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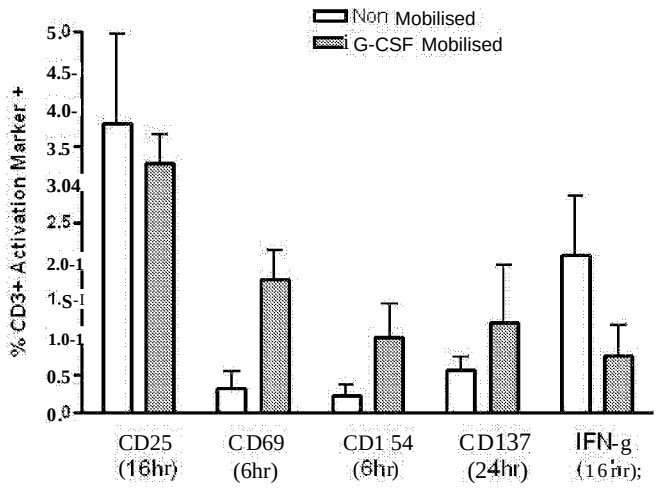
(54) Title: METHOD OF TREATMENT EMPLOYING THERAPEUTIC T CELL PRODUCT FROM MOBILISED DONORS

Figure 3 Optimal time of expression of activation markers in response to CMVpp65 stimulation in G-CSF mobilised (n=5) and non-mobilised (n=5) PBMC.



(57) Abstract: The present disclosure provides a method of treating a human patient in need thereof with immune reconstitution therapy by administering a therapeutically effective amount of therapeutic T cell population selected and/or expanded from a mobilised blood sample or a mobilised apheresis sample, wherein selection is on the basis of a steady state marker and/or an activation marker optionally followed by expansion, or expansion is in the presence of antigen, such as a viral antigen. It also extends to methods of generating said therapeutic T cell populations and the product obtainable therefrom.

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METHOD OF TREATMENT EMPLOYING THERAPEUTIC T CELL PRODUCT FROM MOBILISED DONORS

The present disclosure relates to methods for preparing a T cell immunotherapy product from a mobilised blood sample, for example for immune reconstitution, the T cell population obtained from the said method and pharmaceutical formulations comprising same. The disclosure also provides the T cell population and said pharmaceutical formulation for use in therapy, particularly immune reconstitution therapy, for example in the treatment or prophylaxis of viral infections such as CMV and adenovirus infections.

BACKGROUND

Immune compromised patients are susceptible to opportunistic virus infection. This is a huge problem in bone marrow transplant patients because their immune cells are sometimes intentionally depleted as part of the bone marrow transplant procedure and other times rendered non-functional due to steroid treatment for Graft versus Host Disease (GvHD) which is a common complication of bone marrow transplantation. Latent viruses, such as CMV and adenoviruses, become re-activated and the body is unable to fight the infection.

A practice of immune reconstitution has developed and this involves the transplant (adoptive transfer) into the transplant patient of immune cells from a matched HLA donor, usually the same donor who provided the bone marrow or peripheral stem cell transplantation. These cells appear to engraft in the patient to provide long-term immunity to pathogens or at least interim assistance in fighting infection until the patient's own immune system is fully reconstituted through the engraftment of the donor's haematopoietic stem cells which will then develop into a diverse array of blood cells and immune cells.

Years of clinical research into the adoptive transfer of donor immune cells to achieve immune reconstitution in a patient following a bone marrow transplant has illustrated the benefits of this approach as well as the challenges of optimising the approach to ensure a consistently efficacious and safe result. In some cases, the number of donor immune cells which are necessary to effect immune reconstitution against a specific pathogen cannot be obtained through simple mechanical selection systems. In such cases, the minimum dosing of the therapeutic immune cells, in particular antigen-specific T cells which demonstrate an adaptive memory immune response against the target pathogen, can be obtained by expanding the desired donor T cell population on an *ex vivo* basis using a cell culture system. Prior art indicates that the process of expansion of the cells from the donor sample generally takes about 21 days and the focus has been to expand the specific cells in order to obtain the highest number possible number (yield) of the relevant cell populations as well as the highest possible purity of the relevant cell populations, for example to obtain a population which is as close to 100 per cent of the desired cells as possible.

The starting population of cells is obtained from a donor derived blood sample or dedicated apheresis product. The current practice is that the apheresis product is harvested in a dedicated apheresis when the donor has not undergone GCSF treatment and therefore is not a mobilised blood sample, for reasons discussed below.

Mobilisation by recombinant human granulocyte-colony stimulating factor (G-CSF) is used to increase the number of donor stem cells in circulation prior to donation. This allows peripheral blood stem cell plant transplantation as opposed to bone marrow transplantation. Peripheral blood stem cell transplantation has a number of advantages over bone marrow transplantation.

The current practice is that after a stem cell transplantation from the donor to the patient and the donor is then required to return at a future point in time, when the effects of the mobilisation have

subsided, to provide a further unmobilised blood sample or apheresis which can be used to generate a therapeutic T cell product to augment the patient's immune responses. The T cell product may be selected from a subset of cells from the sample and/or be expanded from a fraction of a blood or apheresis sample.

Having to return for a second procedure is very inconvenient for donors and can result in non-compliance which means that sometimes a blood sample or leukapheresis is not available for generating an expanded T cell product to augment the patient's immune responses.

Currently mobilised blood is not used to generate an expanded T cell product because early work established that mobilised blood does not have the same properties as non-mobilised blood and in particular that there may be reduced activity in T cells in mobilised blood, for example Mielcarek *et al* in Blood, March 1, 1997 vol. 89 no. 5 1629-1634 describe the suppression of alloantigen-induced T-cell proliferation by $CD14^+$ cells derived from granulocyte colony-stimulating factor-mobilised peripheral blood mononuclear cells.

In short after G-CSF stimulation *in vivo,* human and murine T cells show a reduced cytotoxic activity. A reduced proliferative response is also observed upon *in vitro* stimulation.

Reyes *et al* in the British Journal of Cancer (1999) 80 (1/2), 229-235 describes how granulocyte colony-stimulating factor (G-CSF) transiently suppresses mitogen-stimulated T-cell proliferative response.

Murine and human studies have suggested that G-CSF mobilization inhibits type 1 cytokine production by T cells, through inhibition of secretion as a single cell level as well as reducing the fraction of cytokine-secreting cells in the periphery; arguing against the use of these cells in adoptive immunotherapy (Pan et al 1999, Arpinati et al 200 and Tayebi et al 2001).

This reduced functionality of certain cells from mobilised-blood was confirmed by a number of authors see for example Joshi *et al* -Decreased immune functions of blood cells following mobilization with granulocyte colony-stimulating factor: association with donor characteristics Blood, 15 September 2001 Vol 98, No 6, 1963-1970, and Nawa *et al* G-CSF reduces IFN-Y and IL-4 production by T cells after allogeneic stimulation by indirectly modulating monocyte function, Bone Marrow Transplantation (2000) 25, 1035-1040.

G-CSF was also considered to have a role in immune tolerance, see for example Anke Franzke's review in Cytokine & Growth Factor Reviews 17 (2006) 235-244 entitled the role of G-CSF in adaptive immunity and Rutella *et al* granulocyte colony-stimulating factor: a novel mediator of T cell tolerance, The Journal of Immunology 2005 7085-7097. Whist immune tolerance to transplanted cells is desirable general immune tolerance is not desirable when generating a therapeutic T cell product for augmenting a patient's immune response. In fact tolerance may have some links with T cell anergy or hyporesponsiveness.

Other research has suggested that G-CSF may skew the T cell population to the Th2 group, which may be less effective in controlling an intracellular viral infection.

Thus the practice in the field is to not employ mobilised-blood for the preparation of expanded T cell products.

The present inventors believe that whilst *in vitro* T cells from mobilised blood appear less able to secret interferon-gamma (an activation marker for antigen-stimulated T cells) as per Figure 6 the cells nevertheless are suitable for use as T cell therapeutic product. This observation is somewhat counter-intuitive because interferon-gamma is a pro-inflammatory cytokine involved in immune responses and

a skilled person would naturally consider that lower levels of secretion of this cytokine was indicative of generally lower activity of the T cell from mobilised blood. However, it is possible to select and expand antigen specific T cells from mobilised blood and once taken out of the mobilised cell environment these cells are not inferior in function to T cells from non-mobilised blood.

Surprisingly, the present inventors have established that in fact therapeutic T cell products selected and/or expanded from G-CSF mobilised blood or mobilised apheresis are safe and effective when administered *in vivo* to a post-haematopoietic stem cell transplant patient.

SUMMARY OF INVENTION

In one embodiment there is provided a method of treating patient in need thereof with immune reconstitution therapy by administering a therapeutically effective amount of a therapeutic T cell population selected and/or expanded from a G-CSF mobilised blood sample or mobilised apheresis, in particular where the patient is post-haematopoietic stem cell transplantation.

The present disclosure also provides a therapeutic T cell population selected and/or expanded from a G-CSF mobilised blood sample or for use in treatment, in particular the treatment of a post-haematopoietic stem cell transplant patient.

In one embodiment the therapeutic T cell population is an antigen-specific T cell population.

In one embodiment the antigen-specific T cell population is specific for a virus for example selected from the group comprising cytomegalovirus, adenovirus, varicella zoster virus, human papillomavirus, hepatitis B virus, hepatitis C virus, BK virus, Epstein-Barr virus, Kaposi's sarcoma-associated herpes virus and human T-lymphotropic virus, such as cytomegalovirus or adenovirus.

In one embodiment the virus is cytomegalovirus.

In one embodiment the therapeutic T cell population is suitable for treating virus infection, in particular a specific virus infection described herein or a combination of the same.

In one embodiment the T cells are allogeneic i.e. derived from a HLA matched donor, in particular a fully matched donor.

In one embodiment the T cell population is selected on the basis of a steady state marker, for example the T cell receptors (TCR).

In one embodiment the T cell population is selected on the basis of a marker for example a marker that is independently selected from CD25, CD69, CD137, and CD154 and a combination thereof, for example CD69, CD137, and CD154 and a combination thereof, such as CD154.

After selection the cell population may be expanded to increase the dose of cells available for the patient.

Alternatively, a starting population of cells may be expanded in the presence of antigen. This process involved a natural selection element in that the process specifically cultivates cells specific to the antigen and non-target cell populations are reduced or eliminated.

In one embodiment the population of T cells does not comprise significant amounts of the cell surface marker CD25.

In one embodiment the therapeutic T cell product is selected from a G-CSF mobilised apheresis.

In one embodiment the therapeutic T cell product is expanded from a G-CSF mobilised blood sample.

Cells derived from mobilised sample may show reduced levels of interferon-gamma secretion *in vitro.* Nevertheless the inventors have evidence to suggest that these cells are functioning and are suitable for use in the therapeutic product despite *in vitro* property. This generates a practical difficulty in relation to the selection of the relevant populations because selections of the relevant T cell populations based on methods such as gamma-capture are sub-optimal. Therefore, if selection is to be employed a steady state T cell marker and/or an activation marker has to be employed. In one embodiment this employs a stimulation step followed by selection on a cell surface marker such as CD154, in another embodiment this employs a direct selection method such as one based on the T cell receptor-streptamer selection.

Thus in fact mobilised-blood is a suitable starting material for the preparation of T cell products and also provided is a method of selecting and/or expanding a target T cell population which is specific to a virus from a starting T cells population from a mobilised blood sample wherein selection employs direct selection targeting a steady state maker on the surface of the T cells and expansion employs conditions suitable for expansion of target virus specific T cell population.

Given the negative disclosures in relation to the use of mobilised-blood samples for the preparation of T cell products, it is very surprising that the material can in fact be employed successfully. Additionally the methods according to the present disclosure provide a huge advantage to donors, patients and healthcare workers because use of mobilised blood samples ensures the expanded T cell therapy will be available to more patients without the inconvenience and disadvantages caused to donors by the prior art methods.

There are also significant resource savings associated with the present method because the collection, transport and storage of a second sample requires a significant amount of additional human and financial resources.

Furthermore, being able provide an immunotherapy with T cells from mobilised blood may have the further advantage that the therapeutic product can be prepared immediately after the donation thereby avoiding the "lag-time" associated with obtaining an unmobilised sample and then processing the same to provide a therapeutic product.

Finally the donor has not to undergo an additional medical intervention and is therefore not put at the risks associated with an additional leukapheresis procedure.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Functional profile in unpaired G-CSF mobilised (n=6) and non-mobilised (n=6) donors

Quantitative assessment of IL-2, TNF, IFN-γ, IL-10, IL-4 and IL-5 in the supernatant of cultures after 16 hour CMVpp65 stimulation. Concentration of cytokine is expressed as a net value after subtraction of the negative control (unstimulated).

Figure 2 Identification and isolation of IFN-g secreting antigen-specific T cells in unpaired G-CSF mobilised (n=6) and non-mobilised (n=6) donors. (2A) PBMC were stimulated for 16 hours with CMVpp65 and the frequency of IFN-γ secreting cells analysed amongst CD3+ T cells. (2B) IFN-γ secreting cells were isolated using magnetic cell sorting and purity and yield determined within the CD3+ population.

Figure 3 Optimal time of expression of activation markers in response to CMVpp65 stimulation in G-CSF mobilised (n=5) and non-mobilised (n=5) PBMC. PBMC were stimulated over 24 hours and samples analysed for CD25, CD69 CD154 and CD137 expression at 1, 4, 6, 16 and 24

hours. IFN-γ secretion was analysed at 16 hours. Bars represent net expression in the CD3+ population for each activation marker at the optimal time of expression.

Figure 4 Direct comparison between a G-CSF mobilised and non-mobilised donor of CD154 surface expression at 4 and 6 hours. (4A) PBMC were stimulated with either CMVpp65 Peptivator or SEB in the presence or absence of CD40-specific antibody (^g/ml). Cells are gated on CD3+ CD4+ T cells (4B, C) Comparison of CD154 expression in non-mobilised (n=5) and G-CSF mobilised (n=5) donors. Data are presented as means with standard deviation (SD)

Figure 5 Isolation of CMV-specific T cells through CD154 expression in two unpaired donors. (4A) PBMC from non-mobilised and G-CSF mobilised donors were stimulated with CMVpp65 Peptivator for 6 hours in the presence of CD40-specific antibody. Cells were stained for CD154 amongst CD3+ lymphocytes before stimulation after stimulation and after sorting of CD154+ T cells on the MiniMACS. (B) Positive fractions from CD154+ sorts after CMVpp65 stimulation in G-CSF mobilised (n=4) and non-mobilised (n=4) PBMC. Data are presented as means with SD.

