Novel approaches for the isolation and expansion of cytomegalovirus-specific T cells for adoptive immunotherapy

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Declaration

I   confirm that the work presented in this thesis is
my own. Where information has been derived from other sources, I confirm
that this has been indicated in the thesis.

For Sangeeta

Abstract

CMV infections post hematopoietic stem cell transplantation (HSCT) can be effectively controlled through the adoptive transfer of donor derived CMV-specific T cells (CMV-T). Current strategies involve a second leukapheresis collection from the original donor to manufacture CMV-T, which is undesirable in the related donor setting and often not possible in unrelated donor transplants. To overcome these limitations I have investigated using a small aliquot of the original granulocyte-colony stimulating factor (G-CSF) mobilised HSCT graft to manufacture CMV-T. I have explored the T cell response to CMVpp65 peptide stimulation in G-CSF mobilised PBMC and subsequently examined the identification and isolation of CMV-T based on the activation markers CD154 and CD25. CD25+ enriched CMV-T from G-CSF mobilised PBMC were shown to contain a higher proportion of FoxP3 expressing cells than non-mobilised PBMC and also showed superior suppression of T cell proliferation. The successful expansion of CMV-T enriched through CD154 created a product which contained CD4+ and CD8+ cells, demonstrated a high specificity for CMV, secreted cytotoxic effector molecules and lysed CMVpp65 peptide loaded PHA blasts. These data provided the first published evidence that CMV-T can be effectively manufactured from G-CSF mobilised PBMC and that they share the same characteristics as CMV-T isolated in an identical manner from conventional non-mobilised PBMC. This provides a novel strategy for adoptive immunotherapy that abrogates the need for successive donation. The final part of the thesis explored the clinical scale-up of CD154 based enrichment of CMV-T from a G-CSF mobilised donor to assess the feasibility of manufacturing a viable therapeutic cell therapy. This has involved developing a GMP compliant, clinical scale system for the isolation of functional CD154+ CMV-T and has demonstrated the feasibility of translating this process into routine clinical practice, for the prophylactic and pre-emptive treatment of CMV reactivation following allogeneic HSCT.

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Abbreviations

AdV	**Adenovirus**
ALL	**Acute Lymphoblastic Leukaemia**
AML	**Acute Myeloid Leukaemia**
APCs	**Antigen Presenting Cells**
APC	**Allophycocyanin**
BMT	**Bone Marrow Transplantation**
CBA	**Cytometric Bead Array**
CFSE	**Carboxyfluorecein Diacetate Succinimidyl Ester**
CML	**Chronic Myeloid Leukaemia**
CMV	**Cytomegalovirus**
CMV-T	**Cytomegalovirus-specific T Cells**
CTL	**Cytotoxic T Lymphocyte**
CTL-p	**Cytotoxic T Lymphocyte-precursor**
CXCR	**Chemokine Receptor**
Cy	**Cyanine**
DC	**Dendritic Cell**
DFS	**Disease Free Survival**
DLI	**Donor Lymphocyte Infusion**
EBMT	**European Group for Blood and Marrow Transplantation**
EBV	**Epstein-Barr Virus**
ER	**Endoplasmic Reticulum**
FISH	**Fluoresecence in-situ Hybridisation**
FITC	**Fluorescein isothiocyanate**
FoxP3	**Forkhead Box P3**
FSC	**Forward Scatter**
G-CSF	**Granulocyte-Colony Stimulating Factor**
GMP	**Good Manufacturing Practice**
GvHD	**Graft versus Host Disease**
GvL	**Graft versus Leukaemia**
HBSS	**Hank's Balanced Salt Solution**
HLA	**Human Leukocyte Antigen**
HSA	**Human serum Albumin**
HSC	**Haematopoietic Stem Cell**

