cells from T_N cells. However, after a further 2 weeks in culture with only low dose IL-2 (added every 2-3 days) the expression of the three markers was almost completely lost on late T_{Nrev} (Fig. 4c). The profile of early T_{Nrev} cells maintained in long term culture with IL-2 therefore came to almost completely resemble the

profile of T_N cells. The change in the level of expression of specific markers by T_N , T_{CM} and T_{Nrev} is represented by Figure 4d.

Based on our findings, we propose the inclusion of a novel pathway into the T-cell differentiation model that takes into account the reversion process from T_{EM} to T_N . (Fig. 4e). T_{Nrev} can therefore be considered to be the T_{scm} cells of the present invention.

Reverted T_{Nrev} cells proliferate and differentiate rapidly into functional effector cells following secondary stimulation

As $CD8^+$ T_{Nrev} cells are antigen-experienced cells that have undergone activation and expansion, it might be expected that they would have diminished proliferative potential when compared to primary T_N . As such we used CFSE-labelling followed by PHA activation to examine and contrast the proliferative properties of early T_{Nrev} cells and T_N cells. Interestingly, T_{Nrev} cells differentiated more rapidly than T_N into T_{CM} and T_{EM} subsets, and also exhibited a markedly higher proliferation rate (Fig. 5a,b). To investigate if T_{Nrev} cells could acquire effector function following restimulation, $CD8^+$ T_{Nrev} cells generated from SSC-specific TCR-transduced CBMC were re-stimulated with peptide-pulsed DCs and then stained with antibodies to perforin and granzyme B to examine their cytotoxic phenotype. Following stimulation, SSC- $CD8^+$ T_{Nrev} cells acquired a T_{EM} phenotype and expressed high levels of intracellular perforin and granzyme B (Figure 5c). Indeed they were able to exert cytolytic activity against SSC-loaded T2 cells in a ^{51}Cr release assay (Figure 5d).

Discussion

Here we tracked the phenotypic and functional profile of CB $CD8^+$ T_N cells following antigen-activation and demonstrate for the first time that, after differentiating to T_{CM} or T_{EM} , T cells are able to revert back to a T_N -like phenotype. A wide range of phenotypic and functional analyses showed that T_{Nrev} cells are very similar to T_{SCM} . These features include the ability to undergo rapid proliferation and differentiation to effector cells after secondary stimulation. As such, T_{Nrev} cells and T_{SCM} cells can be regarded effectively as the same subset. Replacing IL-7 with IL-2 led T_{Nrev} cells to express a $CD27^+/CD45RA^+/CD45RO^-/CD69^-/CD95^+/CD122^+/CD127^+/CCR7^+/CXCR3^+/CXCR4^+$ phenotype that was indistinguishable from T_{SCM} . T_{Nrev} cells retain phenotypic features typical of activated memory cells, including CD95, CD25, CXCR3 and Integrin $\beta 7$ expression and this correlates with their rapid functional response to stimulation. However, not all the markers associated with T cell activation are

upregulated in early T_{Nrev} cells, with proteins such as CD70, TNFSF10 and TNSF14 expressed at levels that are comparable to naïve cells. A further key feature of $CD8^+ T_{Nrev}$ cells is re-expression of CD45RA, a critical regulator of the signaling threshold in T lymphocytes.

When T_{Nrev} were cultured further in resting conditions they reverted to a phenotype that was very similar to primary T_N cells, with progressive downregulation of both CD95 and Integrin $\beta 7$ and the loss of CXCR3.

The data also show that the ability to revert is intrinsic to $CD8^+$ T cells, since IL-7 can revert isolated $CD8^+$ T cells without other cell types present.

Our results show that T_{Nrev} proliferate and differentiate rapidly into functional effector cells following secondary stimulation. These differentiated cells were able to exert cytolytic activity against SSC-loaded T2 cells in a ^{51}Cr release assay. We have also shown that T_{Nrev} can be generated from TCR-transduced CBMC, and that said TCR-transduced T_{Nrev} can differentiate into a T_{EM} phenotype, and were able to exert cytolytic activity. These TCR-transduced T cells will be particularly useful for therapy, since they can be transduced with a specific TCR or CAR in order to target cells having particular biomarkers.

Our data show a novel method for the *in vitro* generation of T_{SCM} and reveal that the differentiation pathway of $CD8^+$ T cells is not unidirectional but may include reversion back to less differentiated subsets such as T_{SCM} .

EXAMPLE 2

To determine the effect of different cytokines and different activating agents upon the pattern of T cell differentiation, we analysed the effect of incubation (i.e. culture) with a range of different cytokines and/or activating agents on T_{Nrev} (i.e. T_{SCM}) generation. Having demonstrated the ability of IL-7 to drive phenotypic reversion of activated $CD8^+$ T cells back to T_{Nrev} we next went on to assess whether this property was unique to IL-7 or was also shared by other cytokines.

Materials and Methods

Cell separation and culture

CB was obtained from CB collections unsuitable for transplant, provided by the NHS Cord Blood Bank, Colindale, UK. PB was collected from platelet donors undergoing apheresis at the NHS

Blood Donor Centre, Birmingham, UK. Informed consent was sought from each donor prior to sample collection. PB mononuclear cells (PBMC) and CB mononuclear cells (CBMC) were obtained by Ficoll separation.

In some instances, (i.e. to exclude the possibility of the selective death of memory cells, or the selective proliferation of naïve cells) CD8⁺ T_N cells were enriched by immunomagnetic separation, using the Naïve CD8⁺ T Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany), exactly following manufacturer's instructions. However, CBMC were generally activated following Ficoll separation without enrichment for T cells.

Cells were cultured in RPMI 1640 plus 10% FCS (both from Sigma-Aldrich, St. Louis, MO).

TCR gene transduction and re-activation of transduced cells.

CBMC T cells were retrovirally transduced with an HLA A*1101-restricted TCR, specific for the SSCSSCPLSK (SSC) peptide of the LMP2 protein of Epstein Barr virus, as previously described¹. Also previously described is the generation of dendritic cells (DCs) from PBMCs, the loading of said DCs with the peptide, and the re-stimulation of transduced T cells with peptide-pulsed DCs.

Cell activation and treatment

Cells were activated, or re-activated, with either of the following stimuli:

Anti-CD3: cells were incubated in the presence of 66 ng/ml anti-CD3 antibody (OKT3), plus 300 U/ml IL-2 (Miltenyi); cells were activated in this way throughout the work, unless otherwise stated;

Anti-CD3 and crosslinked anti-CD28: cells were incubated in the presence of 66 ng/ml OKT3 antibody, 66 ng/ml LEAF anti-CD28 (BioLegend, San Diego, CA), and 66 ng/ml rat anti-mouse IgG1 (BioLegend), plus 50 U/ml IL-2;

CD3/CD28 beads: Dynabeads T Activator CD3/CD28 beads (Life Technologies, Grand Island, NY) were incubated with CBMC at 1:1 ratio in the presence of 30 U/ml IL-2;

Phytohaemagglutinin (PHA): cells were incubated in the presence of 1% PHA M form (Life Technologies), plus 50 U/ml IL-2;

Staphylococcus enterotoxin B (SEB): cells were incubated in the presence of 1 μ g/ml SEB (Sigma-Aldrich), plus 50 U/ml IL-2.

IL-2 100 U/ml, for soluble anti CD3, or 30 U/ml, for the other cases, was added at day 2. Phenotype was checked every other day, and, unless otherwise stated, when the percentage of CCR7⁺/CD45RA⁺ CD8⁺ T cell dropped below 20% half of the medium was removed from cultures and replaced with fresh medium containing either IL-2, IL-4, IL-6, IL-7, IL-15 or IL-21 (all from Miltenyi), or combinations thereof, at the final concentration of 50 U/ml, 25 n/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml, and 50 ng/ml, respectively. The concentrations were chosen on the basis of titration experiments. Thrice a week, half of the culture medium was removed and replaced with new medium plus cytokine(s). Whenever CD3/CD28 beads were used, an attempt was made at removing the beads from the cells before starting the secondary incubation with IL-7, but after 6 passages on the proper magnet most of the cells had one or more beads attached to the surface.

Flow cytometry

The antibodies used for cell staining are listed in Figure 12. Gating strategy involved selection of single cells and use of a “dump channel” including either 7-aminoactinomycin D (7AAD, BD), and PerCP-conjugated CD14, CD16 and CD19, or Live/Dead Fixable Violet (Life Technologies, Thermo Fisher Scientific, Wilmington, DE, USA) and Pacific Blue-conjugated CD14, CD16 and CD19.

Transduced lymphocytes were identified using HLA A*1101:SSC peptide-specific pentamers and Tag/PE (Proimmune, Oxford, UK).

We performed flow cytometry acquisition on either a violet laser FacsCanto II, or a Fortessa (BD)

Results

Phenotypic reversion of activated CD8⁺ T cells to T_{Nrev} cells is supported by cytokines that maintain cell survival

Parallel cultures of activated CBMCs were incubated with single and multiple combinations of the γ c cytokines IL-2, IL-7, IL-15, IL-4 and IL-21. Cytokines were added 2-3 days post activation (i.e. around day 6). IL-6, an inflammatory cytokine that is known to deliver pro-apoptotic signals, was also incorporated.

Phenotypic reversion was indeed observed for several of these cultures but was both slower to develop and significantly less marked than was seen with IL-7. In particular, at day 15, when maximum reversion was reached, the number of CD8⁺ T cells with T_N phenotype increased by 72% for cells incubated with IL-7, compared to 41%, 25% and 31% for cells incubated with IL-2, IL-4 or IL-15 respectively (Figure 11). Interestingly, CD8⁺ T cells cultured with IL-6 or IL-21 were driven toward more a differentiated memory phenotype with a substantial increase in T_{Eff} cells. No synergistic effect was observed when IL-7 was administered together with IL-2, IL-4 or IL-15.

We further observed that the ability of individual cytokines to promote reversion back to T_{Nrev} cells correlated with their ability to support CD8⁺ T cell survival *in vitro* (Fig. 6a, activated cells incubated from day 4 with the indicated cytokine and medium). Indeed, cell death was low in cultures incubated with IL-2, IL-4, IL-7 and IL-15 whilst the use of IL-6, IL-21, or indeed medium alone, all led to massive cell death.