Figure 6 Re-stimulation of expanded CD154+ T cells. (A) Expanded CD154+ T cells stained for CD3 and CD4 before re-stimulation after 21 days in culture. (B) Expanded CD154+ were co-cultured with autologous PBMC with or without CMVpp65 for 6 hours in the presence of CD40-specific antibody. After stimulation we analysed expression of CD154 versus CD69. (C) CD154+ expanded cells from G-CSF mobilised (n=3) and non-mobilised (n=3) PBMC analysed for CD154, CD69 (D) Expanded CD154+ from G-CSF mobilised PBMC were stimulated as described in the presence of Brefeldin A and CD28-specific antibody. Cells were fixed and permeabilised and analysed for expression of CD154 versus IL-2, TNF and IFN-γ. (E) Analysis of IL-2, IFN-γ, and TNF expression after re-stimulation with autologous CMVpp65 PBMC in G-CSF mobilised (n=3) and non-mobilised (n=3) PBMC. Data are presented as means with SD.

Figure 7 CD154+ CMV-specific T cells isolated from G-CSF mobilised PBMC effectively kill target cells. Specific lysis of autologous PHA blasts loaded with CMVpp65 peptides at E:T ratios from 50:1 to 0.5:1 determined using fluorescent dye Calcein-AM cytotoxicity assay.

Figure 8 A sample of mobilised apheresis product was expanded for 10 days using a rapid expansion process- employing the G-rex40 culture device and IL-4 and IL-7. Cells were then re-stimulated with media alone (un-stimulated) or with CMV pp65 peptides. The amount of IFN gamma production was measured by flow cytometry. Cells are gated on live lymphocytes and CD3. This plots shows in QI that the desired population of expanded cells from mobilised blood are capable of secreting interferon-gamma. The skilled person will know that the profile exhibited in this plot is comparable to the profile obtained under the same conditions for cells expanded from non-mobilised blood.

Figure 9 A sample of mobilised apheresis product was expanded for 10 days using a rapid expansion process- employing the G-rex40 culture device and IL-4 and IL-7. Cells were then re-stimulated with media alone (un-stimulated) or with ADV Hexon V peptides. The amount of IFN gamma production was measured by flow cytometry. Cells are gated on live lymphocytes and CD3. This plots shows in QI that the desired population of expanded cells from mobilised blood are capable of secreting interferon-gamma. The skilled person will know that the profile exhibited in this plot is comparable to the profile obtained under the same conditions for cells expanded from non-mobilised blood.

Figure 10 A sample of mobilised apheresis product was taken from the stem cell harvest and sent to Cell Medica for processing. The cells were exposed to the specific streptamer selection reagent and selected using the CliniMACS. This was then dosed at 3 x 10e4 T cells per Kg for administration to the patient. The percentage of cells expressing the CMV specific T cell receptor (streptamer positive) was measured by flow cytometry. This shows that T cells can be successfully derived from mobilised apheresis samples in doses and purity equivalent to non-mobilised products and can be administered to patients safely.

Figure 11 Shows cells from Figure 10 were gated on live lymphocytes and CD3.

Figure 12 &13 Show that antigen specific T cells are functional even when derived from an original sample which is mobilised

Figure 14 Shows analysis of a sample therapeutic T cells selected by gamma-capture used to treat a patient with refractory CMV infection and the starting material from which it was derived

DETAILED DESCRIPTION

Mobilised blood as employed herein refers to a blood sample from a donor who has been mobilised by treatment with agent such as G-CSF. The process of mobilisation increases the number of stems cells in the peripheral blood.

Apheresis as employed herein is the product of the process where the blood of a donor is passed through an instrument that separates out one of more particular components from the blood and returns the remainder back into the donor's circulation.

Apheresis is employed to generate the leukapheresis employed in stem cell transplantation.

In preparation for the stem cell transplantation the leukapheresis product may undergo a selection for CD34+ stem cells. A bi-product is obtained from this process known as the $CD34^-$ fraction. Advantageously, the present process can employ this bi-product to selected or expand the therapeutic T cell population from. In one embodiment the apheresis is a CD34- fraction.

Apheresis is also advantageous in that it potentially gives access to a large number of cells in the starting material, for example in the region of 1 to 10 billion cells, such as 2, 3, 4, 5, 6, 7, 8 or 9 billion cells. This number of cells is sufficient to generate a suitable therapeutic dose of T cell by selection only, i.e. without the requirement for subsequent expansion.

In contrast a blood sample may only contain in the region of 20 million cells. Therefore if the starting material is a blood sample or a sample containing relatively low number of cells then an expansion step will generally be required generate a suitable therapeutic dose of cells for the patient.

Mobilised apheresis as employed herein refers to a sample from a donor who has been mobilised by treatment with agent such as G-CSF. The process of mobilisation increases the number of stems cells in the peripheral blood.

There are various permutations of the present process and these are summarised below:

1. Direct selection based on a steady state marker, such as the TCR marker, to give a therapeutic dose (starting material an apheresis),
2. Selection based on an activation marker, such as described herein, to give a therapeutic dose (starting material an apheresis),
3. Direct selection based on a steady state marker, followed by expansion to give a therapeutic dose (starting material blood or apheresis)

4. Selection based on an activation marker, followed by expansion to give a therapeutic dose,
5. Expansion in the presence of antigen to generate a therapeutic dose of an antigen specific T cell population (starting material blood or apheresis).

Immune reconstitution as employed herein is intended to refer to providing the host with a mechanism for generating an immune response or augment the host's immune response to approximate that in a healthy individual where otherwise the host's response would be minimal or non-existent due to an impairment

In one embodiment the haematopoietic stem cell transplantation is allogeneic haematopoietic stem cell transplantation (allo-HSCT) including procedures involving stem cell donation from related or unrelated donors or from cord blood, such as peripheral stem cell transplantation unless the context indicates otherwise.

Effective in treatment as employed herein refers to a therapy that is safe for administration to patients and is at least broadly comparable to prior art T cell therapies derived from non-mobilised blood.

Therapy in the context of the present disclosure includes prophylactic therapy, which in the context of immune reconstitution is standard practice.

"T cell" is a term commonly employed in the art and intended to include all CD3+ cells including thymocytes, immature T lymphocytes, mature T lymphocytes, resting T lymphocytes or activated T lymphocytes. A T cell can be a T helper (Th) cell, for example a T helper 1 (Thl) or a T helper 2 (Th2) cell, although other grouping of T cell populations are being discovered based on intensive research. The T cell can be a CD4+ T cell, CD8+ T cell, CD4+CD8+ T cell, CD4-CD8- T cell or any other subset of T cells.

T cell product as employed herein refers to a population of T cells suitable for use in therapy, for example immune reconstitution therapy.

Expanding a target T cell population as employed herein is intended to refer to increasing the number of the target cells in a population of cells as a result of cell division, for example by culturing a starting population of cells in a suitable medium.

T cell expansion may be evaluated by counting viable CD3+ cells (i.e. the target population of cells).

Viable cells can be tested by cell staining with Trypan blue (and light microscopy) or 7-amino-actinomycin D, vital dye emitting at 670nm (or ViaProbe a commercial ready-to-use solution of 7AAD) and flow cytometry, employing a technique known to those skilled in the art. Where the stain penetrates into the cells the cells are considered not viable. Cells which do not take up dye are considered viable. An exemplary method may employ about 5 μL of 7AAD and about 5 μL of Annexin-V (a phospholipid-binding protein which binds to external phospholipid phosphatidylserine exposed during apotosis) per approximate IOOμL of cells suspension. This mixture may be incubated at ambient temperature for about 15 minutes the absence of light. The analysis may then be performed employing flow cytometry. See for example MG Wing, AMP Montgomery, S. Songsivilai and JV Watson. An Improved Method for the Detection of Cell Surface Antigens in Samples of Low Viability using Flow Cytometry. J Immunol Methods 126: 21-27 1990.

An alternative stain is TO-PRO-3 which is a carbocyanine monomer nucleic acid stain with far-red fluorescence similar to Alexa Fluor 647 or Cy 5 dyes. It is useful as a nuclear counterstain and dead cell indicator, and is among the highest-sensitivity probes for nucleic acid detection.

In one embodiment the T cell population is selected from mobilised blood by direct selection based on a steady state marker, such as the T cell receptor (TCR). This process employs HLA: peptide complexes particularly in the form of multimers, such as tetra, penta and/or hexamers which ligate to T cell receptor. These peptides are labelled, for example with a fluorescent label or a magnetic bead which allows then to them to be identified and selected. In one embodiment a magnetic label is employed.

Thus direct selection generally involves the clinical grade enrichment of lymphocytes from a fraction of mobilised apheresis product. This may use a dedicated device such as a Sepax device from Biosafe. The resulting lymphocytes are then incubated with a selection reagent which is a mulimerised MHC/peptide complex attached to a magnetic bead. Where the MHC/peptide complex is matched to the patient and donor and is specific for an antigen specific T cell receptor. Following incubation the cells are washed and the bound cells are selected with a device such as the Miltenyi CliniMACS or any other technology that enables cell selection using magnetic beads where positive cells are retained on a magnetic column or in a bag and the negative cells are washed off. The magnet is then removed and the antigen specific cells are eluted

In one embodiment the multimers are Streptamers. The ligation of the TCR by Streptamers is reversible and after selection of the desired population of cells then treatment with a specific reagent results is removal of the complexes from the cells.

HLA complexes employed need to be matched with the HLA type so that they can ligate a virus specific population of T cells, such that the peptides or multimers are of a specific HLA-type, for example Al, A2, B7, A24, B35, such as A0201 and B0702.

Selection can also or alternatively be based on activation markers. These are makers which are upregulated as a consequence of antigen stimulation. A plethora of these exist and are known to those skilled in the art and include makers such as CD25, CD69, CD137, CD154 and combinations thereof.

These markers can be selected by ligation with monomeric, dimeric, multimeric antibody or binding fragments thereof. These antibodies or fragments are labelled, for example with a fluorescent label or a magnetic bead which allows then to them to be identified and selected. In one embodiment a magnetic label is employed.

In one embodiment the antibody or fragment employed is a fab-streptamer, for example available from IBA GmBH Germany.

The binding of these fab-streptamers is reversible in that after selection of the desired cell population treatment with an appropriate reagent releases them from the cells.

Ligation as employed herein refers to binding.

In one embodiment the T cell population is selected from mobilised blood and then expanded.

In one embodiment selection is not required before expansion because the expansion selectively enriches for the target population of cells which is facet of the expansion process.

A T cell population specific to a virus as employed herein is intended to refer to the fact that the relevant population of cells primarily recognises and at least one viral antigen to which is specific, and for example generates an immunological response after recognition of the target virus. Specificity in this context does not necessarily mean that only the target virus is recognised, although in some instances only the target virus will be recognised, but at least the target virus is recognised with greater affinity, avidity or magnitude of response in comparison to non-target viruses.

Viral antigen as employed herein is intended to refer to those antigens specified by the viral genome (often coat proteins) that can be detected by a specific immunological response. In one embodiment the viral antigen is a surface antigen.

In one embodiment the virus is a DNA virus, for example a double stranded DNA virus.

In one embodiment the virus is an RNA virus.

Typically the PBMCs are obtained from the blood or apheresis product by Ficoll density gradient separation known to those skilled in the art.

The step of obtaining a sample from the patient can be a routine technique of taking a blood sample. This process presents little risk to patients and does not need to be performed by a doctor but can be performed by appropriately trained support staff. In one embodiment the sample derived from the patient is approximately 500ml, 400ml, 300ml, 200ml, 100ml, 50ml, 40ml, 30ml, 20ml, 10ml, 5ml or less of blood.

In one embodiment the starting material is a fraction of the mobilised apheresis product that is taken once it has been ensured that the CD34+ cell dose for the patient has been achieved. For example 4 x 10e6 CD34+ cells per kg patient weight.

In one embodiment the cells which are bi-product of the stem transplantations are employed. Stem cells for transplantation are often selected on the basis of CD34. Those populations which are negative for CD34 are often discarded after selection. However, this deselected population is suitable for generating a therapeutic T cell product, for example employing a method described herein, such as T cell expansion.

As is known to the skilled person expansion of T cells is generally performed in a suitable T cell expansion media. T cell expansion media generally comprises serum, media and any cytokines employed in the expansion step.

In one embodiment the media is Advanced RPMI media or RPMI media 1640, available from Life Technologies.

In one embodiment the cell expansion medium comprises 10% human AB serum, 200 mM L-glutamine, and RPMI-1640.

In one embodiment the medium comprises 45% advanced RPMI, 45% EHAA, 10% FCs and 200 mM L-glutamine.

In one embodiment the cell expansion medium comprises 10% human AB serum, 200 mM L-glutamine, 45% Earle's Ham's amino acids (EHAA or Click's medium) and 45% advanced RPMI or RPMI-1640.

In one embodiment the cytokines employed are discussed below.

In one embodiment the T cell expansion medium employed is not changed or supplemented during the expansion process, especially where a rapid expansion process is employed. Rapid expansion as employed herein refers to a process in a therapeutic product is obtained within less than 18 days, such as 7-10 days.

Thus in one embodiment there is provided according to the present disclosure an *in vitro* expansion process for rapid expansion of antigen specific T cells (such as allogeneic antigen specific T cells) comprising the steps culturing in a gas permeable vessel a population of PBMCs (such as allogeneic

PBMCs) in the presence of a peptide or peptide mix relevant to a target antigen(s), in the presence of an exogenous cytokine characterised in that the cytokine is other than exogenous IL-2.