HSCT	**Haematopoietic Stem Cell Transplantation**
HTL-p	**Helper T Lymphocyte-precursor**
IE-1	**Immediate Early Antigen-1**
IFN-γ	**Interferon-gamma**
IL	**Interleukin**
mHags	**Minor Histocompatibility Antigens**
MHC	**Major Histocompatibility Complex**
MMLR	**Modified Mixed Lymphocyte Reaction**
NK	**Natural killer**
ORFs	**Open Reading Frames**
PBMCs	**Peripheral Blood Mononuclear Cells**
PBSCs	**Peripheral Blood Stem Cells**
PCR	**Polymerase Chain Reaction**
PE	**Phycoerythrin**
PerCP	**Peridinin Chlorophyll Protein**
PHA	**Phytohaemagglutinin**
PI	**Propidium Iodide**
RIC	**Reduced Intensity Conditioning**
RPMI	**Roswell Park Memorial Institute**
RS	**Reed-Sternberg**
SEB	**Staphylococcal Enterotoxin B**
SSC	**Side Scatter**
sIg	**Surface Immunoglobulin**
TAP	**Transporter Associated with antigen processing**
TBI	**Total Body Irradiation**
TCR	**T Cell Receptor**
Th1	**T helper cell 1**
Th2	**T helper cell 2**
TLR	**Toll-like receptor**
TNF-α	**Tumour Necrosis Factor-alpha**
Tregs	**T Regulatory Cells**
UCB	**Umbilical Cord Blood**
vs.	**Versus**

Chapter 1

General Introduction

1.1 Haematopoiesis and the Components of the Immune System

Haematopoiesis, derived from the Ancient Greek, hemat ("blood") and poieo ("to make"), is the formation of blood cellular components. Haematopoiesis starts from a common haematopoietic stem cell (HSC) that resides in the medulla of the bone marrow and is able to differentiate into specialised lineages of blood cells. This ability to give rise to all different types of blood cells means HSCs are often referred to as pluripotent. Approximately $10^{11} - 10^{12}$ new blood cells are produced daily, and HSCs have the capacity for self-renewal so as they proliferate, the overall cellularity of the bone marrow remains in a normal steady state. The maturation of HSCs down erythroid, myeloid and lymphoid lineages occurs under the influence of haematopoietic growth factors that regulate proliferation and differentiation together with the microenvironment of the bone marrow, provided by a stromal matrix on which HSCs grow and divide.

The immune system has evolved into many distinct and complex defence mechanisms against infection by foreign organisms, such as bacterial and viral pathogens. The immune system comprises of two distinct layers, innate immunity and adaptive immunity. The innate immune system is not specific to a single pathogen but recognizes common features of pathogens through receptors such as the family of Toll-like receptors (TLR) which can trigger macrophage activation and subsequent phagocytosis. Innate immunity does not lead to immunological memory and some pathogens are able to overcome this first line of defence. Natural killer (NK) cells are an important cellular component of the immune system, providing rapid response to virally infected cells and are critical to the innate immune response. NK cells kill virus-infected cells by targeting the presence of antibody or changes in receptors on the surface infected cells. However in recent reports, activation of NK cells has been shown to induce memory cell-like

functions, capable of cytokine secretion upon reactivation (Sun *et al*, 2009; Cooper *et al*, 2009), thereby suggestive of a role in the adaptive immune response.

The adaptive immune response is defined by specificity and memory to immune responses, such as antibody production in defence against bacteria. The key effector cells of the adaptive immune system are lymphocytes, both T and B cells which derive from the common lymphoid progenitor and originate in the bone marrow where B cells mature, whereas the precursor T cells migrate to the thymus to undergo maturation. The process of T cell development and maturation occurs in the thymus, where T cell receptor (TCR) genes are randomly rearranged in every T cell to generate a highly diverse repertoire of T cells. TCRs consist of two transmembrane glycoprotein chains, α and β. Each chain consists of a constant region and a highly polymorphic variable region which binds to processed antigenic peptide when presented to the TCR in association with a major histocompatibility complex (MHC) molecule. The expression of co-receptor molecules CD8 and CD4 occurs following TCR gene rearrangement. Immature T cells initially express both CD4 and CD8 molecules, but after interaction with stromal cells in the thymus they mature into single positive CD4+ T helper cells (Th) or CD8+ cytotoxic T cells (CTL) (Zuniga-Pflucker, 2004). T cells undergo clonal activation and proliferation when they encounter antigen and migrate from the thymus to establish the peripheral T cell pool. Some T cells are capable of recognizing self-antigen presented by dendritic cells (DCs) within the thymic microenvironment, termed self-reactive, and undergo deletion in the thymus, thereby preventing autoimmunity. T cells with affinity for self antigens may escape the thymus and are normally inhibited by clonal anergy or by T regulatory cells. A small minority of T cells bear TCRs composed of γ and δ chains, but unlike conventional αβ T cells are able to recognise antigen directly without the requirement for presentation by an MHC molecule and they may bind peptides presented by non-classical MHC-like molecules.