To evaluate if phenotypic reversion could take place following activation by mitogenic stimuli other than T cell receptor (TCR) cross-linking, we activated CBMC with phytohaemagglutinin (PHA), Staphylococcal Enterotoxin B (SEB), anti-CD3/anti-CD28 beads, and anti-CD3 plus cross-linked anti-CD28 antibodies (Figure 6b). IL-7 was added on day 6 post activation. The percentages of CD8⁺ T cells undergoing IL-7-dependent phenotype reversion after activation with PHA and SEB were similar to those found in cells activated with anti-CD3. However, activation with CD3/CD28 beads led to a smaller proportion of cells reverting to a naïve phenotype. No differences were observed in the kinetics of reversion between CD8⁺ T cells that had been activated with anti-CD3 alone and those that had been activated with anti-CD3 plus cross-linked anti-CD28 antibodies, demonstrating that more potent activation with co-stimulation did not prevent the cells from reverting back to a T_{Nrev} phenotype.

T cells can undergo several rounds of reversion

To assess if cells could undergo more than one cycle of phenotypic reversion, CBMC were subjected to several rounds of activation followed by incubation with IL-7. Each successive activation was generated by changing the media of the cells. CBMC were activated initially with anti-CD3 before IL-7 was added to the culture to induce reversion to T_{Nrev}. Serial rounds of anti-CD3 stimulation led to a high rate of cell death and so PHA was used for two further rounds of activation, followed again by IL-7 incubation once the proportion of T_N cells had fallen below 20% (Fig. 6c). After each cycle of activation and IL-7 treatment, a reversion to the T_{Nrev}

phenotype was observed, indicating that recently differentiated CD8⁺ memory T cells can undergo repeated cycles of activation and reversion.

In order to demonstrate that phenotypic reversion is possible after activation with cognate antigen and not just with mitogens, CBMC were transduced with a gene encoding a T-cell receptor (TCR) specific for the SSC peptide of the LMP2 protein of Epstein-Barr virus. Following activation and retroviral transduction, cells acquired a predominantly T_{CM}/T_{EM} phenotype. These cells were then incubated with IL-7 and underwent prompt reversion to a T_{Nrev} phenotype. Cells were then re-challenged with SSC-pulsed dendritic cells (DCs) and differentiated again to CD8⁺ T_{EM} within 5 days (Fig. 2d). At this point IL-7 was re-added, and after a further 9 days a second reversion to the T_{Nrev} phenotype was attained. Our findings demonstrated that reversion to CD8⁺ T_{Nrev} was also possible after physiological stimulation with cognate antigen presented by professional antigen-presenting cells.

Discussion

The results reveal an important role of IL-7 in inducing reversion of recently differentiated effector CD8⁺ cells back in to T_{Nrev}. IL-7 was the most potent independent driver of this reversion but other members of the γ c cytokine family, namely IL-2, IL-4 and IL-15, were also able to induce reversion in a proportion of cells. The ability to mediate reversion correlated with relative capacity of cytokines to support cell survival. We have found that phenotypic reversion can occur following activation by peptide pulsed (SSC)- DCs, phytohaemagglutinin (PHA), Staphylococcal Enterotoxin B (SEB), anti-CD3/anti-CD28 beads, or anti-CD3 plus cross-linked anti-CD28 antibodies. Therefore, phenotypic reversion can occur following activation by TCR specific (e.g. peptide) or TCR-non specific (for example PHA, SEB or anti-CD3/anti-CD28 stimulation) mitogenic stimuli. We have also observed that cells can undergo more than one cycle of phenotypic reversion. CBMC underwent three rounds of reversion when subjected to one cycle of anti-CD3 activation + IL-7 treatment, followed by two further cycles of PHA activation and IL-7 incubation. We have also shown that transduced CBMC can undergo at least two cycles of phenotypic reversion.

EXAMPLE 3

We further investigated distinct markers that could be used for discriminating CD8⁺ T_{Nrev} from T_N subsets, in particular CD117.

Materials and Methods

Cell separation and culture

Cells were obtained, separated (if necessary) and cultured as described in the above Examples.

Cell activation and treatment

- 5 Cells were activated, or re-activated, with the following stimuli:

Anti-CD3: cells were incubated in the presence of 66 ng/ml anti-CD3 antibody (OKT3), plus 300 U/ml IL-2 (Miltenyi); cells were activated in this way throughout the work, unless otherwise stated.

- 10 IL-2 100 U/ml was added at day 2. The phenotype was checked every other day, and, unless otherwise stated, when the percentage of CCR7⁺/CD45RA⁺ CD8⁺ T cell dropped below 20% half of the medium was removed from cultures and replaced with fresh medium containing IL-7, (Miltenyi) at the final concentration of 25 ng/ml. The concentration was chosen on the basis of titration experiments. Thrice a week, half of the culture medium was removed and replaced with new medium plus cytokine(s).

- 15 The antibodies used for cell staining are listed in Table 3. Gating strategy involved selection of single cells and use of a “dump channel” including either 7-aminoactinomycin D (7AAD, BD), and PerCP-conjugated CD14, CD16 and CD19, or Live/Dead Fixable Violet (Life Technologies, Thermo Fisher Scientific, Wilmington, DE, USA) and Pacific Blue-conjugated CD14, CD16 and CD19.

- 20 We performed flow cytometry acquisition on either a violet laser FACS Canto II, or a Fortessa (BD)

Results

CD117 is expressed by T_{Nrev}

- 25 CD117 expression was assessed by flow cytometry after activation and during phenotype reversion (day 0, day 6, day 12 and day 20). CD117 was highly expressed by T_{Nrev} and distinguished T_{Nrev} from naïve T cells (Figure 7). IL-7 was added to the culture medium when the proportion of T_N CD8⁺ T cells dropped below 20%; in this instance on day 5 after activation.

Discussion

Our results identify CD117 as a distinctive marker of T_{Nrev} (i.e. T_{SCM}). This is of particular interest, since CD117, encoded by the *kit* gene, is the receptor for Stem Cell Factor (SCF). CD117 is expressed by Haemopoietic Stem Cells but has not been previously described in
5 mature circulating lymphocytes.

EXAMPLE 4

Having demonstrated the ability of $CD8^+$ T cells to be phenotypically reverted back to T_{Nrev} , we next went on to assess whether this property was unique to cord blood lymphocytes and $CD8^+$ T cells or was also shared by other lymphocytes, for example $CD4^+$ T cells and/or T cells obtained
10 from adult lymphocytes.

Materials and Methods

Cell separation and culture

Adult lymphocytes were obtained as per standard methods known to those skilled in the art. CB was obtained from CB collections unsuitable for transplant, provided by the NHS Cord Blood
15 Bank, Colindale, UK. $CD4$ or $CD8^+$ cells were obtained, separated (if necessary) and cultured as described in the above Examples.

Cell activation and treatment

Cells were activated, or re-activated, with the following stimuli:

Anti-CD3: cells were incubated in the presence of 66 ng/ml anti-CD3 antibody (OKT3), plus 300
20 U/ml IL-2 (Miltenyi); cells were activated in this way throughout the work, unless otherwise stated.

IL-2 100 U/ml was added at day 2. The phenotype was checked every other day, and, unless otherwise stated, when the percentage of $CCR7^+/CD45RA^+$ $CD8^+$ or $CD4^+$ T cells dropped below 20% half of the medium was removed from cultures and replaced with fresh medium
25 containing IL-7, (Miltenyi) at the final concentration of 25 ng/ml. The concentration was chosen on the basis of titration experiments. Thrice a week, half of the culture medium was removed and replaced with new medium plus cytokine(s).

Flow cytometry

Flow cytometry was performed as in Example 3.

Results

The reversion of CD4⁺ T cells (isolated from cord blood) was assessed by phenotypic analysis of CD45RA and CCR7 expression. These markers are used conventionally to categorize T-cells into naïve (CD45RA⁺/CCR7⁺, T_N), central memory (CD45RA⁺/CCR7⁺, T_{CM}), effector memory (CD45RA⁺/CCR7⁻, T_{EM}) and effector (CD45RA⁺/CCR7⁻, T_{Eff}) subsets. CD95 expression was also assessed in the CD8⁺ cells.

CD4⁺ T_N cells from CB were activated with anti-CD3 and the phenotype was monitored over time. At day 5, when the percentage of T_N dropped below 20%, IL7 was added to the cultures. Numbers indicate the percentage of each cell type as calculated from flow cytometry (Figure 8). Between day 0 and day 5 there was an expansion of T_{CM} and T_{EM} subsets with a concurrent reduction in the T_N population. However, during further incubation with IL-7, CD4⁺ T cells reverted back to a phenotype resembling T_N cells, as shown by the increased percentage of cells with a naïve phenotype on day 13.

To evaluate if cells obtained from adult lymphocytes could also revert to a T_{Nrev} phenotype, CD8⁺ T_N cells were isolated from adult lymphocytes and activated with anti-CD3. The phenotype was monitored over time (Figure 9). As expected, there was an expansion of T_{CM} and T_{EM} subsets with a concurrent reduction in the T_N population after activation. However, following further incubation with IL-7, a high percentage of CD8⁺ T cells reverted back to a phenotype resembling T_N cells. T_N cells on day 0 lacked CD95 expression; by day 2 onwards nearly all of the activated T_N cells were CD95⁺.

Discussion

Our data show that T_{Nrev} can be generated, not only from T cells isolated from cord blood, but also from T cells obtained from adult lymphocytes. In addition, we have shown that T_{Nrev} can be generated from CD4⁺ T cells.

EXAMPLE 5

Expression of Stem Cell Factor Receptor (CD117) by human early lineage CD8 T cells

We have found for the first time that mature T cells can be induced to express CD117 (Stem Cell Factor Receptor). Except for a small proportion of early thymocytes, CD117 expression has never been described in mature T cells. We describe how CD117 identifies early lineage T cells and how these cells could be selected using CD117 as a selection marker.

5

Materials and Methods

The cells were obtained, separated (if necessary) and cultured as described above.

T cells with naïve phenotype $CCR7^+/CD45RA^-$ from cord and adult peripheral blood were cultured with anti-CD3 and IL-2 followed by IL-7. Cells from cord blood were cultured without prior separation as they are largely all naïve. With adult T cells, cells with naïve phenotype were immunomagnetically isolated from healthy donor adult peripheral blood. The T cells were activated with soluble anti-CD3 antibodies and IL-2, driving the cells to central memory ($CCR7^+/CD45RA^-$) and effector memory ($CCR7^-/CD45RA^+$) phenotypes. After 6 days, IL-7 was added to induce reversion to T_{Nrev} phenotype. During activation and reversion the cells were analysed for expression of CD117 (Stem Cell Factor Receptor).