In one embodiment there is provided according to the present disclosure *in vitro* expansion process for rapid expansion of antigen specific T cells, such as allogeneic antigen specific T cells comprising the steps culturing in a gas permeable vessel a population of PBMCs (such as allogeneic PBMCs) in the presence of antigen, for example a peptide or peptide mix relevant to a target antigen(s), in the presence of an exogenous cytokine characterised in that the expansion to provide the desired population of T cells is 14 days or less, for example 9, 10, 11 or 12 days, such as 10 days.

Cytokines that may be employed in the process of the current disclosure include IL-1, IL-2, IL-4, IL-6 IL-7, IL-12 and IL-15.

A large amount of, as yet non-definitive, literature underlines how IL-2, IL-7 and IL-15 play non-redundant roles in shaping the representation of memory cells. IL-2 controls T-cell clonal expansion and contraction, and promotes lymphocyte differentiation. IL-2 and IL-15 can also support memory cell division and have been used in combination with antigen-driven stimulation, for the expansion of CTL.

IL-7 regulates peripheral T-cell homeostasis, and contributes to the generation and long-term survival of both CD41 and CD81 memory T lymphocytes *in vivo.*

In one embodiment the cytokines employed in the expansion process according to the present disclosure are independently selected from IL-4, IL-7 and IL-15, especially IL-4 and IL-7.

In one embodiment the cytokines employed are IL-4 and/or IL-7. Whilst not wishing to be bound by theory the inventors believe that these cytokines have a role to play in shaping the frequency, repertoire and expansion of viral antigen-specific T cells.

In one embodiment the method according to the present disclosure provides a T cell population which has a repertoire of antigen-specific T cells.

The repertoire of T cells may be determined by ELISPOT analysis after stimulation with peptide libraries aliquotted into pools such that each peptide is uniquely represented in two pools (Kern, F., N. Faulhaber, C. Frommel, E. Khatamzas, S. Prosch, C. Schonemann, I. Kretzschmar, R. Volkmer-Engert, H. D. Volk, and P. Reinke. 2000. Analysis of CD8 T cell reactivity to cytomegalovirus using protein- spanning pools of overlapping pentadecapeptides. Eur J Immunol. 30:1676-1682 and Straathof, K. C , A. M. Leen, E. L. Buza, G. Taylor, M. H. Huls, H. E. Heslop, C. M. Rooney, and C. M. Bollard. 2005. Characterization of latent membrane protein 2 specificity in CTL lines from patients with EBV-positive nasopharyngeal carcinoma and lymphoma. J. Immunol. 175:4137-4147) or by intracellular cytokine staining by plating 200,000 of the final T cell product in a round bottomed 96 well plate and using peptides as above to re-stimulate the cells at a concentration of lug/ml. This is performed overnight in the presence of 5ug/ml of Brefeldin A which prevents secretion of cytokine and therefore is used to ensure build-up of IFNg inside the cells for enumeration using flow cytometry.

IL-4 is generally employed at a final concentration of 250 ng/ml of culture or less, such as 200 ng/ml or less.

IL-7 is generally employed at a final concentration of 50 ng/ml of culture or less, such as 20 ng/ml or less, in particular 10 ng/ml.

If IL-15 is employed a suitable final concentration is 50 ng/ml of culture or less, such as 20 ng/ml or less, in particular 10 ng/ml.

In one embodiment in about 20 mis per GRex-10 (for example 20 x 10^8 PBMCs) a further lOmls medium containing IL-4 (1666 units per mL) and IL-7 (lOng per ml) is added.

IL-12 has a role in Thl focussing and exogenous IL-12 may be omitted if a balanced Thl/Th2 is desired. In one embodiment the process of the present disclosure does not employ exogenous IL-12. However, in the context of the present T cell product a Thl response in the CD4+ population is thought to be desirable.

In one embodiment when IL-4 is employed the in the expansion process of the present disclosure. At day 10 or day 11 the number of expanded cells may be 10, 20, 30, 40 50, 60, 70, 80, 90, 100 or 200% higher than cells expanded employing a similar protocol replacing IL-4 with IL-2.

When exogenous IL-2 is employed in the rapid expansion system hyper-proliferation of T cells is generated. When this hyper-rapid expansion occurs then the balance of desirable T cells and the residual cells is suboptimal in that the expansion happens so rapidly that many that the residual cells have not died and thus remain present in the total cell population. Thus the present inventors have reconciled the inherently incompatible factors of rapid expansion with the selectivity of culturing the cells for a period of time which allows death of the non-target cells and have found that the omission of IL-2 improves the ratio of desired cells to residual cells. What is more in the period 7 to 14 days such as 10 days the ratio of desired cells to residual cells is a cross-over point where the cultured product becomes suitable for use in therapy. This cross-over point is defined as when a sufficient minimum dose of therapeutic T cells is achieved within a dose formulation which falls within the safety threshold of no more than 5 x 10^5 CD3+ T cells per kg of patient body weight.

In one embodiment the T cell population as allogeneic, that is to say the T cell population is derived from a donor who is not the patient.

Generally the donor will be fully HLA matched.

The human leukocyte antigen (HLA) system is the name of the major histocompatibility complex (MHC) in humans. The super locus contains a large number of genes related to immune system function in humans. This group of genes resides on chromosome 6, and encode cell-surface antigen-presenting proteins and many other genes. The HLA genes are the human versions of the MHC genes that are found in most vertebrates (and thus are the most studied of the MHC genes). The proteins encoded by certain genes are also known as antigens, as a result of their historic discovery as factors in organ transplants. The major HLA antigens are essential elements for immune function. Different classes have different functions:

HLAs corresponding to MHC class I (A, B, and C) present peptides from inside the cell (including viral peptides if present). These peptides are produced from digested proteins that are broken down in the proteasomes. In general, the peptides are small polymers, about 9 amino acids in length. Foreign antigens attract killer T-cells (also called CD8 positive- or cytotoxic T-cells) that destroy cells.

HLAs corresponding to MHC class II (DP,DM, DOA,DOB, DQ, and DR) present antigens from outside of the cell to T-lymphocytes. These particular antigens stimulate the multiplication of T-helper cells, which in turn stimulate antibody-producing B-cells to produce antibodies to that specific antigen. Self-antigens are suppressed by suppressor T-cells.

In one embodiment the selection of cells is based on the interferon-gamma secretion or a cell surface activation marker, after stimulation of the cells with antigen, in particular peptides of a relevant antigen.

In one embodiment the mobilised blood sample obtained from the donor may be cryopreserved before processing.

In one embodiment after expansion optionally one or more components such as stabilising agents and/or cryopreservants are added to the formulation, for example human serum albumin, glycerol, DMSO or similar.

The present invention also extends to compositions comprising the allogeneic antigen-specific T cell populations according to the invention. These compositions, may comprise a diluent, carrier, stabilizer, surfactant, pH adjustment or any other pharmaceutically acceptable excipient added to the cell population after the main process steps. An excipient will generally have a function of stabilizing the formulation, prolonging half-life, rendering the composition more compatible with the in vivo system of the patient or the like.

In one embodiment a protein stabilizing agent is added to the cell culture after manufacturing, for example albumin, in particular human serum album, which may act as a stabilizing agent. The amounts albumin employed in the formulation may be 10 to 50% w/w, such as about 12.5% w/w.

In one embodiment the formulation also contains a cryopreservative, for example glycerol or DMSO. The quantity of DMSO is generally 12% or less such as about 10%w/w.

In one embodiment the process of the present invention comprises the further step of preparing a pharmaceutical formulation by adding a pharmaceutically acceptable excipient, in particular an excipient as described herein, for example diluent, stabilizer and/or preservative.

Excipient as employed herein is a generic term to cover all ingredients added to the T cell population that do not have a biological or physiological function.

In one embodiment the pharmaceutical composition is adapted for administration by infusion.

In one embodiment the target virus to which an antigen specific T cell population is generated is CMV and, for example the antigen employed to the target the virus is pp65. The sequence for human cytomegalovirus (strain AD169) is in the UniProt database under number P06725. The recombinant protein can be purchased from Miltenyi Biotech. The latter company also provide PepTivator® CMV pp65 which is a peptide pool that consists mainly of 15-mer peptides with 11-amino acid (aa) overlap, covering the complete sequence of the pp65 protein of human cytomegalovirus.

In aspect the disclosure extends to a T cell product obtained or obtainable from the present method.

In one aspect the disclosure extends a virus specific expanded T cell product

In one embodiment the disclosure extends treatment or prophylaxis of a patient with a T cell product according to the present disclosure or a composition comprising the same 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 weeks or more after receiving a bone marrow transplant or peripheral stem cell transplant.

All citation and documents referred herein are specifically incorporated by reference. All references to literature and patent documents are incorporated by reference.

Comprising in the context of the present invention means including.

Described above are embodiments comprising certain integers. Embodiments of the invention described above can be combined as technically appropriate. The present disclosure also extends to corresponding embodiments consisting of said integers as herein described.

EXAMPLES

Example 1

Blood Donors and Cell Preparation

We obtained blood samples from both G-CSF mobilised and non-mobilised healthy donors after a 3-5 hour leukapheresis. Informed consent was obtained in accordance with the Declaration of Helsinki and studies were approved by the Royal Free NHS Trust Research and Development review board. Peripheral blood mononuclear cells (PBMC) were generated using Ficol (Axis Shield Diagnostics) density gradient separation and cultured in RPMI 1640 Medium (Gibco) supplemented with 1% antibiotic (Gibco) and 10% heat inactivated human AB serum (Biosera) at a concentration of 1×10^7/ml. Excess PBMC were cryopreserved at 1:1 with human serum albumin (HSA) 4.5% (Bio Products Laboratory) containing 20% DMSO (WakChemie) as a future source of feeder cells. PBMC were stimulated for up to 24 hours in 6 well culture plates (Nunc) with CMVpp65 Peptivator (Miltenyi Biotec) at 37°C/5% $C0_2$. For CD154 experiments cultures were stimulated in the presence of ^g/ml anti-CD40 antibody (BioLegend)

Flow Cytometry Analysis

Flow cytometry experiments consisted of four to six colour panels where a minimum of 50,000 CD3+ were acquired after gating of viable lymphocytes using FSC and SSC signals on a FACScan flow cytometer (Cytek UK) and data analysed using FlowJo version 7.6 (TreeStar). For isotype control staining of cytokines and activation markers we used PE-conjugated mouse IgGl κ antibodies (BD Bioscience). Cells were stained for 15 minutes in the dark, washed in 2ml of HBSS for 5 minutes and resuspended in 200μl of FACS Flow (BD Bioscience) before acquisition. Cytokine analysis of supernatants from CMVpp65 stimulated and untouched PBMC were performed on a FACS Aria flow cytometer (BD Biosciences) and a minimum of 20,000 events collected.

Cytokine analysis by cytometric bead array (CBA)

Supernatants were collected from CMVpp65 stimulated and non-stimulated control cultures at 16-24 hours from both mobilised and non-mobilised donors and stored at -80°C. Analysis by Cytometric Bead Array Kit (BD Biosciences) was used to quantify the level of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF. Analysis of acquired data was performed using FCAP Array Software version 1.0.1 (Soft Flow Hungary Ltd.).

Time course Assay

PBMC isolated from mobilised and non-mobilised donors were stimulated in 96 well plates at a concentration of 1×10^7/ml for 24 hours with either CMVpp65 Peptivator or 1 μg/ml SEB (Sigma) or left untouched. Samples were taken at 1, 4, 6, 16 and 24 hours and stained with APC-conjugated anti-CD3, FITC-conjugated anti-CD4, PerCP-conjugated anti-CD8 and either PE-conjugated anti-CD154, anti-CD25, anti-CD69 or anti-CD137 (all BD Bioscience).

Isolation of Antigen Specific T cells

For the isolation of antigen-specific T cells following CMVpp65 stimulation cells were either stained with PE-conjugated anti-CD25 after 16 hours or PE-conjugated anti-CD154 after 6 hours (both BD Bioscience). Labelling was performed for 20 minutes using 10μl of antibody per 10^7 cells in IOOμl of CliniMACS buffer. After 20 minute incubation with PE-conjugated microbeads (20μl/10^7 cells) in 80μl of CliniMACS buffer the cell suspension was enriched using MS columns on a MiniMACS (all Miltenyi Biotec). All incubation steps were performed at 4-8°C in the dark. Antigen-specific T cells were also isolated using the IFN-γ secretion assay according to the manufacturer's recommendation (Miltenyi Biotec) and isolation was identical to that of CD154 and CD25 separation.

This was also performed on steady state specificity marders such as the specific T cell receptor whereby Stage/I BA product- streptamers were used as the selection reagent and on a clinical scale using the CliniMACS for selection.

Expansion of antigen-specific T cell lines

After a 6 hour incubation, we cultured up to 0.25 x 10^5 isolated CD154+ cells in the presence of 50:1 γ-irradiated (30 Gy) autologous PBMC to act as feeder cells in 24 well plates with RPMI 1640 medium containing 10% human AB serum, 1% antibiotic and supplemented with lOng/ml of IL-7 and IL-15 (Cell Genix). Culture medium was replenished every 2-3 days and cells split when necessary. Cells were expanded up to a maximum of 23 days before harvest.

Where cells were not selected prior to expansion, cells were seeded at 2 x 10e6 PBMC per ml in 20ml in the G-rexlO expansion system from Wilson Wolf. The cells were seeded with the specific peptide, IL-4 and IL-7 in RPMI 10% human serum and were cultured un-touched for 10 days.

Re-stimulation of Expanded antigen-specific T cell lines

We restimulated expanded cells for a period of 5-6 hours with either CMVpp65 Peptiavtor, CMV IE-1 (JPT) loaded autologous PBMC or untouched autologous PBMC as a control, all labelled with IμM CFSE (Sigma) at a ratio of 2.5: 1 at a concentration of 1 x 10^7/ml in 48 well plates. For analysis of intracellular cytokines and CD154 we incubated cells in the presence of anti-CD28 antibody (BD Bioscience) and added ^g/ml of Brefeldin A (Sigma) after 2 hours. Cells were fixed and permeabilised using Intrastain (DakoCytomation) according to the manufacturer's instructions and stained with APC-conjugated anti-CD154, PerCP-conjugated anti-CD4 either PE-conjugated anti-IL-2, anti-TNF or anti IFN-γ (all BD Biosciences). For surface staining cells were incubated in the presence of anti-CD40 antibody and then stained for 10 minutes with FITC-conjugated anti-CD4, PE-conjugated anti-CD154, PerCP-conjugated anti-CD8, APC-conjugated anti-CD3 and APC Cy7-conjugated CD69 (all BD Biosciences).