B cells mature in the bone marrow where they receive signals from local stromal cells that initiate their division and maturation, and enter the bloodstream to migrate to the peripheral lymphoid organs. B cells express surface immunoglobulin (sIg) antigen receptors and respond to antigens together with T cell help from CD4+ T cells by undergoing plasma cell differentiation which is followed by the secretion of antibody,

identical to sIg, into the extracellular space. This process is referred to as the humoral immune response as opposed to the T cell response which is generally referred to as the cell-mediated immune response.

1.2 Antigen Processing and Presentation

T cell activation and the subsequent destruction of foreign pathogens is a process that requires the recognition of antigens displayed in the peptide-binding groove of the MHC Class I and Class II molecules on the cell surface by specific T cell receptors (TCR). MHC molecules acquire peptide antigens for presentation in two distinct pathways, endogenous and exogenous, which determines the class of MHC they are assigned to. MHC Class I molecules are composed of heavy chains and an invariant light chain, known as the ß$_2$-microglobulin, and report on intracellular events such as viral or bacterial infection via endogenous processing and the presentation of peptides to CD8+ T cells. Peptides (8-10 amino acids) are loaded onto MHC class I molecules in the endoplasmic reticulum (ER) from proteins degraded by proteasomes.The peptides are transported to the ER by the transporter associated with antigen processing (TAP) complex. Peptides are loaded onto MHC class I molecules with the aid of members of the peptide-loading complex, proteins such as tapasin, calreticulin and ERp57. Peptide loaded MHC class I molecules are subsequently trafficked to the cell surface via the Golgi apparatus where they are recognized by specific TCRs on CD8+ T cells.

MHC Class II molecules present peptides that are taken up by the cell exogenously from the extracellular environment via the endocytic pathway and present these peptides to CD4+ T cells. MHC class II molecules are synthesised in the ER where the α- and ß-chains associate with the invariant chain (Ii; CD74), a protein that enables correct folding, trafficking and protection of the peptide binding groove. MHC class II molecules are then transported from the ER where they fuse with lysosomes containing peptides. Prior to fusing, proteins are degraded in the lysosomes by acidic proteasomes (cathepsins) to peptides 12-25 amino acids in length. Peptide-loaded MHC class II molecules are subsequentlytransported to the cell surface, where they engage CD4+ T cells.

MHC class II expression is mostly restricted to antigen presenting cells (APCs), which include macrophages, B cells and DCs. Immature DCs reside at high density in tissues that interface with the external environment, like the skin, lungs and gastrointestinal tract, where they encounter antigen. Following antigen take-up by endocytosis, DCs traffic to the lymph nodes where they are able to present antigen to T cells. This mechanism of presentation of antigen to T cells by DCs is central to the activation of naïve T cells and directs the adaptive immune response against invading microorganisms. The presentation of antigen to naïve T cells requires both the interaction of MHC loaded with peptide on APCs with the specific TCR as well as a second signal to initiate activation of the T cell. The most important of these signals, often referred to as the co-stimulatory signal, occurs when CD28 on APCs ligates with B7.1 (CD80) and B7.2 (CD86) molecules on T cells. The membrane proteins CD4 and CD8 also enhance T cell responses by helping to stabilise weak TCR and MHC interactions and augmenting T cell signalling. Lack of co-stimulation during antigen presentation to T cells can lead to a state of unresponsiveness known as T cell anergy.

In addition to the processing of antigen via the endocytic pathway for loading onto MHC class II molecules, DCs have the ability to process these exogenous antigens and transfer them to the MHC class I pathway, a mechanism often referred to as cross-presentation (Ackerman & Cresswell, 2004). The cross presentation of peptides processed in this manner is critical for initiating CD8+ T cell responses to viruses that do not infect APCs and therefore allows access to the MHC class I presentation pathway. Recent studies have indicated that cross-presentation generally results from the uptake of proteins by DCs from the extracellular milieu that require further processing (Jensen, 2007). One such mechanism involves the transport of proteins, rather than peptides, from phagosomes into the cytoplasm where they enter the MHC class I pathway as substrates for proteasomes. Although there is still some controversy regarding the mechanics of cross-presentation with regard to antigen processing, it is evident that there are several overlapping pathways in antigen presentation through MHC class I and class II molecules.