15

Results

In cord blood, the CD8 T cell population is largely homogenous and the vast majority of activated cells revert to a naïve phenotype (i.e. T_{SCM} or reverted T_N). CD117 expression increased during activation and reversion and by the end of the culture the vast majority of cells become CD117⁺ (Figure13).

20

The adult-derived T_N cells were similarly treated. The plots in Figure 14a show in the three donors the frequency of the differentiation subsets at different time points of the culture, i.e. at the beginning of the test, immediately before IL-7 addition, and at the end of the test. The different differentiation subsets present in the culture were gated on the subsets as defined by the differential expression of CCR7 and CD45RA (Figure 14b). The reversion to naïve phenotype in adults is less pronounced than in cord blood and at the end of the cultures, the T cell population become considerably diverse containing all differentiation subsets, despite starting off with an isolated population of mostly naïve cells. CD117 is expressed only by early differentiation subsets with phenotype of revertant naïve T cells and central memory T cells, but not by cells in late differentiation stages.

25

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Conclusion

Whereas most of the treated cord T cells revert to T_{Nrev} and become CD117 positive, only a subpopulation of adult T cells reverted to a naïve phenotype. Although not all cells with naïve phenotype expressed CD117, the marker was exclusively expressed by early differentiation T cells.

Unlike cord T cells which have largely a homogenous phenotype and function, adult naïve T cells are known to be functionally heterogenous, with differing TREC content (Kimmig et al , JEM 2002). It is therefore not unexpected, that reversion and CD117 expression was heterogenous.

Early differentiation T cells (T_{SCM} and T_{CM}) have superior *in vivo* proliferative and survival capacity, and are considered as ideal target T cells for chimeric antigen receptors or TCR transduction for immunotherapy use. Studies are in progress to isolate these cells, as their efficacy is inhibited in the presence of more differentiated memory T cells.

CD117 could therefore be a useful marker to isolate these early lineage T cells for T cell engineering. As a single marker, CD117 would permit the direct and positive enrichment of early lineage T cells after CAR transduction, with immunomagnetic or other selection methods. CD117 expression has to date not been described in mature T cells. Although its function remains unclear, its distinct expression amongst early lineage T cells provides a tool for selection and analysis.

References

Frumento, G. Cord blood T cells retain early differentiation phenotype suitable for immunotherapy after TCR gene transfer to confer EBV specificity. *Am J Transplant* **13**, 45-55 (2013).

CLAIMS

1. A method of sorting T cells, the method comprising isolating T cells which express CD117, wherein the T cells which express CD117 comprise a nucleotide sequence encoding a TCR and/or a CAR.
2. The method of claim 1, wherein the T cells which express CD117 have undergone reversion.
3. The method of claim 1 or claim 2, the method comprising:
 - (a) optionally activating naïve T cells to generate central memory and/or effector memory T cells;
 - (b) transducing activated central memory and/or effector T cells with a nucleic acid encoding a T cell receptor (TCR) or a chimeric antigen receptor (CAR);
 - (c) subjecting the transduced T cells to reversion; and
 - (d) isolating the T cells which express CD117, wherein step (d) is carried out between step (a) and step (b), between step (b) and step (c), before step (c) and/or after step (c).
4. The method of claim 2 or claim 3, wherein the reversion is carried out in accordance with the method of any one of claims 5 to 14 or 17 to 20.
5. A method of generating T memory stem cells (Tscm), the method comprising culturing central memory and/or effector memory T cells in a culture medium comprising one or more cytokines selected from the group consisting of IL-2, IL-4, IL-7 and IL-15.
6. The method according to claim 5, wherein the culture medium comprises IL-7.
7. The method according to claim 6, wherein the concentration of IL-7 in the culture medium is from 10 to 50 ng/ml.
8. The method according to claim 5 or claim 6, wherein the Tscm have a CD45RA⁺,CCR7⁺,CD95⁺ phenotype.
9. The method according to any one of claims 5 to 8, wherein the method comprises culturing the central memory and/or effector memory T cells in the culture medium for from 4 to 25 days.
10. The method according to any one of claims 5 to 9, wherein the central memory and/or effector memory T cells are CD8⁺.
11. The method according to any one of claims 5-9, wherein the central memory and/or effector memory T cells are CD4⁺.

12. The method according to any one of claims 5 to 10, wherein the method further comprises the step of activating naïve T cells by culturing the naïve T cells in the presence of an activating agent, wherein the activating agent is selected from the group consisting of antigen-pulsed APCs, anti-CD3 and/or anti-CD28 antibodies, anti-CD3 and/or anti-CD28 antibody-coated beads, phytohaemagglutinin (PHA), concanavalin A (ConA), phorbol myristate acetate (PMA) and/or ionomycin, 4.1BB and *Staphylococcus* enterotoxin B (SEB), thereby generating the central memory and/or effector memory T cells.
13. The method according to claim 12, wherein the naïve cells are obtained from cord blood.
14. The method according to claim 12, wherein the naïve cells are obtained from adult lymphocytes.
15. The method according to any one of claims 5 to 14, wherein the Tscm express CD117.
16. The method according to any one of claims 5 to 15, further comprising identifying and/or isolating cells which express CD117.
17. A method of generating naïve T cells, the method comprising culturing Tscm in a culture medium comprising one or more cytokines selected from the group consisting of IL-2, IL-4, IL-7 and IL-15.
18. The method according to claim 17, wherein Tscm are cultured for from 7 days to 28 days or until at least 50% of the cells obtain a CD95⁺ phenotype.
19. The method according to claim 17 or 18, wherein the method comprises transducing the T cells with a nucleic acid encoding a T cell receptor (TCR) or a chimeric antigen receptor (CAR).
20. The method according to claim 6, wherein the method comprises
- (a) activating naïve T cells by culturing the naïve T cells in the presence of an activating agent, thereby generating central and/or effector memory T cells;
- (b) culturing the central and/or effector memory T cells generated in (a) in a culture medium comprising one or more cytokines selected from the group consisting of IL-2, IL-4, IL-7 and IL-15;
- wherein the culture medium is added to the same vessel as step (a); and
- wherein the culture medium is added when the proportion of naïve T cells in the cell population is less than 20%.
21. A population of Tscm, wherein the population comprises at least 5×10^4 /ml Tscm.

22. The population according to claim 21, wherein the Tscm comprises a nucleotide sequence encoding a TCR and/or CAR.
23. A population of naïve T cells obtainable by the method of any one of claims 17 to 19, wherein the population comprises at least 5×10^4 /ml naïve T cells.
- 5 24. The population according to claim 23, wherein the naïve T cells comprise a nucleotide sequence encoding a TCR and/or CAR.
25. A Tscm cell which comprises a transgenic nucleotide sequence encoding a TCR or a CAR.
- 10 26. A naïve T cell which comprises a transgenic nucleotide sequence encoding a TCR or a CAR.
27. The Tscm cell according to claim 25, or the naïve T cell according to claim 26, wherein the cell expresses CD117.
28. The Tscm cell or the naïve T cell according to any one of claims 20-22, wherein the TCR or CAR encoded by the transgenic nucleotide sequence is overexpressed by
- 15 the Tscm or naïve T cell.
29. The Tscm cell or the naïve T cell according to any one of claims 25-28, wherein the transgenic nucleotide sequence encoding the TCR or CAR is operably linked to a non-TCR promoter which is functional in T cells.
30. Tscm or naïve T cells for use in immunotherapy or cancer therapy.