Cytotoxicity Assay

Autologous PBMC were stimulated with 3μg/ml of PHA (Sigma) for 24 hours and then 20U/ml of IL-2 (Miltenyi Biotec) at a concentration of 1 x 10^6/ml in RPMI 1640 with 10% AB serum. PHA blasts were then loaded with CMVpp65 Peptivator to use as target cells. Loaded target cells were labelled with Calcein-AM (Molecular Probes) at a concentration of 10μM and incubated for 1 hour at 37°C. After four washes in complete medium cells were adjusted to 7 x 10^4/ml and added to effector cells at E:T ratios ranging from 20:1 to 0.5:1, in triplicate, in U bottom 96 well plates (Corning). Triplicate wells were also set up to measure spontaneous release (target cells only), maximal release (target cells plus 2% Triton X-100) and medium alone. After incubation at 37°C/5% $C0_2$ for four hours, IOOμI of supernatant was harvested and transferred into new plates. Samples were measured using a BMG FLUOstar Galaxy microplate fluorescence spectrophotometer (MTX Lab Systems Inc.) (excitation filter: 485 ± 9 nm: band-pass filter: 530 ± 9nm). Data were expressed as arbitrary fluorescent units (AFU) and percent lysis was calculated using the formula [(test release-spontaneous release/maximal release-spontaneous release) x 100].

Statistical analysis

Analyses were conducted using GraphPad Prism 4.0. The nonparametric Mann-Whitney test was used to determine the statistical significance between G-CSF mobilised and non-mobilised PBMC and a Paired t test for analysing the effect of CD40 blocking on CD154 expression. Statistical significance was achieved when ***P*** was less than 0.05.

Results

Cytokine Profile of CMVpp65 stimulated G-CSF mobilised PBMC

Initial experiments aimed to investigate the cytokine profile of CMVpp65 stimulated PBMC from G-CSF mobilised PBMC to determine whether there was equivalence with non mobilised PBMC. PBMC from CMV+ healthy individuals were stimulated with CMVpp65 overlapping peptides in 16 hour cultures. After 16 hours aliquots of supernatant were taken from stimulated and untouched cultures and frozen at -80°C. Supernatants were assayed for the cytokines released during the culture period using a flow cytometric based assay, the cytokine bead array (CBA). IL-2, TNF, IFN-γ, IL-10, IL-4 and IL-5 secretion were analysed (Figure 1). No significant difference was observed between G-CSF mobilised and non-mobilised PBMC in terms of the THi cytokines IL-2, TNF and IFN-γ, but a significant decrease in IL-10 secretion from G-CSF mobilised PBMC (P=0.01) was detected and this trend was also evident in the low levels of IL-4 and IL-5 secretion.

Next we evaluated whether CMV-specific T cells could be isolated from G-CSF mobilised PBMC based on IFN-γ secretion, as we have used this system previously for the manufacture of CMV-specific T cells from non-mobilised PBMC and demonstrated their clinical efficacy. Cells secreting IFN-γ in response to CMVpp65 stimulation were captured using IFN-γ specific antibodies and selected using magnetic beads. IFN-γ was measured before and after magnetic enrichment to assess purity and yield between mobilised and non-mobilised PBMC. Although not significant we showed that IFN-γ secretion was decreased after CMVpp65 stimulation (Figure 2A) and that purity and yield (Figure 2B) were also negatively affected in G-CSF mobilised PBMC. The ratio of CD4+ to CD8+ IFN-γ secreting cells appeared to be unchanged in G-CSF mobilised PBMC. In summary PBMC from G-CSF mobilised PBMC are capable of secreting IFN-γ and other effector cytokines at a level similar to non mobilised PBMC, but isolation and detection after CMVpp65 stimulation on a per cell basis appears to be impaired. These results are in line with previously published data suggesting that G-CSF mobilisation impairs the potential for IFN-γ production at a single cell level.[25]

Analysis of activation marker expression after CMVpp65 stimulation

We next investigated the kinetics of activation induced CD25, CD69, CD154 and CD137 expression on CMVpp65 specific T cells in G-CSF mobilised PBMC to determine the optimal duration of stimulation in comparison to non-mobilised PBMC. We stimulated PBMC over a 24 hour period with CMVpp65 peptides and removed PBMC populations from cultures at 1, 4, 6, 16 and 24 hours and then analysed for surface expression of activation markers by flow cytometry (Figure 3). Antigen triggered expression of CD25 was optimal at 16 hours and was of the same intensity in G-CSF mobilised and non-mobilised PBMC. CD69 and CD154 were optimal at 6hrs and expression of both was elevated in G-CSF mobilised PBMC. CD137 expression reached peak intensity at 24 hours and was also increased in G-CSF mobilised PBMC. In line with previous results we observed a reduction in the level of IFN-γ secretion from G-CSF mobilised PBMC at 16 hours after CMVpp65 stimulation.

Assessment of antigen specific expression of CD154 in G-CSF mobilised PBMC

Previously published data have demonstrated that CD154 is a suitable marker for the detection and isolation of CMV-specific T cells. We therefore investigated whether CD154 expression in G-CSF mobilised PBMC was consistent with non-mobilised PBMC, using a CD40-specific antibody to preserve CD154 at the cell surface by preventing ligation with CD40. PBMC were stimulated with either SEB or CMVpp65 peptides for 4-6 hours in the presence or absence of CD40-specific antibody, and then analysed for CD154 expression amongst the CD4+ T cell population (Figure 4A).

Low background CD154 expression in resting CD4+ T cells was comparable between G-CSF mobilised PBMC (0.30%) and non-mobilised PBMC (0.22%). CD154 expression in the presence of CD40-specific antibody at the optimal time point of 6 hours, showed no statistical significant difference between G-CSF mobilised PBMC (1.86%) and non-mobilised PBMC (1.22%)but was in fact elevated in the G-CSF mobilised donor setting(Figure 4B-C), without any unspecific activation induced CD154 expression.

Isolation of antigen-specific T cells from G-CSF mobilised and non-mobilised PBMC through CD154 expression

We next performed a single enrichment step of CMVpp65 stimulated PBMC in the presence of CD40-specific antibody from G-CSF mobilised and non-mobilised PBMC (Figure 5A-B) using magnetic cell separation in 4 CMV+ healthy unpaired donors. We observed no significant difference in the purity of CD154+ CMV-specific T cells (Figure 5C) between G-CSF mobilised (48.94%) and non-mobilised (58.08%) PBMC. CD154 positive fractions were subsequently expanded in short term culture to determine in vitro proliferation and CMV specificity of isolated cells.

Re-stimulation of in-vitro expanded antigen-specific from G-CSF mobilised PBMC

CD154+ CMV-specific T cells were cultured over 21 days in complete medium containing IL-7 and IL-15 in the presence of autologous irradiated feeder cells. CD154+ responder populations showed a mean amplification factor of 74.6-fold (range 48-84) in G-CSF mobilised PBMC (n=3) compared to 103.6 (range 18-168) in non-mobilised PBMC (n=3). Expanded cells were predominantly CD3+ CD4+ in all cultures (Figure 6A) All cultures showed high specificity for CMVpp65 determined by up regulation of CD154+ and CD69+ expression upon re-challenge with autologous CMVpp65 loaded PBMC. In control re-challenge experiments with autologous PBMC alone, low to undetectable levels of CD154 expression was observed (Figure 6B). We observed an increase in the up-regulation of CD154+ CD69+ expression upon re-challenge in cells expanded from G-CSF mobilised PBMC (mean, 93.13%) compared to non-mobilised PBMC (mean, 63.0%) after flow cytometric analysis (Figure 6C). In some experiments expanded cells were re-challenged with CMV IE-1 peptides and no CD154 activation was observed confirming specificity (data not shown).

To analyse the functionality of expanded cells we also tested for production of IL-2, TNF and IFN-γ by intracellular cytokine staining (ICS) (Figure 6D). Expanded cells were capable of synthesising and secreting all three cytokines, but predominantly IFN-γ. In experiments where expanded cells were unstimulated or incubated with CMV IE-1 peptides, minimal cytokine secretion was observed. No significant differences were detected in IL-2, TNF or IFN-γ secretion between G-CSF mobilised PBMC and non-mobilised PBMC (Figure 6E). We have demonstrated that the CD154 assay allows for specific isolation of both expandable and functional CMV-specific T cells from G-CSF mobilised PBMC that is equivalent to published data in non-mobilised PBMC.

Cytotoxic activity of expanded cells

Finally we investigated whether expanded CD154+ CMV-specific T cells isolated from G-CSF mobilised PBMC are able to lyse target cells. Autologous PHA blasts loaded with CMVpp65 peptides and labelled with Calcein-AM dye were used as targets. Targets were effectively killed by expanded cells (Figure 7) at all E:T ratios.

Example 2 Analysis of Cells Obtained from the Negative Fraction of CD34 Selection

The starting material was the negative fraction from a CD34 selection from mobilised HPC-A (also referred to herein as an apheresis sample).

Cells underwent density gradient centrifugation prior to being cultured for 10 days with ADV peptide, IL-4 and IL-7. On day 10 cells were harvested, washed, counted, dosed and cryopreserved. Potency testing for gamma production and phenotyping for purity and viability was also performed.

Doses of 1 x 10^4 and lxlO ^{5} T cell per Kg were frozen (12Kg).

7.56% of T cells produced IFNy following re-stimulation with ADV peptide (release criteria states 1%) and all other release criteria (T cell purity, viability, microbiology, mycoplasma , endotoxin) were met. The scatter plot for this analysis is shown in Figure 11.

PBMC derived from mobilised and non-mobilised material can be accepted as starting material for the process.

No paired samples have been analysed however 9 production runs have been performed on each starting material. Below is a table showing the % IFNg production upon re-stimulation for both mobilised and non-mobilised product

Mobilised product	Non mobilised product
1.32	2.22
2.45	1.85
5.26	0.24
1.95	13.86
6.53	21.19
1.31	1.95
7.56	2.71
3.62	4.89
1.54	17.38
Mean 3.5	Mean 7.3
SD 2.4	SD 7.9

Example 3

The data in Figure 12 and 13 show that antigen specific T cells are functional even when derived from an original sample which is mobilised. PBMC derived from mobilised apheresis were stained with CFSE -a dye that is taken up by cells and when a cell divides the brightness of the cells is reduced and this can be detected by flow cytometry. The cells were cultured for 5 days at 37deg C with either no stimulation (nil) or with the antigen specific peptide, prior to being stained for streptamer, CD3, CD8 and run on a flow cytometer. This shows that the cells can proliferate despite being mobilised as long as there is the sufficient stimulus and they will not proliferate unless the stimulus is there- showing function.

Example 4 Treatment of a Patient

Cells were selected by gamma catch from a frozen mobilised apheresis sample and were used for the treatment of a 72Kg patient with refractory CMV (at least 2 months) with CMV retinitis involvement. A dose of about 22,000 CMV specific T cells was administered by infusion. Following treatment CMV and retinitis resolved and the patient was discharged from hospital. Thus despite the mobilisation the cells administered were functional. The Figure 14 shows that some gamma was produced in the pre selection population, it was reduced in the negative fraction and the positive fraction was the product that was actually administered to the patient

Claims

1. A method of treating a human patient in need thereof with immune reconstitution therapy by administering a therapeutically effective amount of therapeutic T cell population selected and/or expanded from a mobilised blood sample or a mobilised apheresis sample, wherein selection is on the basis of a steady state marker and/or an activation marker optionally followed by expansion, or expansion is in the presence of antigen, such as a viral antigen.
2. A method according to claim 1, wherein the patient is post-haematopoietic stem cell transplantation.
3. A method according to claim 1 or 2, wherein the T cell population is an antigen-specific T cell population.
4. A method according to claim 3, wherein the antigen-specific T cell population is specific a virus for example selected from the group comprising cytomegalovirus, adenovirus, varicella zoster virus, BK virus, human papillomavirus, hepatitis B virus, hepatitis C virus, Epstein-Barr virus, Kaposi's sarcoma-associated herpes virus and human T-lymphotropic virus, such as cytomegalovirus or adenovirus.
5. A therapeutic T cell population selected and/or expanded from a mobilised blood sample or mobilised apheresis or pharmaceutical composition comprising same, for use in human therapy, such as immune reconstitution therapy, wherein selection is on the basis of a steady state marker and/or an activation marker optionally followed by expansion, or expansion is in the presence of antigen, such as a viral antigen.
6. A therapeutic T cell population or pharmaceutical composition comprising same according to claim 5, wherein the therapy is immune reconstitution therapy for a patient post-haematopoietic stem cell transplantation.
7. A therapeutic T cell population or pharmaceutical composition comprising same according to claim 5 or 6, wherein the T cell population is an antigen specific T-cell population.
8. A therapeutic T cell population or pharmaceutical composition comprising same according to claim 7, wherein the antigen-specific T cell population is specific a virus for example selected from the group comprising cytomegalovirus, adenovirus, varicella zoster virus, human papillomavirus, hepatitis B virus, hepatitis C virus, BK virus, Epstein-Barr virus, Kaposi's sarcoma-associated herpes virus and human T-lymphotropic virus, such as cytomegalovirus or adenovirus.
9. A therapeutic T cell population or pharmaceutical composition according to any one of claims 5 to 8, wherein the population is directly selected on the basis of a steady state marker namely the T cell receptor, for example by reversible ligation of the T cell receptor by specific HLA:peptide complexes, in particular Tetra, Penta and/or Hexa streptamers.
10. A therapeutic T cell population or pharmaceutical composition according to any one of claims 5 to 9, wherein the activation marker is a cell surface marker that is upregulated as a consequence of antigen stimulation, for example selected from the group comprising CD25, CD69, CD137 and CD154.
11. A therapeutic T cell population or pharmaceutical composition according to any one of claims 5 to 10, wherein the T cell product is an expanded T cell product, in particular expanded in an antigen specific manner.
12. A therapeutic T cell population or pharmaceutical composition according to any one of claims 5 to 11, wherein the population is substantially negative for cells with the CD25 marker.