1.3   The Biology of Haematological Malignancy

Haematological malignancies arise from the transformation of rare progenitors in the bone marrow or thymus by way of genetic alteration which leads to clonal expansion of abnormal cells. Chronic myeloid leukaemia (CML) was the first human disease in which a specific chromosomal abnormality, the Philadelphia (Ph) chromosome, could be linked to pathogenic leukemogeneis (Nowell & Hungerford, 1960). Leukaemia can be classified into four distinct categories; acute and chronic leukaemia which can be further sub-divided into lymphoid or myeloid malignancies.

Acute leukaemias are usually aggressive diseases, categorised by the accumulation of early bone marrow hematopoietic progenitors, commonly referred to as blast cells, which arise after malignant transformation. In acute lymphoblastic leukaemia (ALL) the bulk of the leukaemia cells resemble lymphoid precursors whereas the origin of leukaemic transformation in acute myeloid leukaemia (AML) has in the past been the subject of some controversy (Russell, 1997). In some AML patients leukemic clones have been shown to originate from erythroid, myeloid and megakaryocytic lineages confirmed by conventional cytogenetics and fluorescence in-situ hybridisation (FISH) (Keinanen *et al*, 1988). In a minority of cases of acute leukaemia, blast cells show characteristics of both myeloid and lymphoid lineages and are termed bi-phenotypic. The accumulation of these abnormal blast cells in the bone marrow and the blood can lead to anaemia, neutropenia and thrompbocytopenia due to bone marrow failure and infiltration of the organs such as the liver, spleen and lymph nodes leading to organ failure. The accumulation of blast cells is driven from their failure to progress through the expected programme of differentiation and to undergo apoptosis coupled with the retention or aquisition of stem cell characteristic of self-renewal. The most common molecular mutation associated with AML occurs within the FLT3 ligand. This is highly expressed in CD34+ HSC playing an important role in normal haematopoiesis by supporting the viability of candidate HSCs (Sitnicka *et al*, 2003). FLT3 ligand mutations lead to constitutive activation that results in cell proliferation (Rocnik *et al*, 2006).

Chronic leukaemias are distinguished from acute leukaemias by their slow progression and paradoxically are almost always more difficult to cure. CML involves myeloid, monocytic, erythroid, megakaryocytic, B-lymphoid and occasionally T-lymphoid lineages (Fialkow *et al*, 1977) and was among the first neoplastic diseases in which therapy with a biological agent (interferon) was shown to suppress the leukaemic clone and prolong survival (Talpaz *et al*, 1987). Diagnosis of CML is assisted by the presence, in the overwhelming majority of patients, of the Philadelphia chromosome (Ph), characterised by the t(9; 22)(q34; q11) translocation between chromosomes 9 and 22. The Ph translocation creates a hybrid BCR-ABL gene encoding a fusion protein which induces tyrosine kinase activity leading to increased cell proliferation. The leukaemia stem cell in CML gives rise to increased numbers of myeloid progenitor cells that fill the marrow, suppressing haematopoiesis and leading to neutropenia, thrombocytopenia and concomitant infections and bleeding.

Malignant lymphomas are a significant classification of haematological malignancy, arising by the accumulation of malignant lymphocytes that accumulate in lymph nodes, leading to the characteristic clinical feature of lymphadenopathy. The accumulation of malignant lymphocytes in the lymph nodes can lead to spill over into the blood, known as the 'leukaemic phase'. Lymphomas can be divided based on the presence of Reed-Sternberg (RS) cells determined by histology. In lymphomas where RS cells are identified, Hodgkin's disease is diagnosed, whereas the absence or RS cells characterises non-Hodgkin's lymphoma. Non-Hodgkin's lymphomas are a diverse group of diseases that can manifest as highly proliferative and rapidly fatal to indolent and well tolerated malignancies.