20

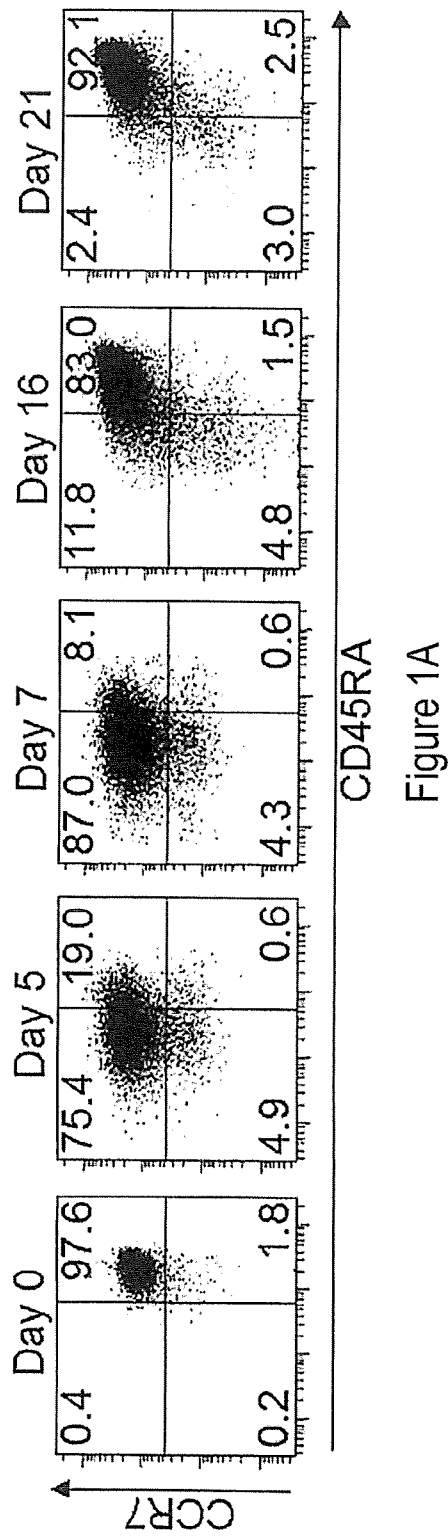


Figure 1A

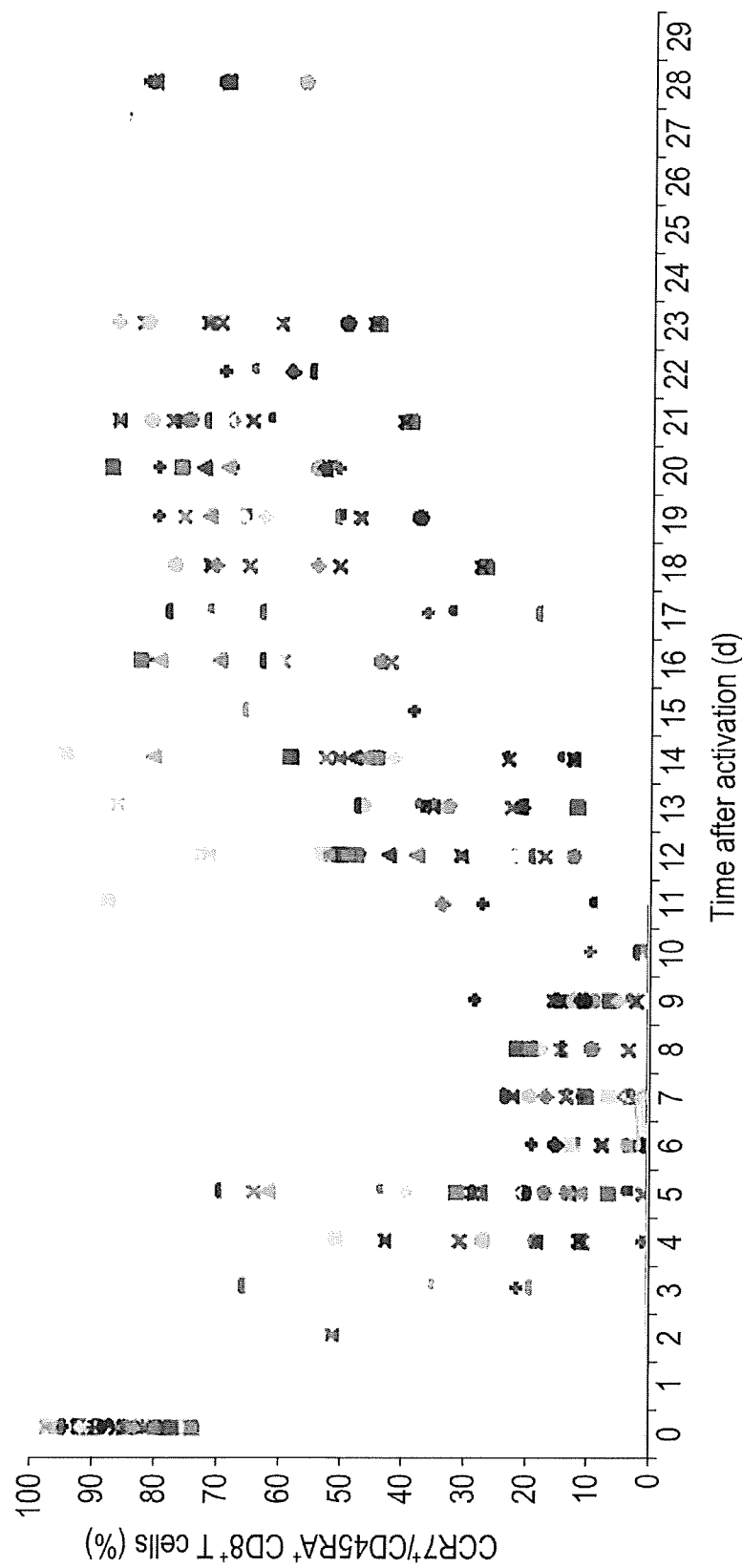


Figure 1B

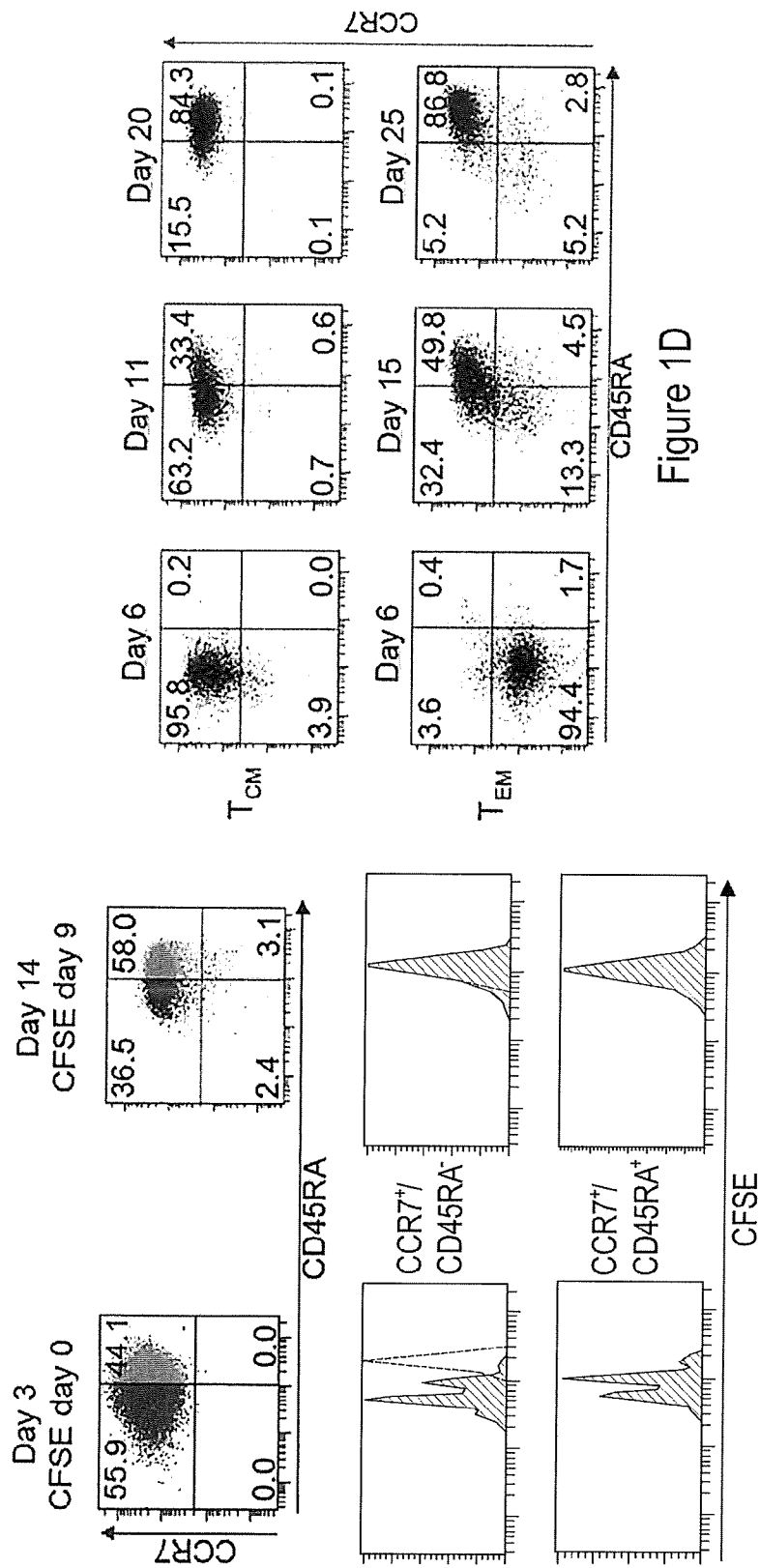


Figure 1C

Figure 1D

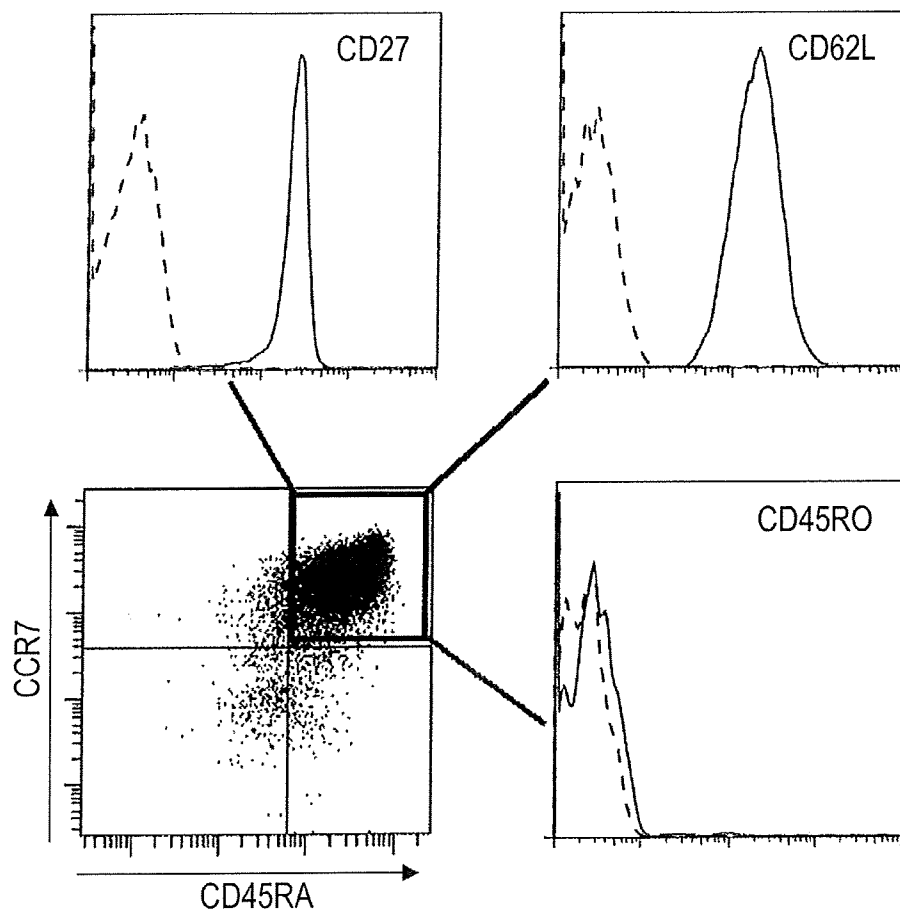


Figure 2

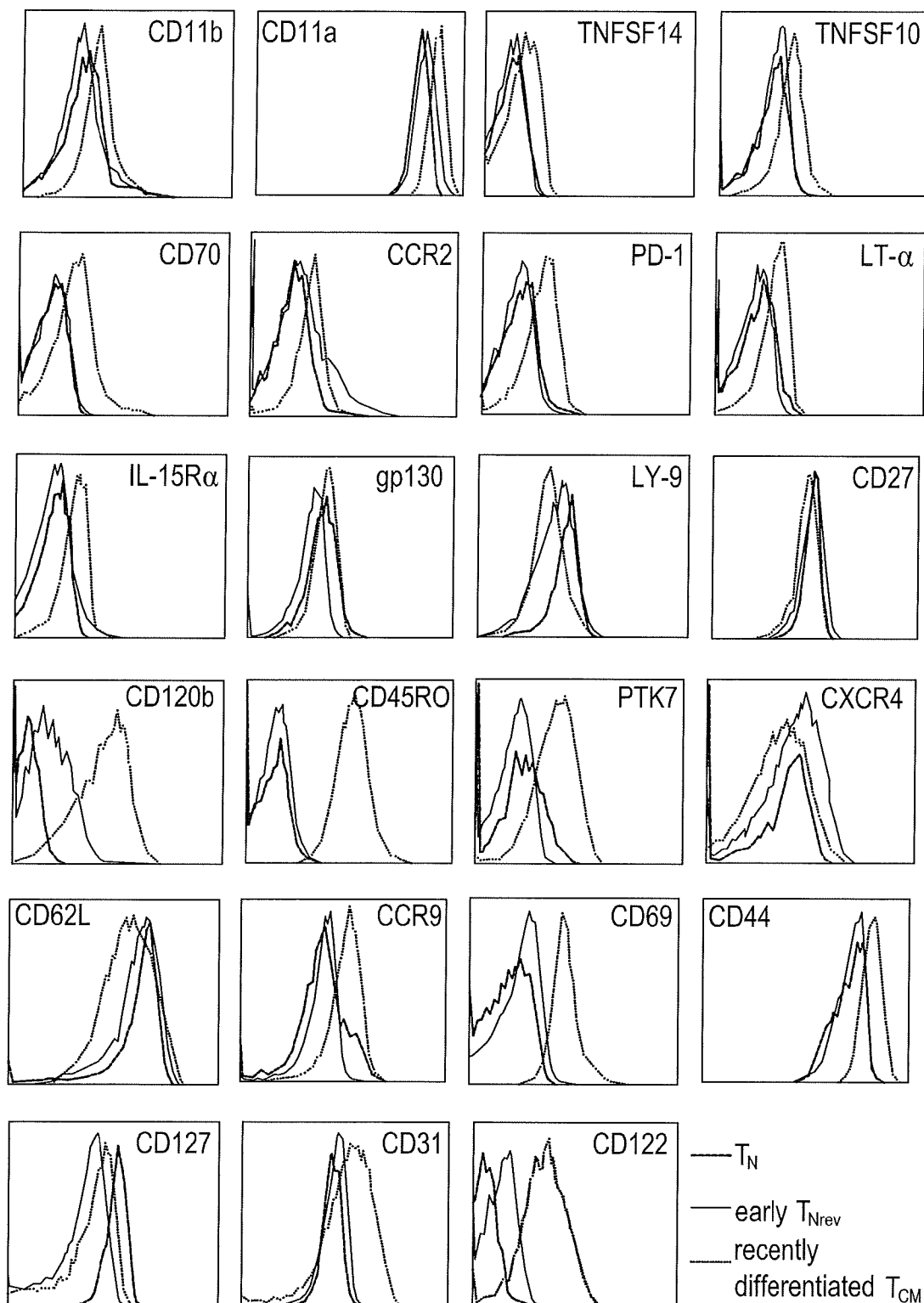


Figure 3

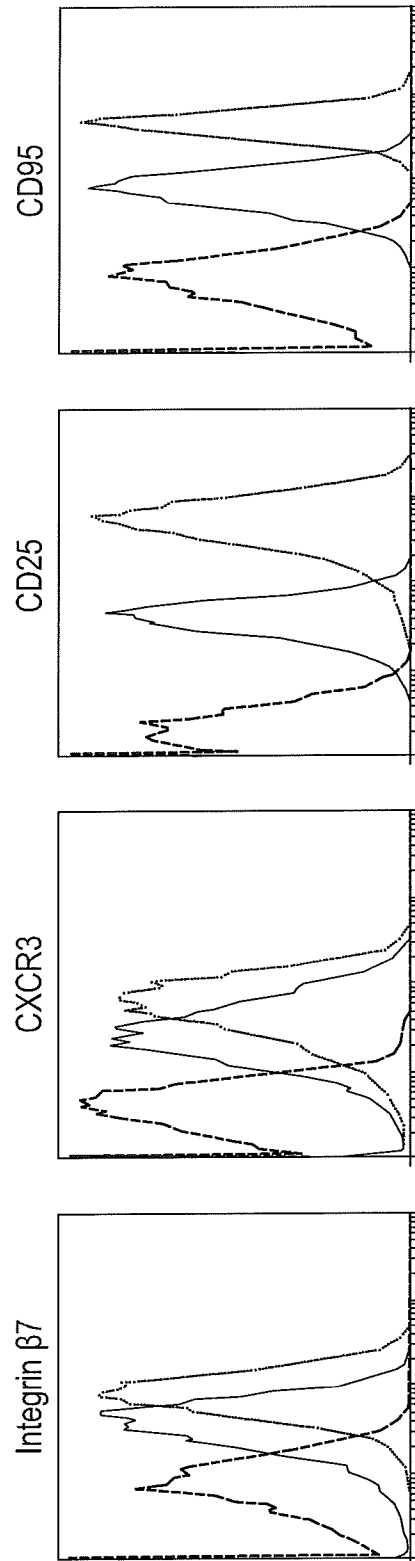


Figure 4A

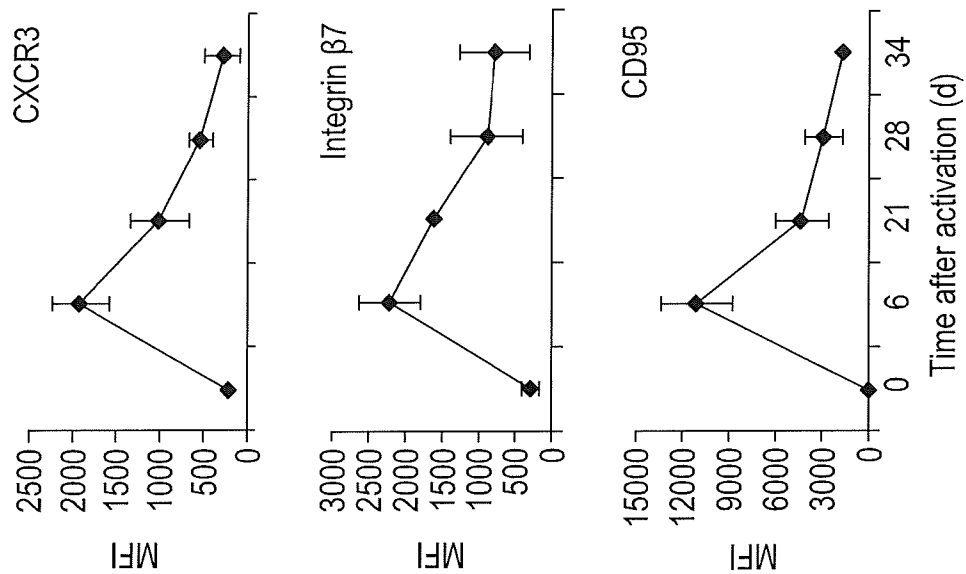