13. A therapeutic T cell population or pharmaceutical composition according to any one of claims 5 to 12, wherein the T cell population is allogeneic.
14. Use of a therapeutic T cell population or pharmaceutical composition as defined in any one of claims 5 to 13, for manufacture of a medicament for immune reconstitution therapy.
15. A method of selecting and/or expanding a target T cell population which is specific to a virus from a mobilised blood or mobilised apheresis sample wherein selection employs targeting a steady state maker or an activation marker on the surface of the T cells and expansion employs conditions suitable for expansion of the antigen specific T cell population, in particular derived from an allogeneic donor.
16. A method according to claim 15, wherein the target antigen is a viral antigen, for example selected from a DNA virus or an RNA virus, such as a DNA virus.
17. A method according to claim 16, wherein the virus is selected from the group comprising cytomegalovirus, adenovirus, varicella zoster virus, human papillomavirus, hepatitis B virus, hepatitis C virus, BK virus, Epstein-Barr virus, Kaposi's sarcoma-associated herpes virus and human T-lymphotropic virus, such as cytomegalovirus or adenovirus.
18. A method according to any one of claims 15 to 17, wherein the steady state marker is the T-cell receptor (TCR), for example wherein the T cell population is selected by reversible ligation of the TCR by specific HLA:peptide complexes in a process of direct selection.
19. A method according to claim 18, wherein the direct selection employs multimeric HLA:peptide complex, for example Tetra-, Penta-, Hexa- and Streptamers
20. A method according to claim 18 or 19 wherein the HLA peptides or multimers are of a specific HLA-type, for example Al, A2, B7, A24, B35, such as A0201 or B0702.
21. A method according to any one of claims 15 to 20, wherein the activation marker is a cell surface marker that is upregulated as a consequence of antigen stimulation, for example selected from the group comprising CD25, CD69, CD137, CD154 and a combination thereof.
22. A method according to claim 21, wherein the T cell population is selected by reversible ligation of the activation marker by a monomeric, dimeric or multimeric antibody or antibody fragment directed against such activation marker.
23. A method according to claim 21 or 22, wherein the steady state marker is expressed between 5 and 25 hours after exposure with stimuli relevant to a target virus, such as 6 hours or 24 hours.
24. A method according to any one of claims 15 to 23, wherein the target T cell population is expanded by culturing in a T cell expansion media, in particular in the presence antigen.
25. A method according to any one of claims 15 to 17, where the target cell population is selectively expanded from the mobilised apheresis without prior selection through a steady state or activation marker.
26. A method according to claim 24 or 25, wherein the expansion in a rapid expansion process, e.g. 15 days or less, such as 14, 13, 12, 11, 10, 9, 8 or 7 days, such as approximately 10 days.
27. A method according to claim 26, wherein the media remains unchanged and unsupplemented over the period of expansion.
28. A virus specific expanded T cell product obtained or obtainable from a method according to any one of claims 15 to 27 or pharmaceutical composition comprising the same.

Figure 1. Functional profile in unpaired G-CSF mobilised (n=6) and non-mobilised (n=6) donors.

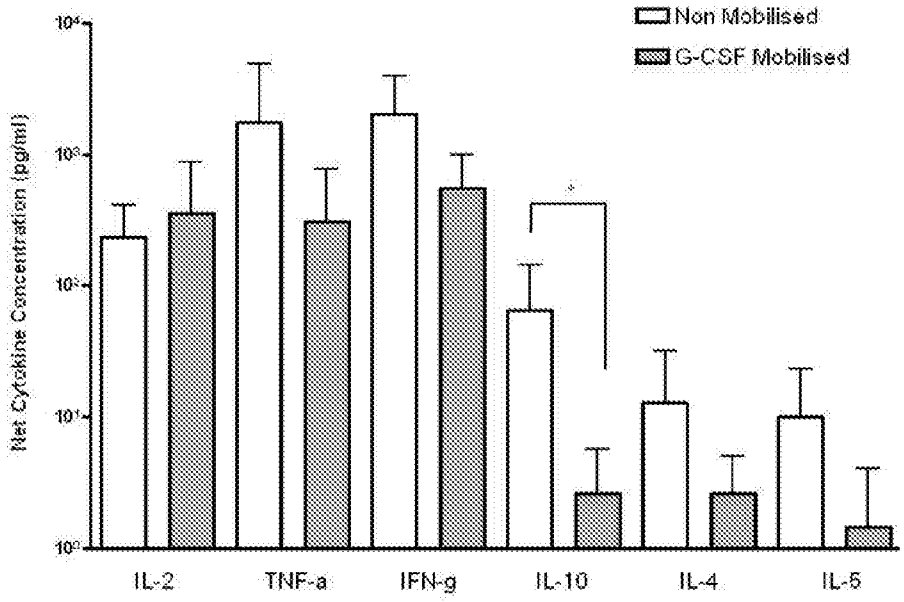


Figure 2 Identification and isolation of IFN-g secreting antigen-specific T cells in unpaired G-CSF mobilised (n=6) and non-mobilised (n=6) donors.

2A

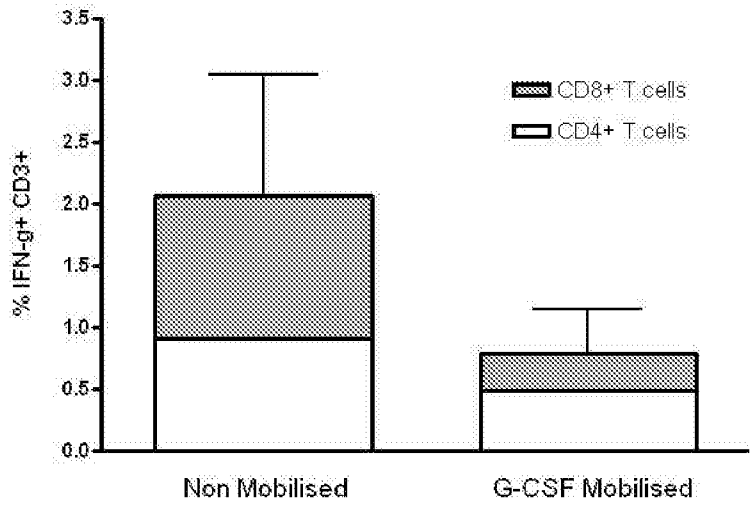


Figure 2B

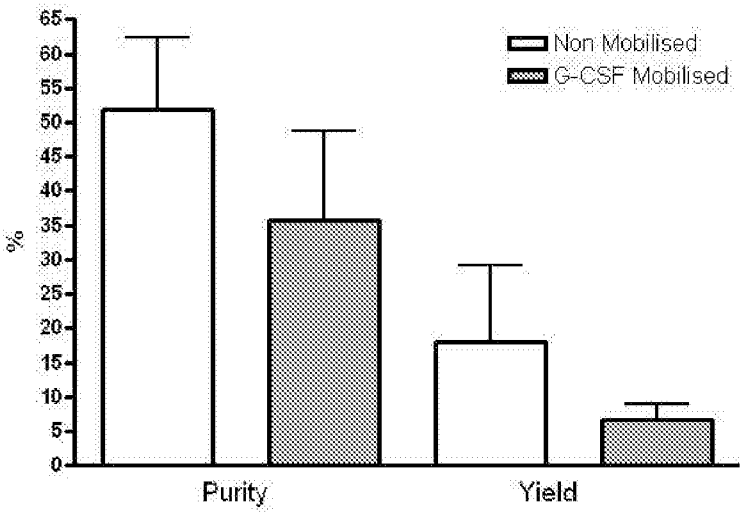


Figure 3 Optimal time of expression of activation markers in response to CMVpp65 stimulation in G-CSF mobilised (n=5) and non-mobilised (n=5) PBMC.

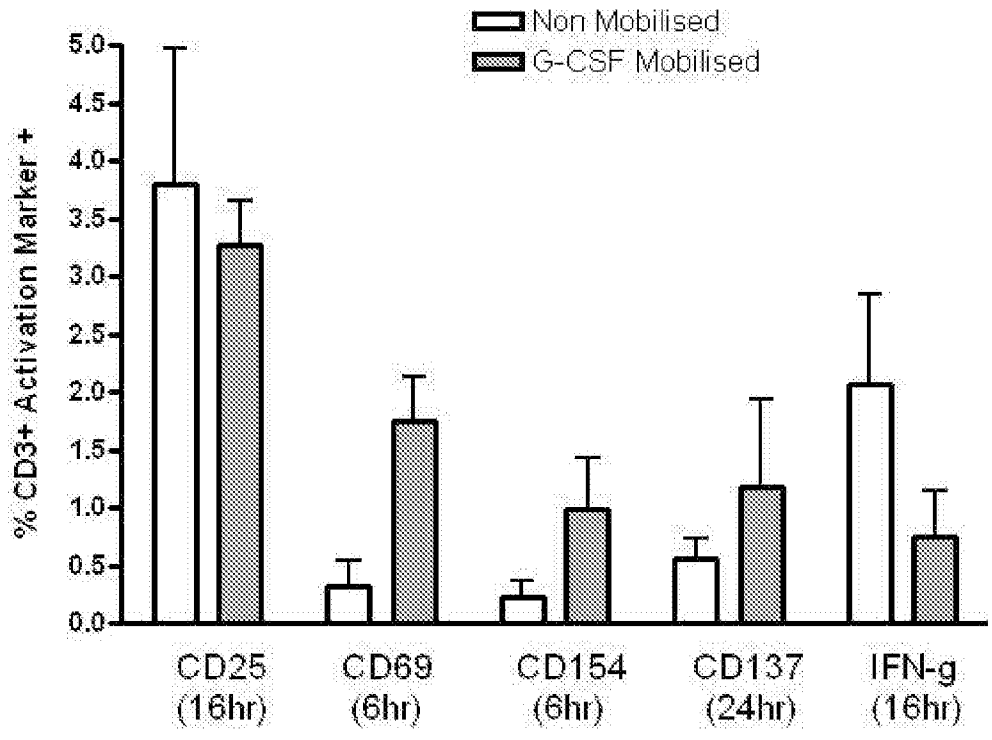
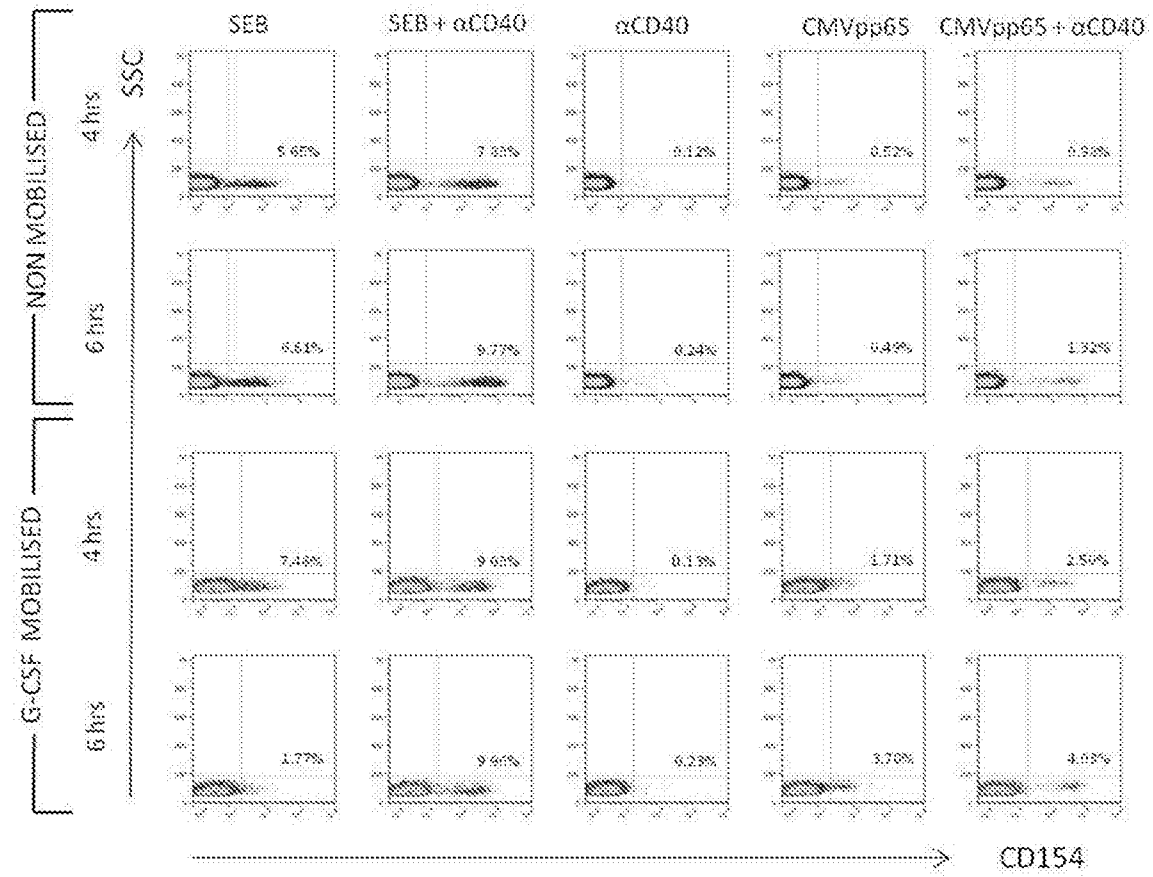
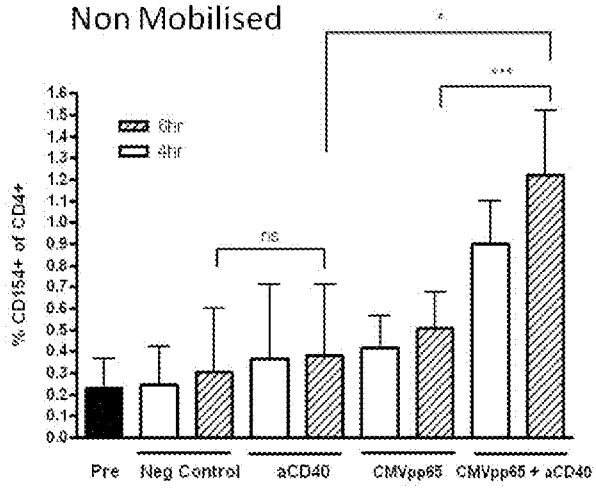


Figure 4 Direct comparison between a G-CSF mobilised and non-mobilised donor of CD154 surface expression at 4 and 6 hours.