Over the last 30 years HSCT has become a therapeutic option in the treatment of most haematological malignancies and is indeed standard practice in many leukaemias and lymphomas. HSCT can take the form of autologous, where the patient's own HSCs are collected during a period of remission, frozen down, and then infused once the patient has undergone a course of conditioning. Alternatively HSCT can be allogeneic, where either a sibling or unrelated donor undergo HSC donation after which they are infused fresh into the patient without the need for cryopreserving. The indications for autologous or allogeneic HSCT in haematological malignancy are dependent on the

stage of the disease and are based on certain risk factors, such as cytogenetics in the case of AML.

1.4   Allogeneic Haematopoietic Stem Cell Transplantation

The treatment of a variety of haematological malignancies through allogeneic haematopoietic stem cell transplantation (HSCT) is now regarded as common practice. The procedure of allogeneic HSCT requires the elimination of the recipient's haematopoietic and immune system through pre-transplant chemotherapy and/or radiotherapy, which provides space for incoming cells to engraft. They also act to kill most residual cancer cells and to lymphodeplete the recipient, preventing graft rejection. The treatment-related mortality from this myeloblative conditioning has largely restricted the use of allogeneic HSCT to healthier younger patients whose tolerance of the radiotherapy makes this approach feasible. However in recent years, allogeneic HSCT has been extended to older patients through the introduction of reduced intensity conditioning (RIC) regimens (Jimenez *et al*, 2007). RIC regimens avoid much of the toxicity associated with myeloblative regimens but require stronger immunosuppression to allow engraftment of incoming cells as the conditioning is not sufficient to eradicate the endogenous bone marrow cells.

The first allogeneic HSCT were performed in patients with acute leukaemia (Thomas *et al*, 1971) and aplastic anaemia (Pillow *et al*, 1966) whose life expectancy was deemed to be limited without such intervention. Allogeneic HSCT was successfully extended to patients with chronic myeloid leukaemia (CML) in the early 1980's (Goldman *et al*, 1986) and was quickly followed with the use of unrelated donor bone marrow for allogeneic HSCT (Beatty *et al*, 1989;McGlave *et al*, 1990).

With the successful establishment of HSCT for a wide spectrum of haematological malignancies has come the quest to resolve the associated allogeneic immune response between donor and recipient that is responsible for both the beneficial effects of graft-versus-leukaemia (GvL) response as well as graft-versus-host disease (GvHD). The delicate balancing act associated with the favourable effects of GvL and the morbidity

and mortality associated with GvHD is one which transplant physicians and scientists have long been attempting to resolve.

1.4.1 Immunological basis for GvHD

The phenomenon of GvHD describes the alloreactivity that results from allogeneic HSCT. Allogeneic haematopoietic stem cell grafts contain immunocompetent cells that are capable of recognising the host as foreign and attempt to reject the host tissues, most commonly the tissues of the skin, gut and liver. GvHD was first documented when irradiated mice were infused with allogeneic marrow and although they recovered from radiation injury and marrow aplasia they subsequently died with secondary disease consisting of diarrhoea, weight loss, skin changes and liver abnormalities (Van Bekkum *et al*, 1967), the hallmarks of GvHD. In 1966, Billingham formulated the necessary requirements for the development of GvHD. First, the graft must contain immunocompetent cells; second, the recipient must not have the capacity to mount an immune response to eradicate the transplanted cells; and third, the recipient must express tissue antigens i.e. HLA or minor histocompatibility antigens (mHags) that are not present in the donor (Billingham, 1966).

GvHD that occurs earlier than 100 days post HSCT is commonly referred to as acute GvHD and can develop within days following HSCT, whereas chronic GvHD is defined as GvHD occurring more than 100 days following HSCT. The development of acute GvHD has been proposed to involve a three-step process where donor immune cells are responsible for the initiation and subsequent injury to host tissues after complex interactions with cytokines. The three-step model first described by Ferrara (Ferrara *et al*, 1996) is initiated by the recipient's conditioning regimen (irradiation and/or chemotherapy) which leads to damage and activation of host tissues, inducing the secretion of inflammatory cytokines. These pro-inflammatory cytokines cause the increased expression of MHC antigens and co-stimulatory/adhesion molecules on APCs, thereby enhancing the recognition of host tissue by donor T cells after HSCT. Following this first phase, donor T cell activation occurs and is characterised by their proliferation and secretion of IL-2 and IFN-γ. This phase in the immunopathophysiology of GvHD, is initiated by donor T cells encountering activated

host APCs bearing recipient allogeneic antigens (alloantigens) which drives their proliferation and differentiation, leading to cytokine dysregulation. The final step incorporates the resulting damage and necrosis of healthy recipient tissues in the form of GvHD. It has recently become evident that GvHD is a result of naïve donor T cell responses and that central and effector memory T cells do not appear to induce GvHD but may however mediate GvL responses (Zheng *et al*, 2008).