Figure 4C

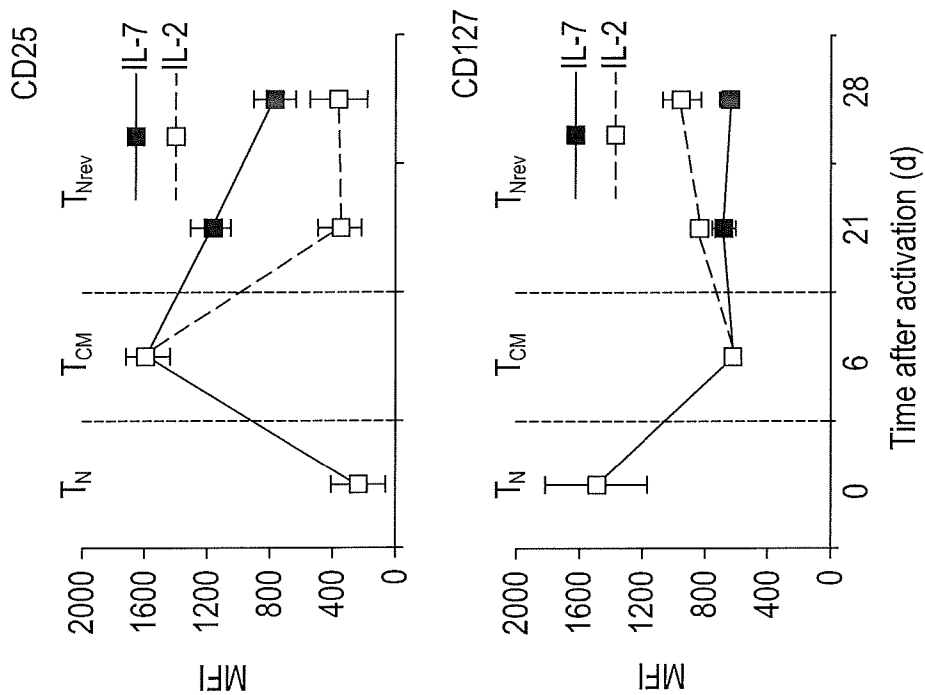


Figure 4B

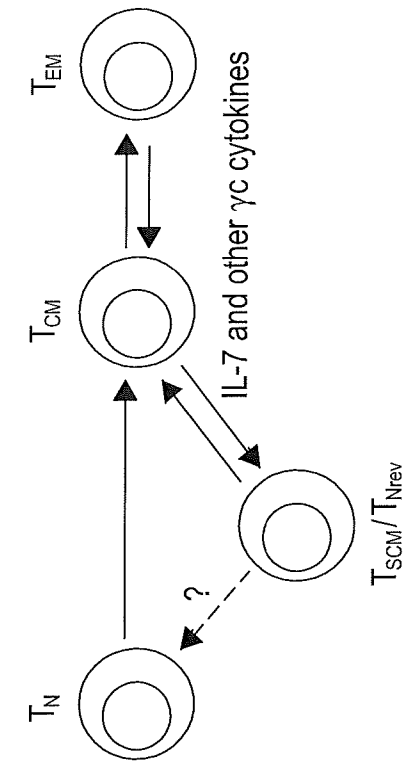


Figure 4E

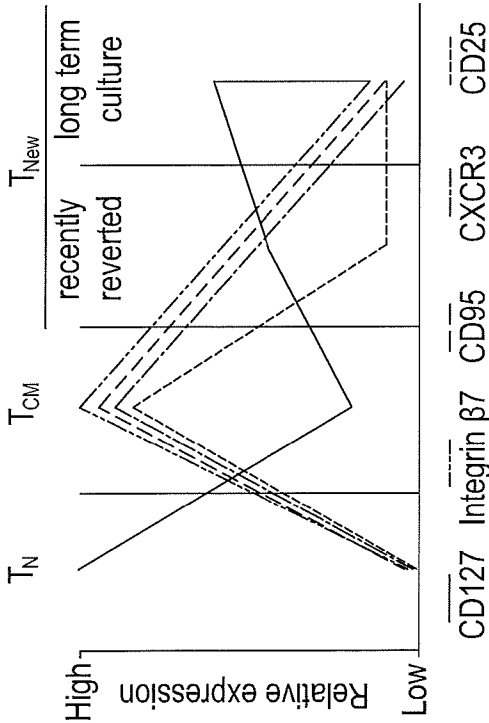


Figure 4D

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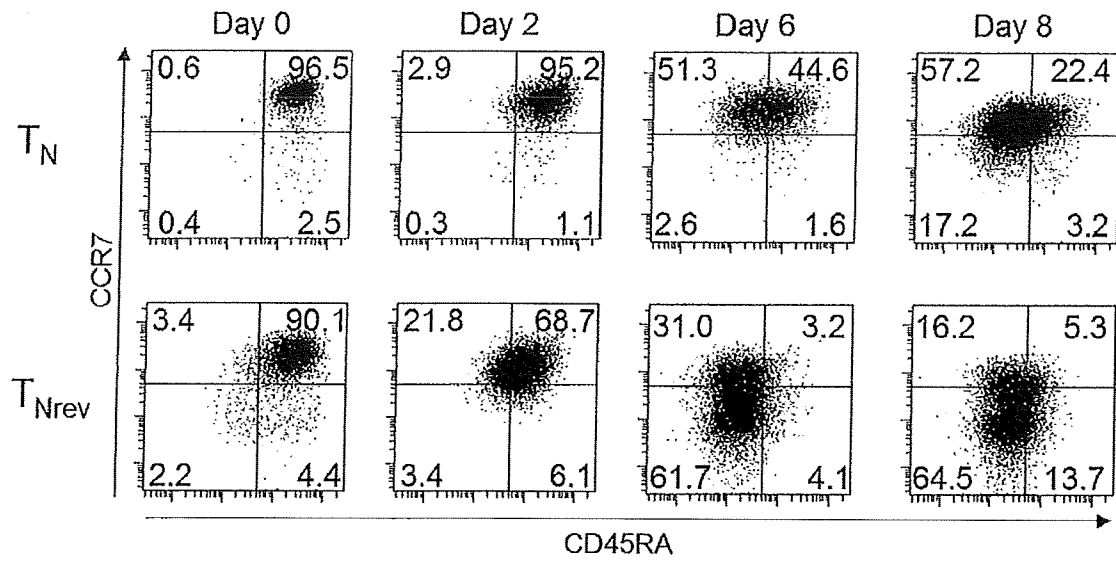


Figure 5A

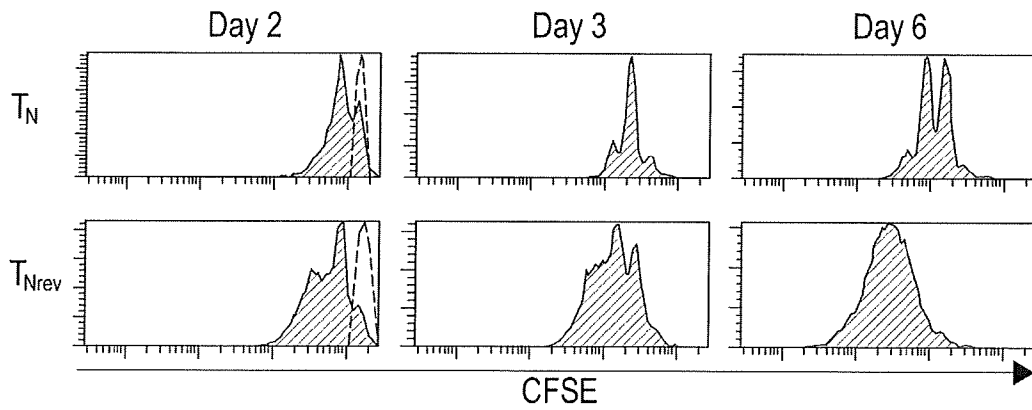


Figure 5B

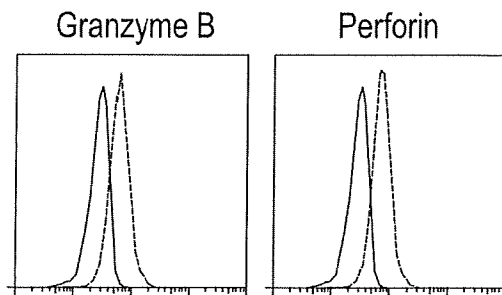