A



B



C

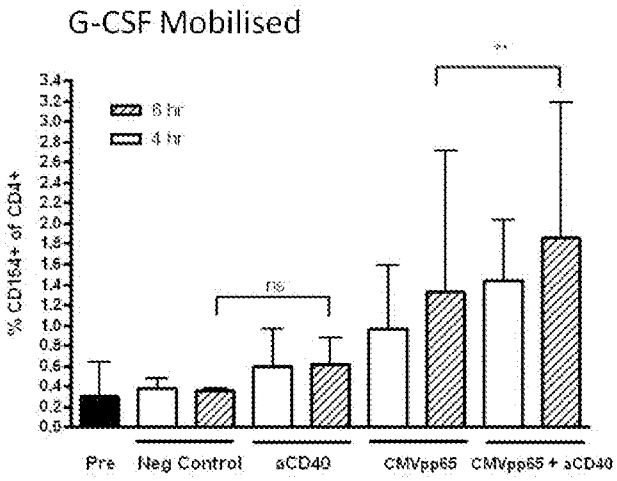
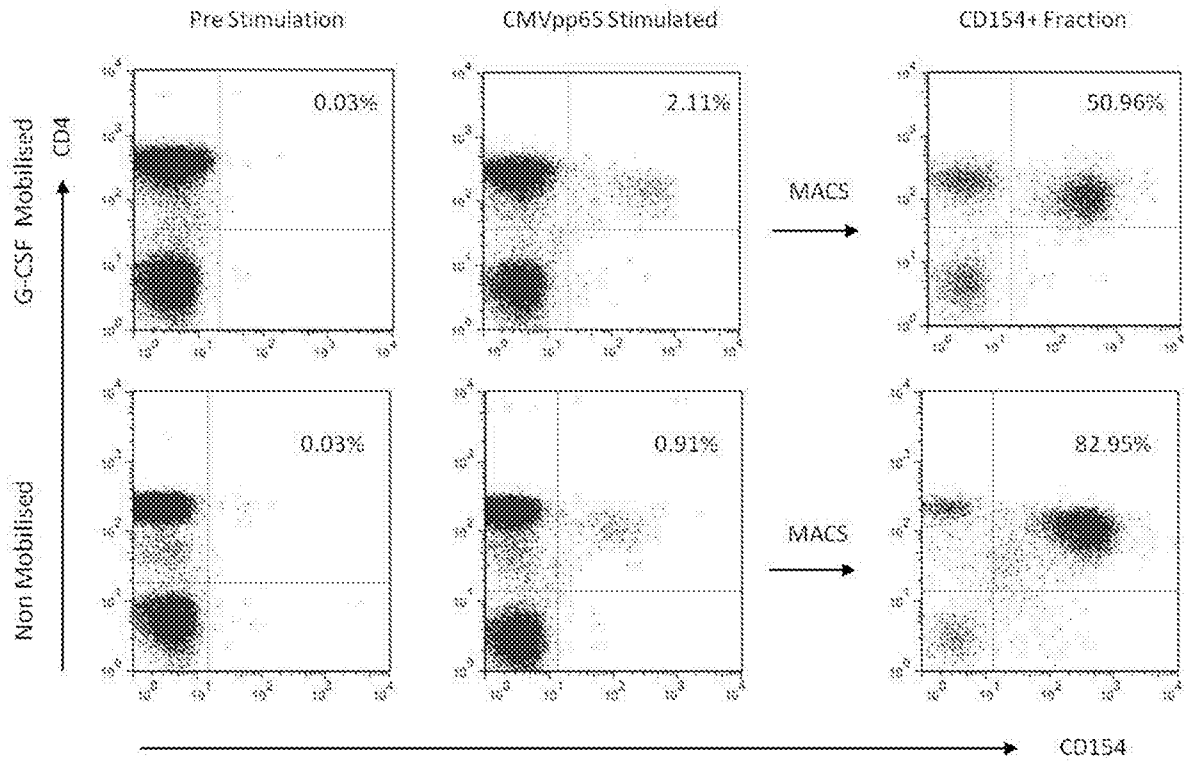


Figure 5 Isolation of CMV-specific T cells through CD154 expression in two unpaired donors.

A



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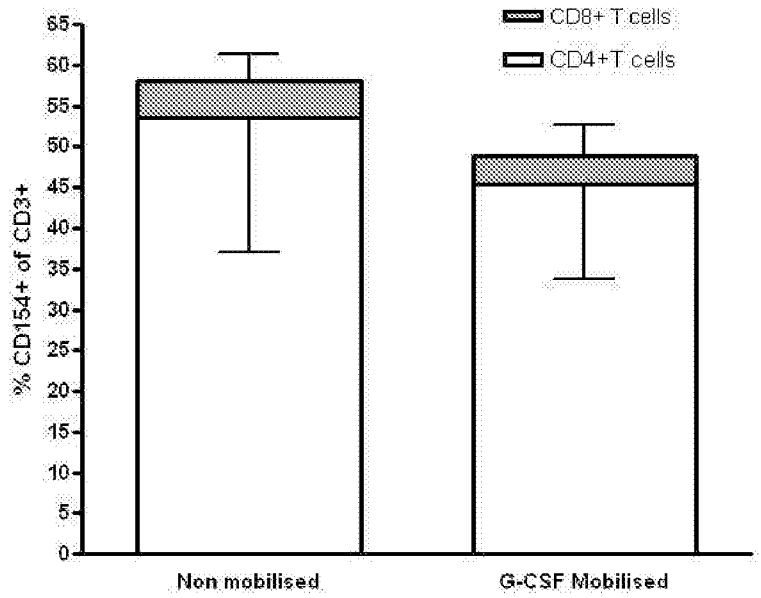
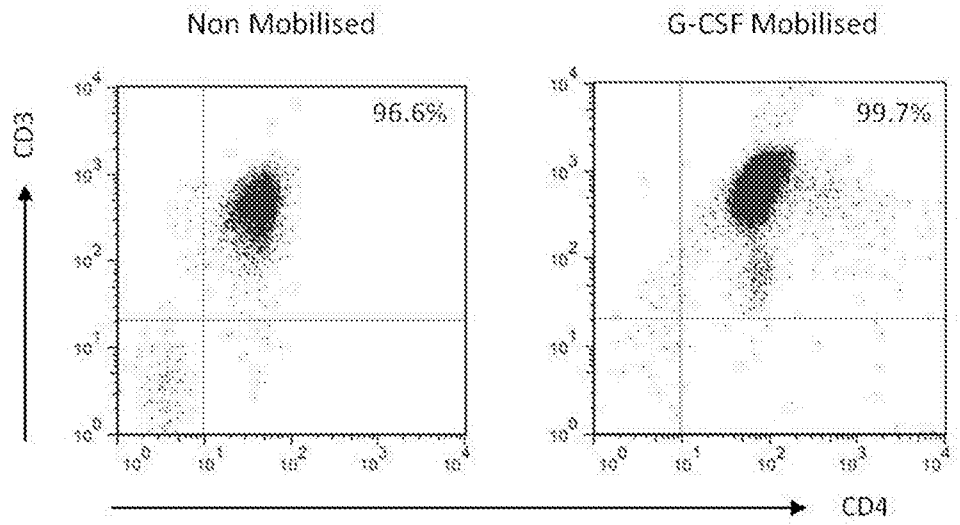


Figure 6 Re-stimulation of expanded CD154+ T cells.

A



B

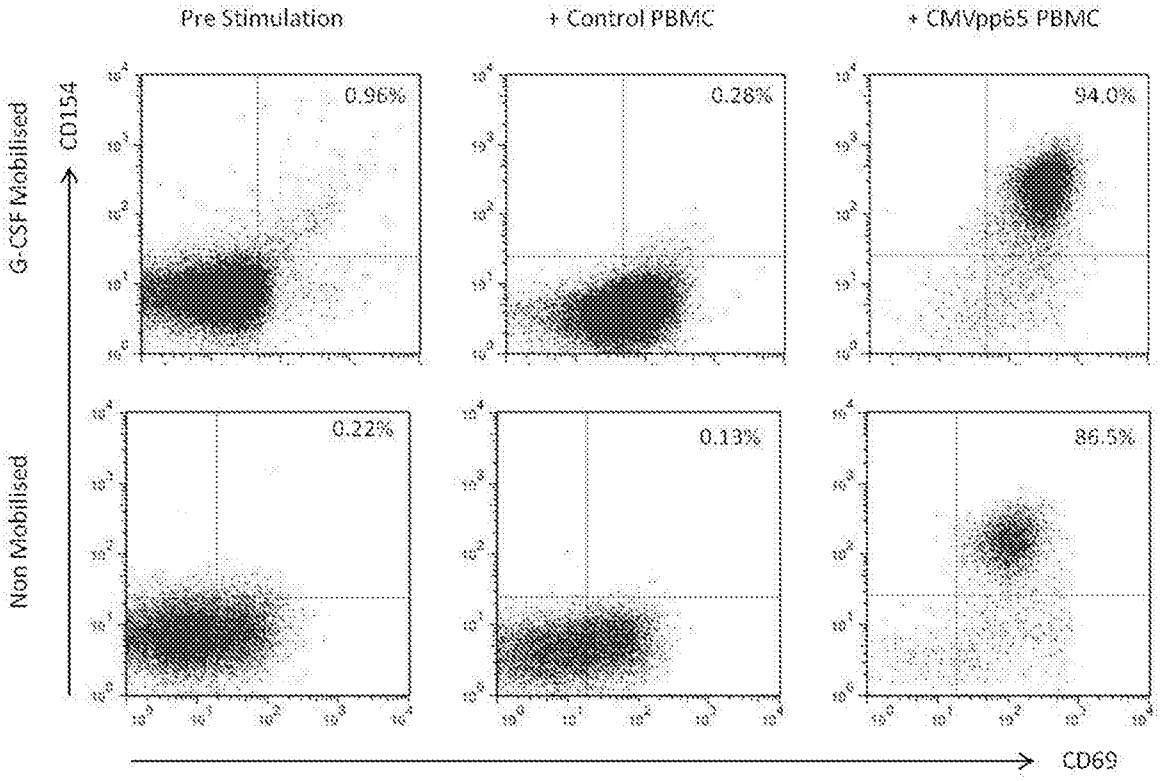
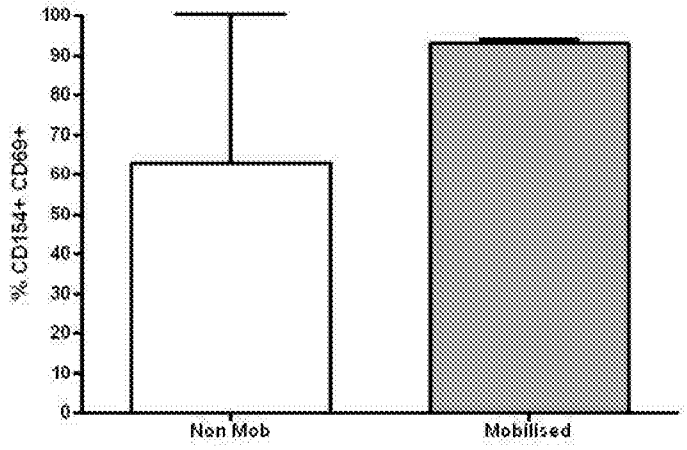