In allogeneic HSCT, where donor and recipient are mismatched at MHC level, GvHD is driven predominantly by donor T cells being activated by allogeneic MHC molecules present on recipient APCs. In the MHC-matched setting, the alloresponse is driven by mHags, most notably HA-1 (den Haan *et al*, 1998) presented in the context of MHC which can induce potent responses in donor T cells, often manifested as GvHD.

Several strategies have been employed by transplant physicians to minimise the debilitating effects of GvHD following allogeneic HSCT. Pharmacological agents such as corticosteroids, cyclosporine A and methotrexate are commonly used prophylactically to suppress donor T cell activation through inhibition of cell signalling pathways. T cell depletion of the HSC graft using monoclonal antibodies such as Campath 1H, which ligates CD52 on T cells and causes their destruction via the complement cascade upon infusion of the graft, is also common practice. This strategy, however, also eliminates donor T cells responsible for anti-leukaemia responses and can lead to increased rates of patient relapse (Horowitz *et al*, 1990). Approaches to reduce the conditioning induced morbidity through the use of non-myeloblative regimens have been successful in AML (Alyea *et al*, 2006) by reducing the incidence of GvHD, whilst relying on the beneficial effects of GvL from the graft to eliminate residual malignant cells. Reduced intensity conditioning regimens commonly consist of a combination of the purine analog fludarabine with either alkylating agents like busulfan, melphalan or cyclophosphamide or in conjunction with low dose total body irradiation (TBI). These protocols reduce the inflammation in the host often seen in myeloblative regimens, thus preventing step 1 in the 3-step GvHD induction process.

In more recent years, studies to minimise GvHD have also focussed on the removal of alloreactive donor T cells from the graft by selective depletion prior to infusion as an

alternative strategy, whilst retaining donor T cells that are capable of mounting a GvL response. Strategies have focussed on negative selection of donor T cells expressing activation markers such as CD25 and CD69 (Koh *et al*, 1999; Fehse *et al*, 2000), killing activated T cells by photodynamic purging (Chen *et al*, 2002) or inducing Fas-mediated apoptosis (Hartwig *et al*, 2002).

1.4.2   Immunological basis for GvL

The GvL response is the mechanism by which immunocompetent transplanted cells mount a sustained response directed towards malignant cells of the recipient and is vital for both long term and disease-free survival. The hypothesis that HSCT was associated with an anti-leukaemic effect was first proposed as early as the 1950's (Barnes *et al*, 1956). Evidence that donor cells are capable of exerting a GvL effect was first provided by a large retrospective study of more than 2,000 recipients of allogeneic HSCT for the treatment of haematological malignancy. Results showed that recipients were at an increased risk of relapse if they had received a graft that had been depleted of T cells to reduce the risk of GvHD (Horowitz *et al*, 1990). Direct evidence of the GvL effect mediated by donor T cells was first provided by the use of donor lymphocyte infusions (DLI) for the treatment of relapse in patients with CML, where subsequent complete cytogenetic remissions was demonstrated (Kolb *et al*, 1990). This was an early example of adoptive immunotherapy post-allogeneic HSCT. DLI has subsequently been extended to different haematological malignancies, and dose escalation protocols established (Mackinnon *et al*, 1995) but DLI still remains most effective in the treatment of CML. The major effector cells of GvL are considered to be donor T cells with both CD4+ and CD8+ cytotoxic anti-leukaemia T cell lines or clones reported (Faber *et al*, 1995;Jiang *et al*, 1991). There is also evidence for the implication of other cell subsets in the GvL effect, most notably NK cells (Ruggeri *et al*, 1999).