Figure 5C

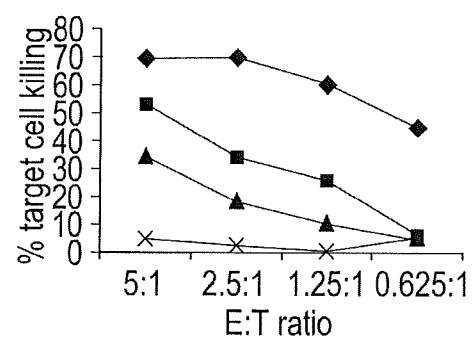


Figure 5D

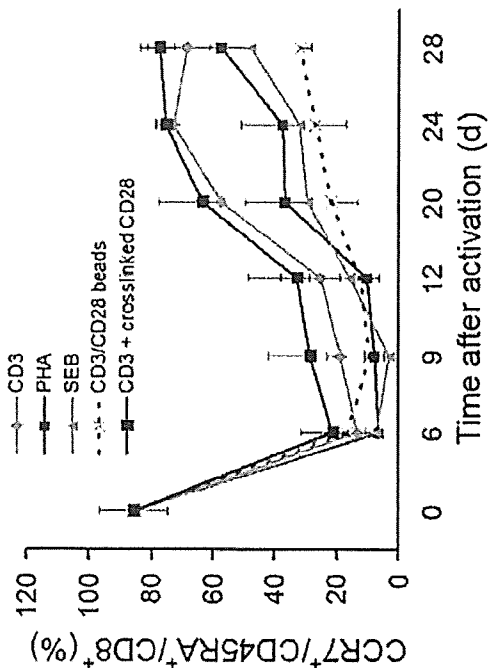


Figure 6B

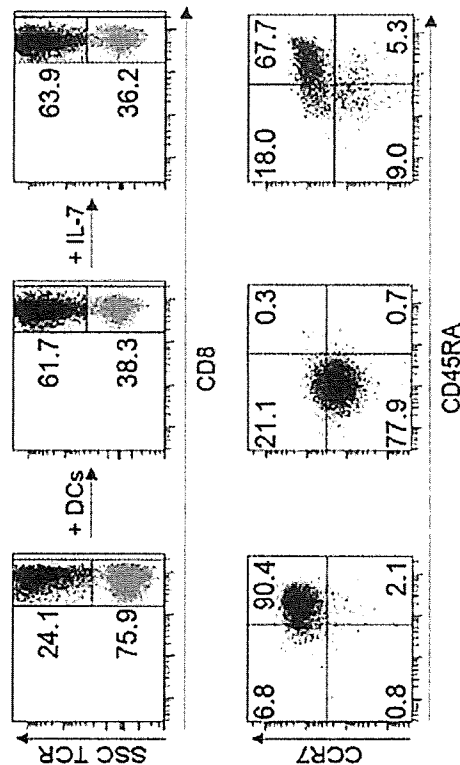


Figure 6D

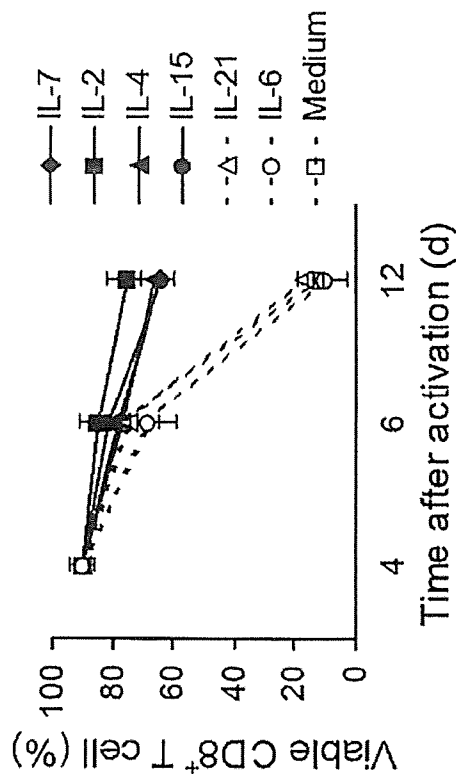


Figure 6A

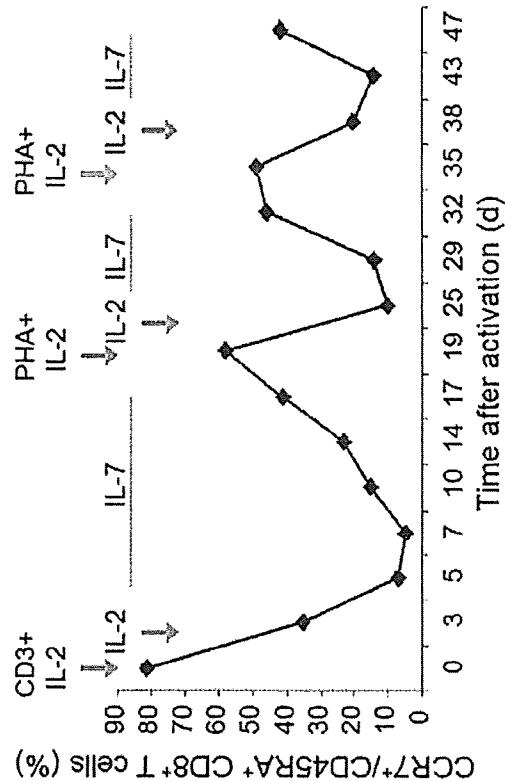


Figure 6C

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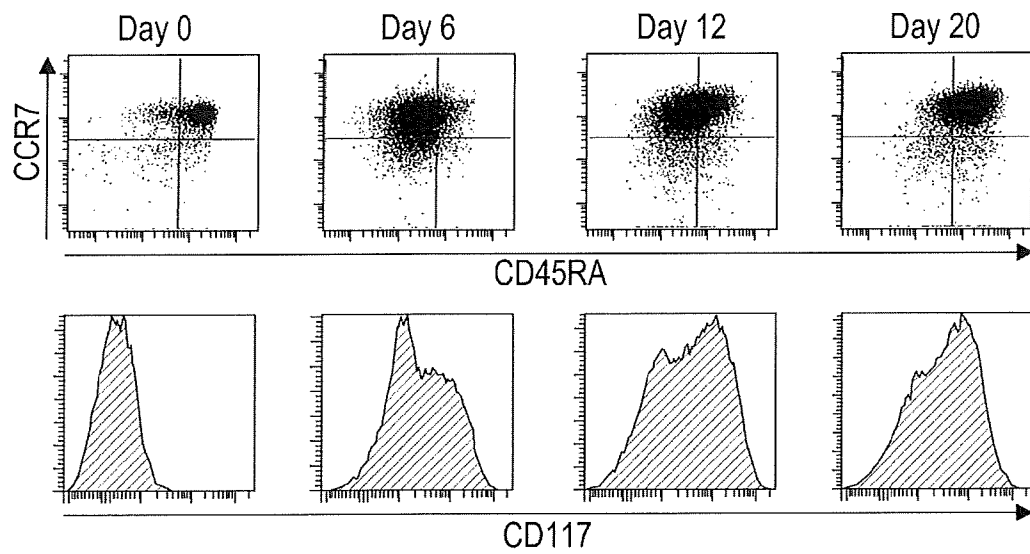