Figure 6 continued

C



D

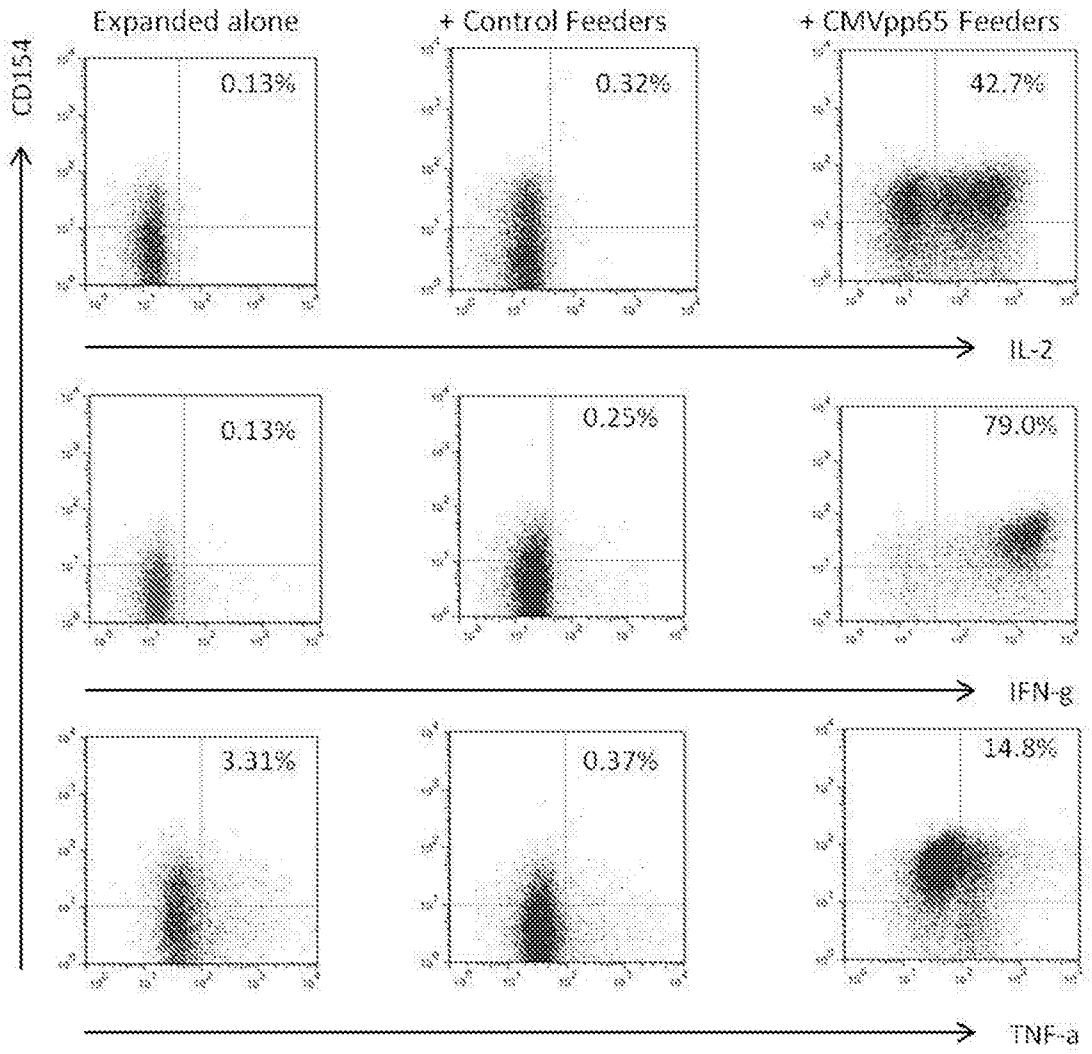


Figure 6 continued

E

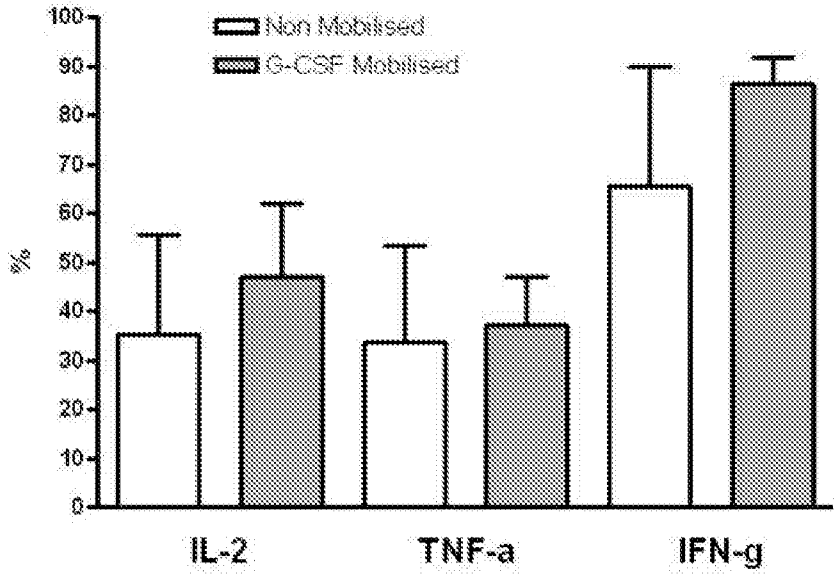
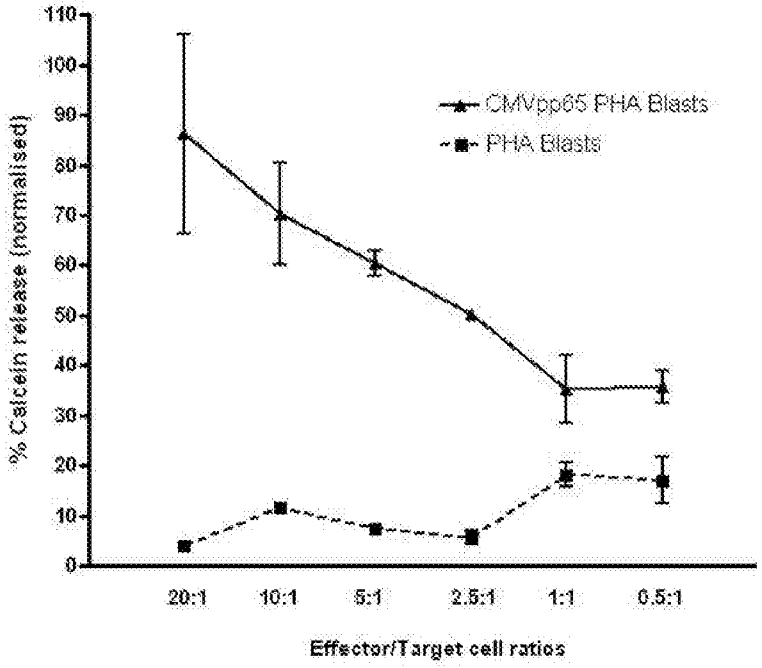


Figure 7 CD154+ CMV-specific T cells isolated from G-CSF mobilised PBMC effectively kill target cells.



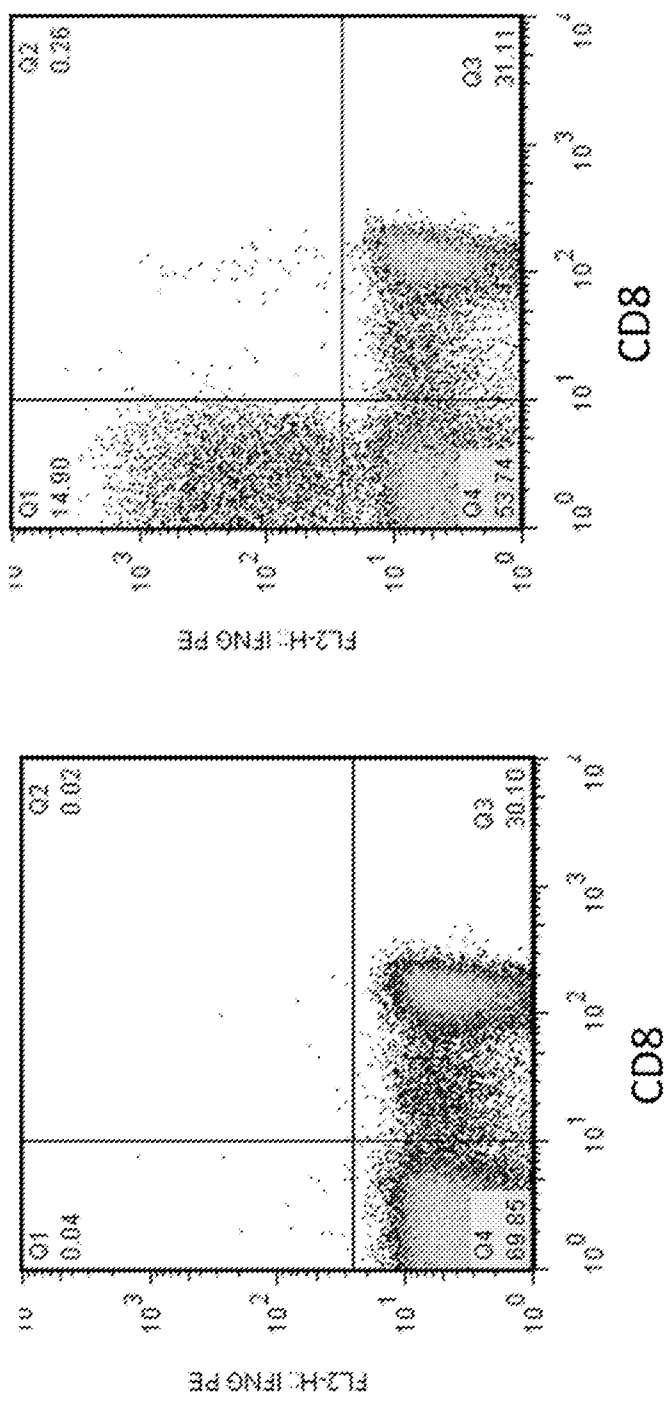
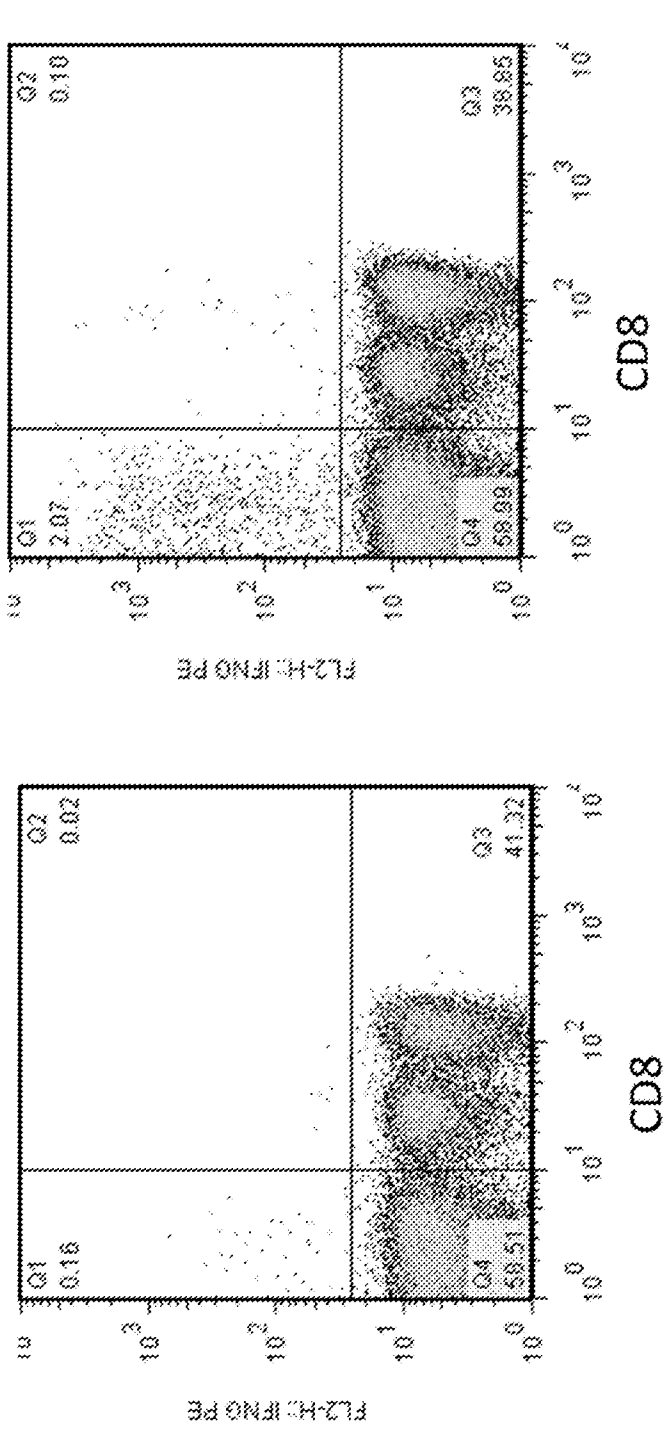


Figure 8 IFN gamma production measured by flow cytometry after 10 day expansion and restimulation with CMV pp65 peptide

Figure 9 IFN gamma production measured by flow cytometry after 10 day expansion and restimulation with Ad Hexon V peptides



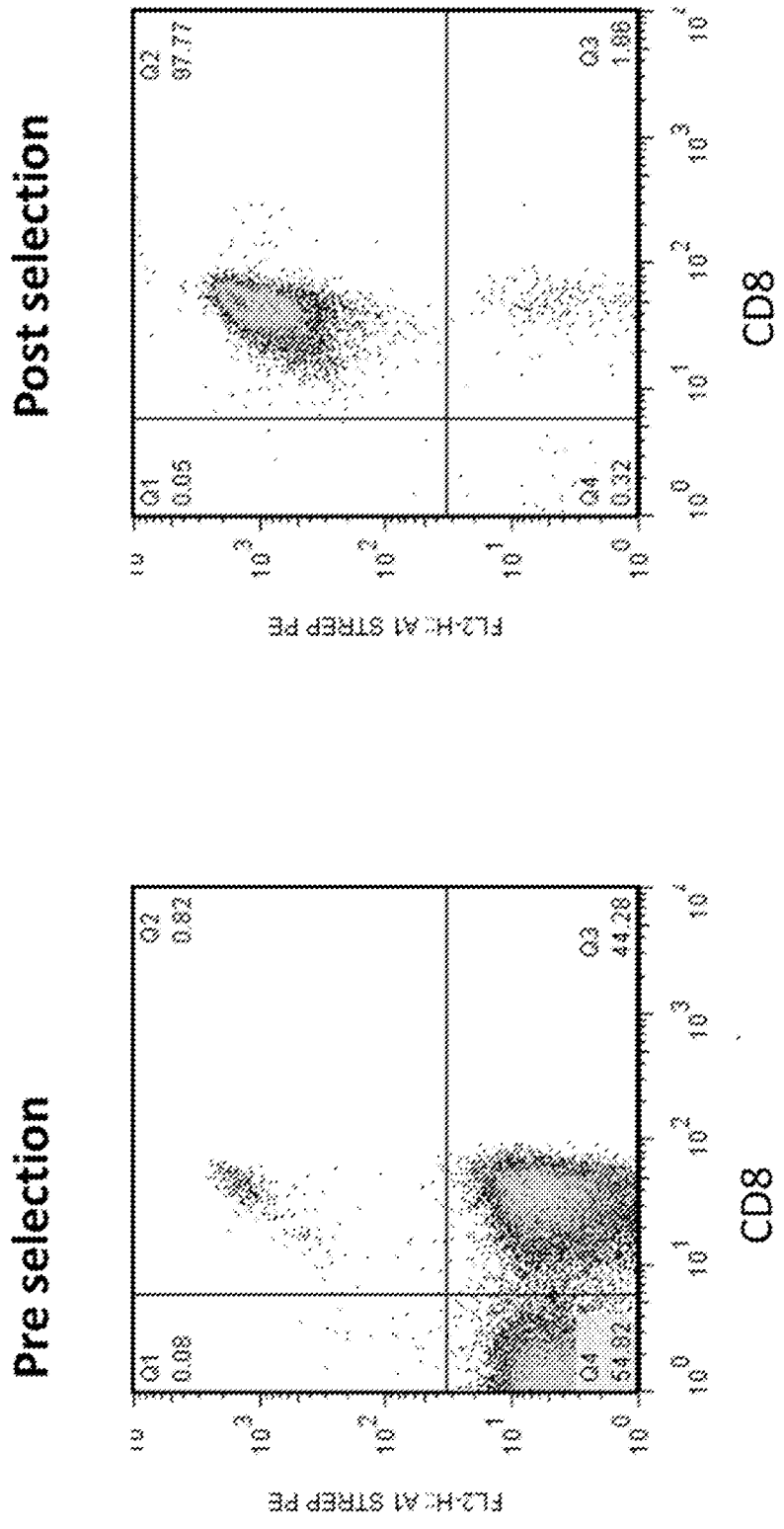


Figure 10 % of cells expressing CMV specific T cell receptors measured by flow cytometry