Figure 7A

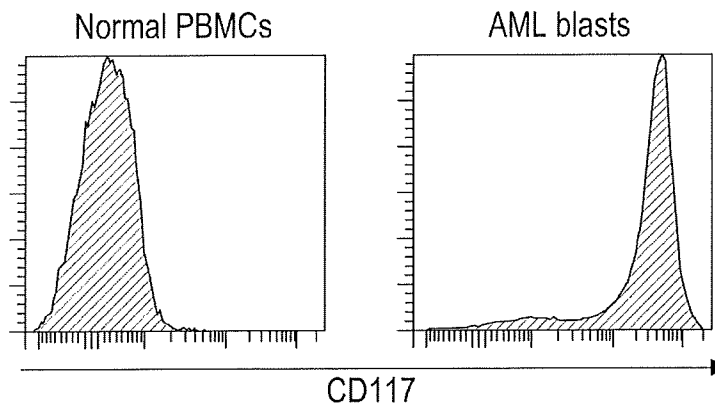


Figure 7B

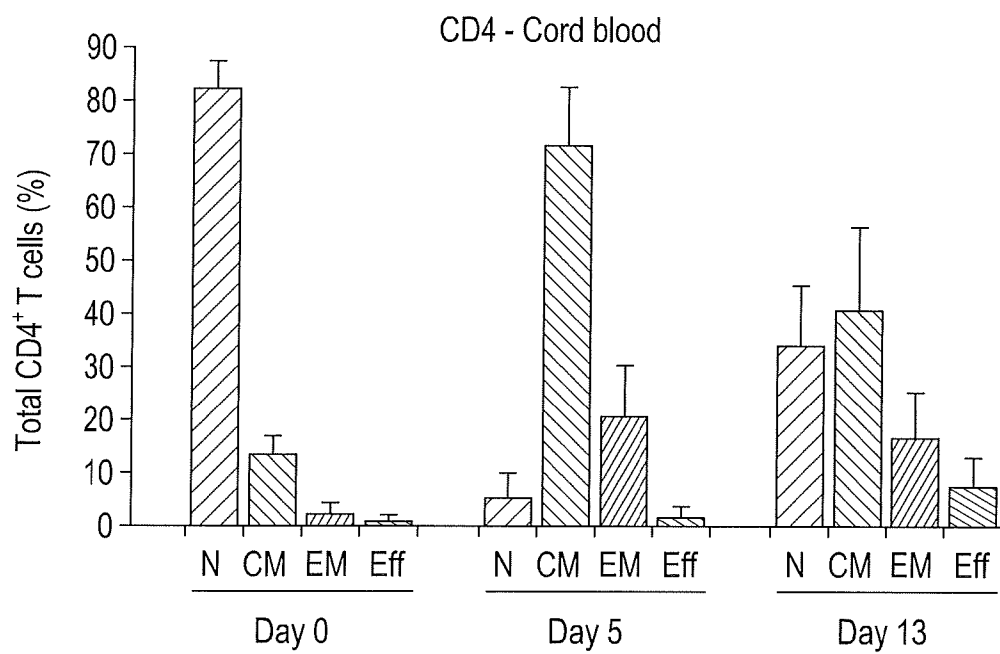


Figure 8

13 / 19

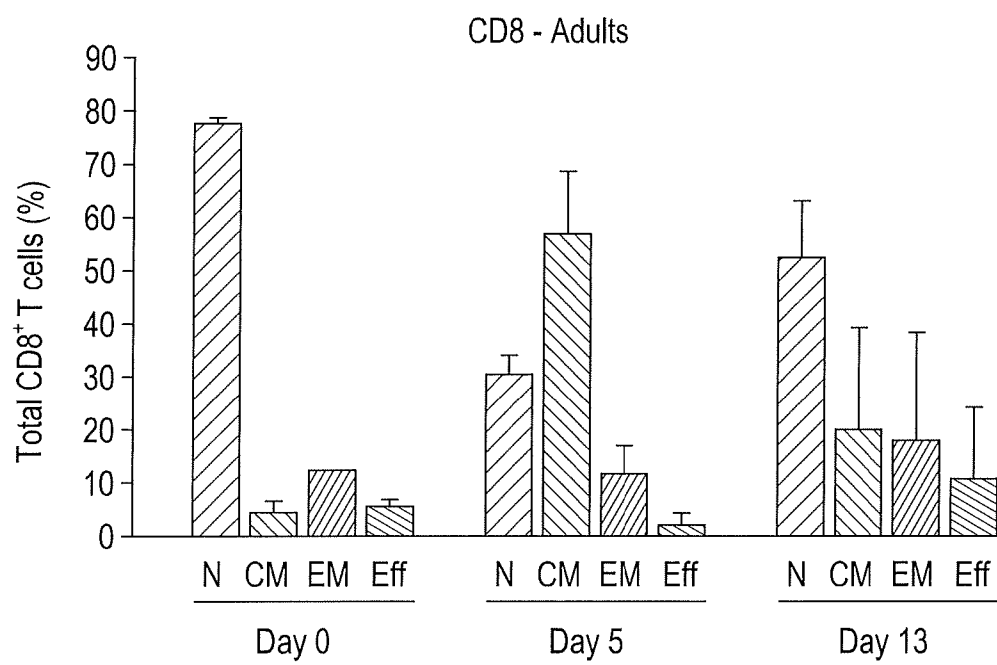


Figure 9

	CB 1		CB 2		CB 3	
	Day6	Day16	Day8	Day18	Day6	Day20
CCR7 ⁺ /CD45RA ⁺	32,1	872,1	9	345,5	21,3	1769,3
CCR7 ⁺ /CD45RA ⁻	982,2	256,6	782,2	303,2	1560,9	487,9
CCR7 ⁻ /CD45RA ⁻	308	79,2	183,7	101,4	213,2	33,1
CCR7 ⁻ /CD45RA ⁺	5,4	72	1,4	32	2,9	43
TOTAL	1327,7	1279,9	976,3	782,1	1798,3	2333,3

Figure 10

	IL-7	IL-2	IL-4	IL-15	IL-21	IL-6	Medium	IL-7 + IL-2	IL-7 + IL-15	IL-7 + IL-4
T_N nadir	3.5 ± 3.6	0.7 ± 0.8	5.0 ± 2.4	1.2 ± 1.8	5.3 ± 5.1	1.0 ± 0.9	7.9 ± 5.9	5.1 ± 5.2	9.1 ± 3.6	9.3 ± 3.1
	60.5 ± 8.5	42.5 ± 8.8	60.4 ± 10.5	37.0 ± 12.5	52.2 ± 12.3	72.6 ± 16.4	54.6 ± 19.2	66.1 ± 15.0	71.2 ± 8.9	64.4 ± 7.6
	34.5 ± 8.5	55.7 ± 12.1	31.7 ± 8.8	58.9 ± 14.6	38.6 ± 6.6	26.3 ± 13.5	34.8 ± 14.0	24.7 ± 7.7	18.8 ± 8.8	21.3 ± 5.7
	1.6 ± 0.7	1.1 ± 0.8	1.9 ± 0.8	2.9 ± 1.7	3.9 ± 4.5	0.2 ± 0.3	2.7 ± 2.4	4.2 ± 6.4	0.9 ± 0.7	5.0 ± 3.8
T_{nrev} plateau	75.8 ± 9.2	41.3 ± 8.4	30.1 ± 4.5	32.1 ± 5.6	6.4 ± 6.4	4.2 ± 3.2	10.1 ± 4.5	68.3 ± 11.2	73.1 ± 3.9	74.7 ± 3.6
	15.0 ± 5.5	22.9 ± 7.9	47.4 ± 7.0	16.6 ± 7.7	22.1 ± 12.8	13.2 ± 6.5	54.9 ± 14.9	21.7 ± 12.7	21.2 ± 4.5	17.6 ± 4.7
	3.1 ± 2.9	23.7 ± 6.2	11.3 ± 5.2	45.1 ± 9.1	66.9 ± 5.9	72.7 ± 8.8	27.9 ± 13.3	6.7 ± 7.9	2.4 ± 2.1	2.3 ± 1.7
	6.0 ± 4.0	12.1 ± 4.7	11.2 ± 5.0	26.3 ± 7.8	14.6 ± 8.9	9.9 ± 9.0	7.1 ± 6.3	3.4 ± 6.4	4.3 ± 4.4	5.1 ± 1.8