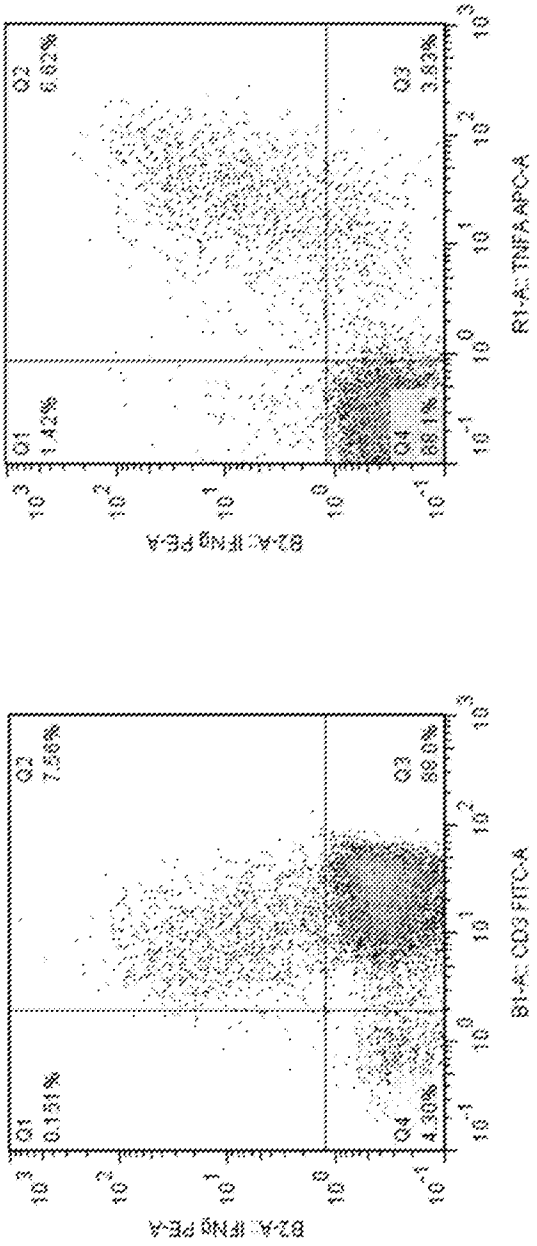


Figure 11 Cells gated on lymphocyte gate (left) or CD3 gate (right).

Figure 12

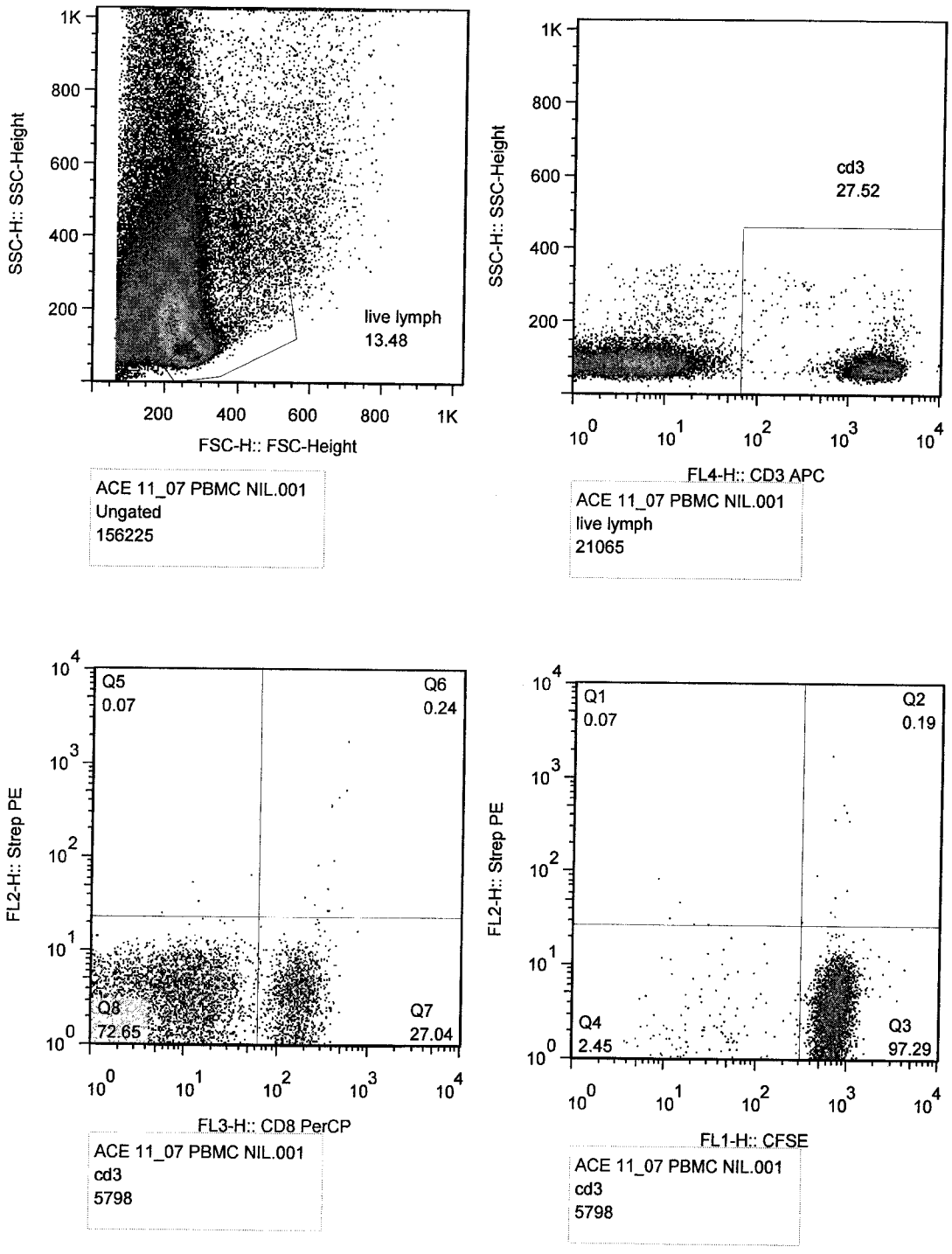
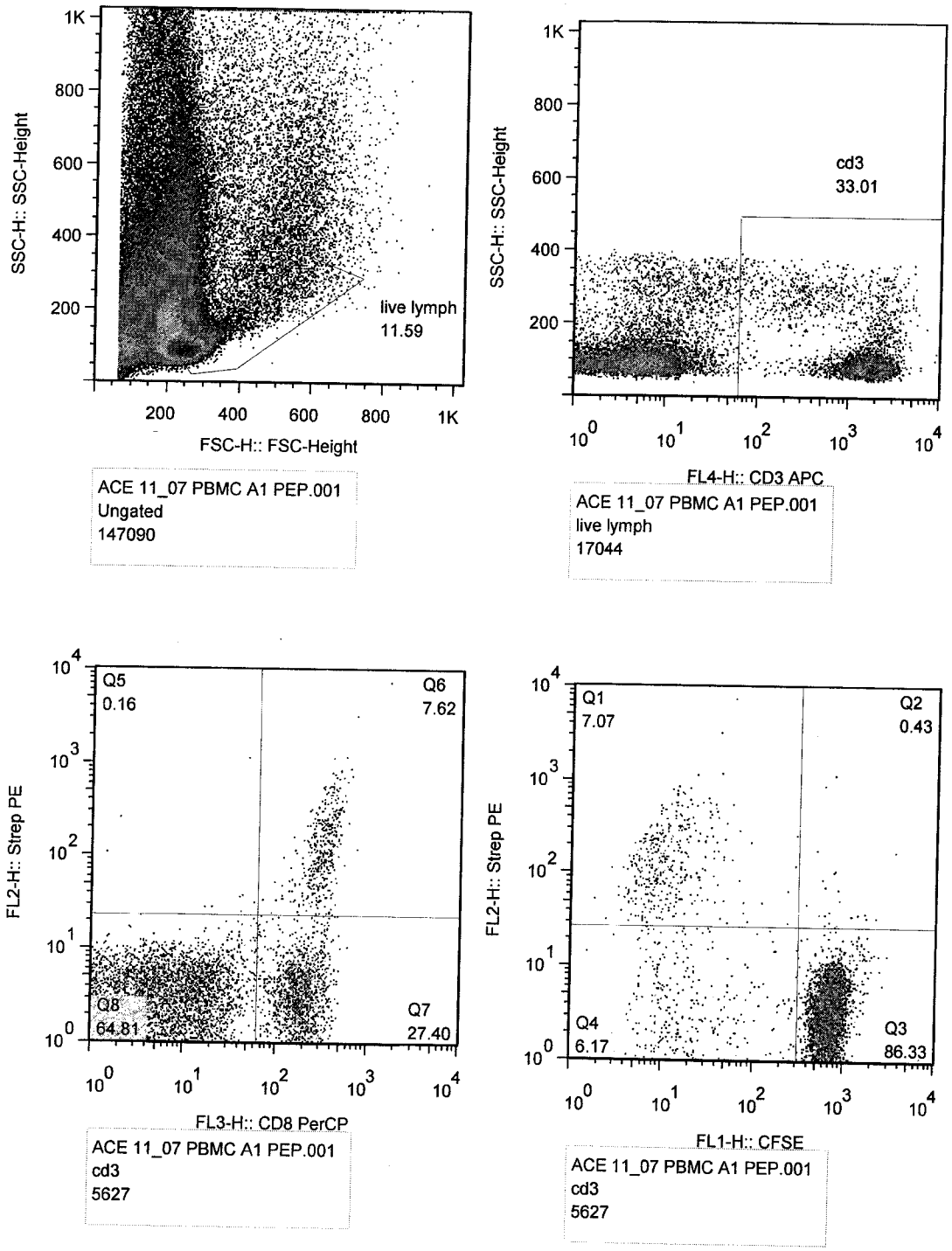

SSC-H:: SSC-Height
FSC-H:: FSC-Height
live lymph
13.48
ACE 11_07 PBMC NIL.001
Ungated
156225
cd3
27.52
FL4-H:: CD3 APC
ACE 11_07 PBMC NIL.001
live lymph
21065
FL2-H:: Strep PE
FL3-H:: CD8 PerCP
Q5
0.07
Q6
0.24
Q8
72.65
Q7
27.04
ACE 11_07 PBMC NIL.001
cd3
5798
Q1
0.07
Q2
0.19
Q4
2.45
Q3
97.29
FL1-H:: CFSE
ACE 11_07 PBMC NIL.001
cd3
5798

Figure 13


SSC-H:: SSC-Height
FSC-H:: FSC-Height
live lymph
11.59
ACE 11_07 PBMC A1 PEP.001
Ungated
147090
cd3
33.01
FL4-H:: CD3 APC
ACE 11_07 PBMC A1 PEP.001
live lymph
17044
FL2-H:: Strep PE
FL3-H:: CD8 PerCP
Q5
0.16
Q6
7.62
Q8
64.81
Q7
27.40
ACE 11_07 PBMC A1 PEP.001
cd3
5627
Q1
7.07
Q2
0.43
Q4
6.17
Q3
86.33
FL1-H:: CFSE
ACE 11_07 PBMC A1 PEP.001
cd3
5627

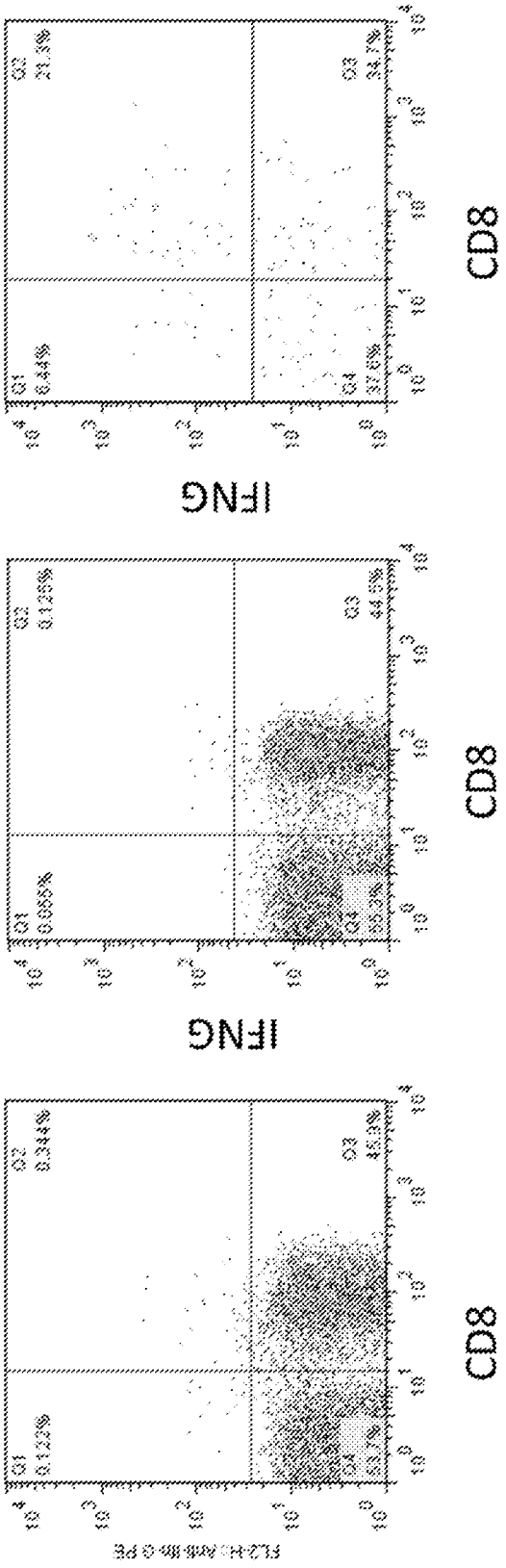


Figure 14