Figure 11

Table 3 List of the antibodies used for cell staining			
Antibody used	Purchased from	Antibody used	Purchased from
CCR2/PE	Biolegend	CD45RA/Fitc	BD
CCR7/Fitc	BD	CD45RA/Pe-Cy7	BD
CCR7/PE	R&D	CD45RA/BV510	BD
CCR7/BV605	BioLegend	CD45RO/PE	BD
CCR9/PE-Cy7	eBioscience	CD62L/PE	BD
CD11a/PE	BD	CD69/efluor 450	eBioscience
CD11b/PE	BD	CD70/APC	BioLegend
CD120b/APC	R&D	CD8/Alexa Fluor 700	eBioscience
CD122/APC	BioLegend	CD8/Fitc	BD
CD127/PE	BioLegend	CD8/Pacific Blue	BD
CD127/Pacific Blue	BioLegend	CD95/APC	BD
CD14/Pacific Blue	Invitrogen	CXCR3/APC	BD
CD14/PerCP	R&D	CXCR4/APC	eBioscience
CD16/Pacific Blue	Invitrogen	gp130/PE	R&D
CD16/PerCP	R&D	Granzyme B/Fitc	BD
CD19/Pacific Blue	Invitrogen	IL-15Ra/PE	Biolegend
CD19/PerCP	R&D	Integrin b7/PE	R&D
CD25/APC-Cy7	BD	LT-a/PE	BioLegend
CD27/APC	R&D	LY-9/PE	BioLegend
CD3/PE	BioLegend	PD-1/Fitc	BD
CD3/V500	BD	Perforin/Fitc	eBioscience
CD31/APC-eFluor 780	eBioscience	PTK7/PE	Miltenyi
CD4/Fitc	eBioscience	TNFSF10/PE	BioLegend
CD44/PE	BioLegend	TNFSF14/APC	R&D
CD117	Miltenyi		

Figure 12

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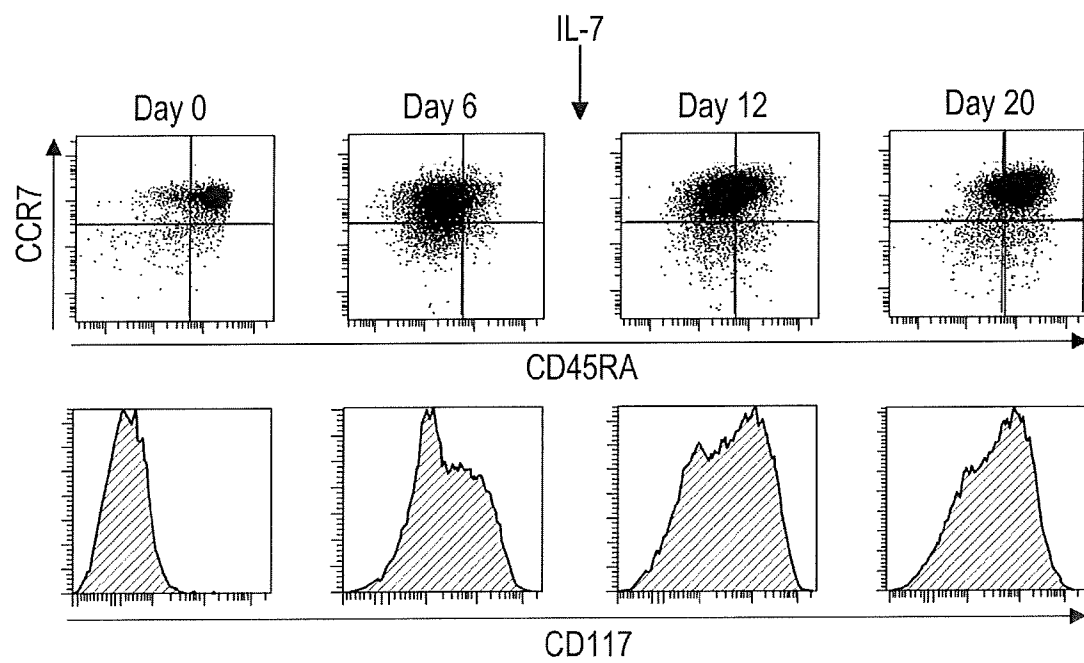


Figure 13

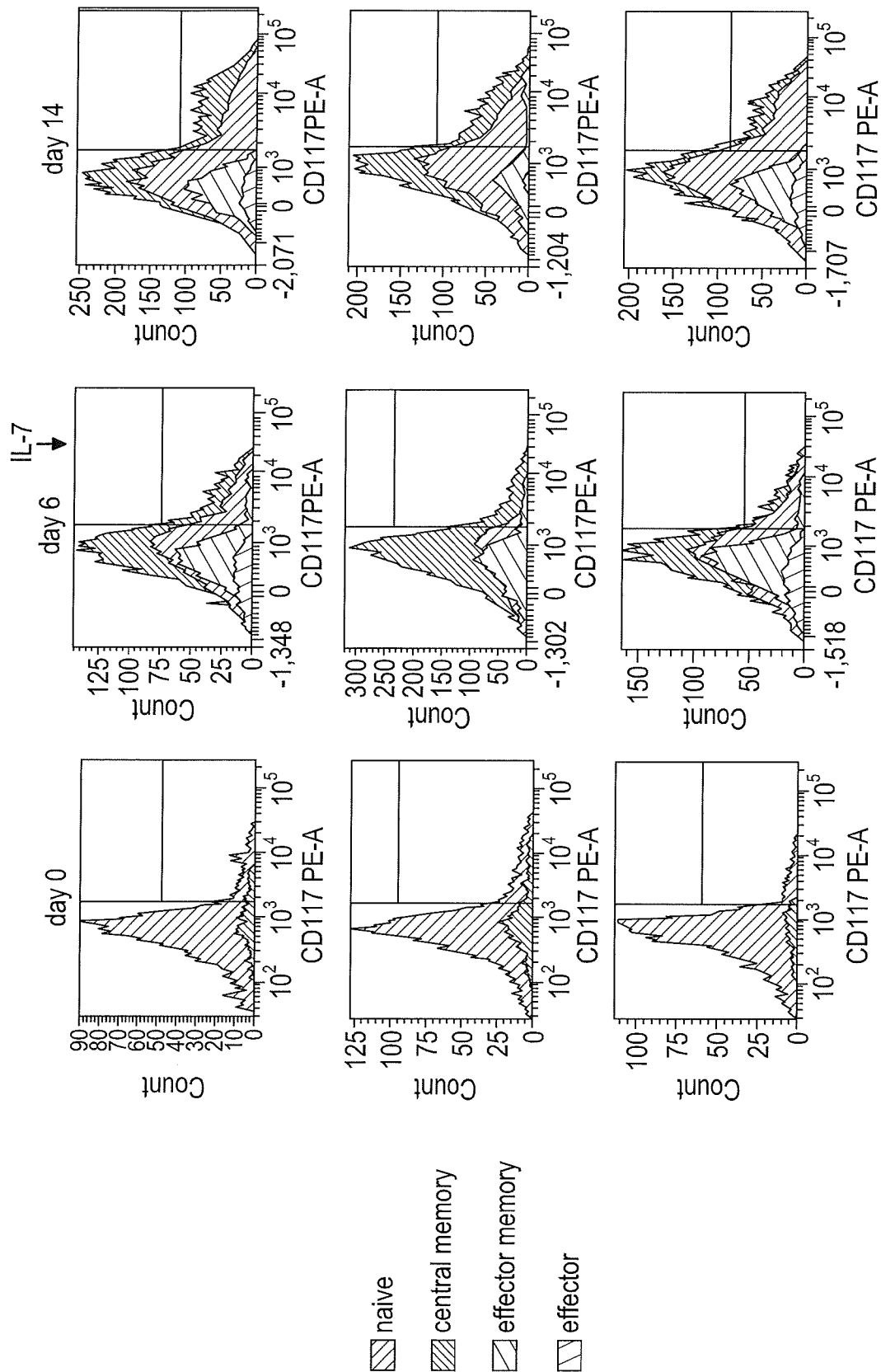


Figure 14A

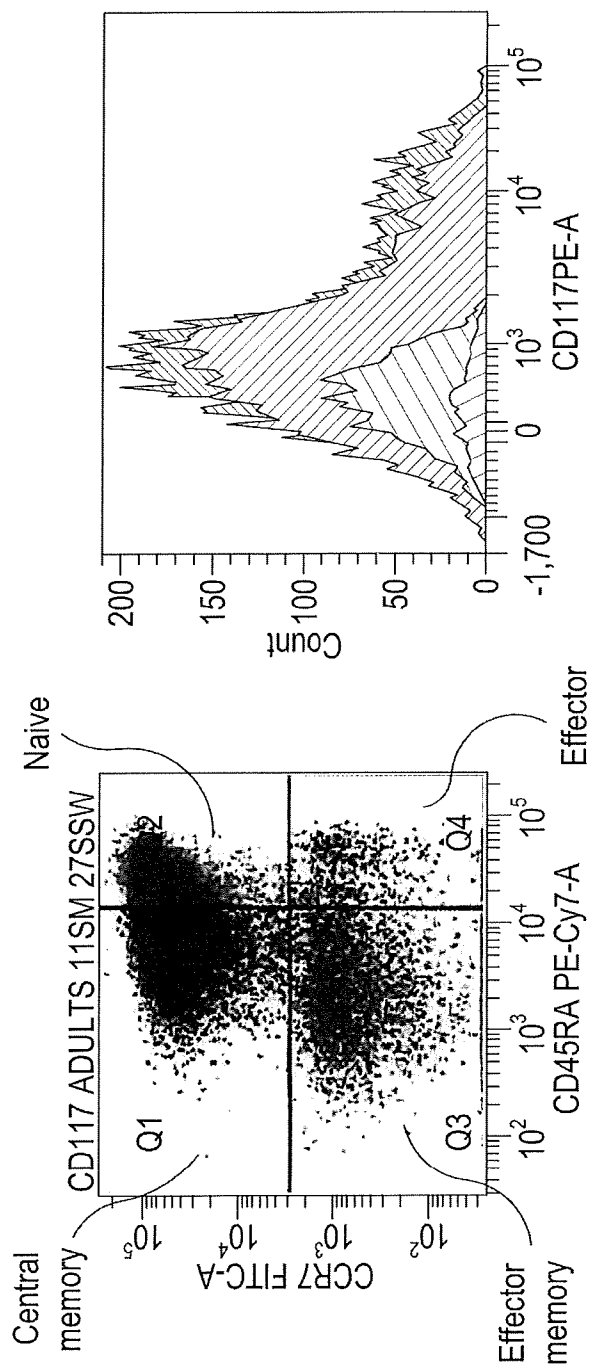


Figure 14b

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/051282

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0783
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)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015/299656 A1 (GATTINONI LUCA [US] ET AL) 22 October 2015 (2015-10-22) paragraph [0002] paragraph [0003] paragraph [0181] paragraph [0091] -----	5-14, 17-26, 28-30
X	WO 2015/189357 A1 (POLYBIOCEPT AB [SE]) 17 December 2015 (2015-12-17) claims 1-38; example 26 ----- -/--	5,8-12, 17,21,30



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

18 July 2017

Date of mailing of the international search report

28/07/2017

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/051282

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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