Studies have demonstrated a strong association between the presence of GvHD and protection from relapse after HSCT through the GvL effect (Horowitz *et al*, 1990; Bacigalupo *et al*, 1991) . A fundamental question in the field of HSCT is whether the mechanism or effectors of GvL are fundamentally different from those of GvHD, or whether GvL simply represents a subset of GvH reactions. The elusive goal in HSCT

continues to be the ability to induce GvL to cure the patient whilst eliminating GvHD. Despite the development of several strategies to separate GvHD and GvL effects, it remains unclear whether they indeed can be separated. Most of these approaches have attempted to separate GvL and GvHD by either the use of RIC regimens to abrogate the release of pro-inflammatory cytokines, usually in conjunction with DLI. Or through advances in cellular therapy including increasing specificity of donor cells towards tumour associated antigens by gene-therapy. Tumour-associated antigens preferentially expressed on malignant cells have been identified as potential targets for incoming donor T cells, most commonly in CML (Schmitt *et al*, 2006), which offer the potential for manipulation of DLI for clinical benefit. A relevant example of this approach includes the Wilms' Tumour antigen-1 (WT1) targeted immunotherapy (Xue *et al*, 2004). WT-1 is expressed at elevated levels in most leukaemias and can be effectively killed by allorestricted CTL.

1.4.3   Stem cell source

There are three main sources of HSCs for clinical transplantation – bone marrow, peripheral blood stem cells (PBSC) and umbilical cord blood (UCB). The principle source of haematopoietic stem cells in both autologous and allogeneic HSCT continues to be PBSCs collected by apheresis after granulocyte colony stimulating factor (G-CSF) mobilisation. The original source of HSCs was bone marrow but its use in HSCT has declined over the years, as the requirement for anaesthesia and hospitalisation in order to obtain a sufficient volume of bone marrow (10-15ml/kg recipient weight) is seen as a strong disadvantage of this approach. In 2009, The European Group for Blood and Marrow Transplantation (EBMT) survey of over 600 transplant centres reporting a total of 31,322 HSCT found that over 70% of allogeneic HSCT were conducted using PBSCs (Baldomero *et al*, 2011). The move away from bone marrow harvests as a source of CD34+ stem cells is largely due to the fact that G-CSF mobilisation enables the collection of significantly larger numbers of CD34+ cells compared to traditional bone marrow harvests, as well as an increase in the numbers of committed myeloid, erythroid and megakaryocytic progenitors (Russell *et al*, 1996). Furthermore a meta-analysis of nine randomized trials comparing allogeneic PBSCs with bone marrow transplantation concluded that mobilised stem cells are associated with faster engraftment, reduced

rates of relapse in haematological malignancy, and improvement in overall and disease-free survival in patients with late-onset disease. However, the use of mobilised stem cells is also associated with more acute GvHD grades 2-4 and a significant risk of extensive, chronic GvHD (Stem Cell Trialists' Collaborative Group, 2005), than traditional bone marrow grafts; thought to be related to the higher T cell dose contaminating the graft.

UCB is also a rich source of HSCs and is associated with a decreased incidence of GvHD and reduced histocompatibility requirements due to the naïve immunological state of lymphocytes within the UCB graft. This has led to the establishment of a number of cord blood banks over the past decade. The first human UCB transplant was performed in 1988 in a child with Fanconi anaemia, where the UCB from an HLA-identical sibling was used (Gluckman *et al*, 1989). HSCT using UCB has largely been restricted to paediatrics and small adults due to the dose limitations of UCB. UCB collections contain a significantly reduced total number of CD34+ cells due to the collection volume (50-200ml), which when used in adults can have the impact of delayed or failed engraftment compared with PBSCs. In more recent years, dual cord blood transplants have been performed to maximise CD34+ cell numbers and therefore reduce the time to engraftment in the adult setting. To overcome the limitation of UCB in terms of progenitor cell numbers, several studies (McNiece *et al*, 2000) have investigated the expansion of CD34+ cells in short term culture from UCB. However, these studies have yet to be translated into clinical practice.

1.4.4   Human Leukocyte Antigens

The recognition and destruction of virally infected and tumour cells by T cells is a process controlled through the binding of peptide fragments originating from foreign antigens to molecules termed the MHC. The MHC is polygenic, containing several MHC Class I and MHC Class II genes coding for a diverse range of peptide binding specificities; and polymorphic, meaning that there are multiple variants of each gene within the population. The genes coding the MHC are located on chromosome 6 in humans is an area known as the human leukocyte antigen (HLA) region. The genomic region on chromosome 6 contains the class I subregion at one end which contains genes

encoding the class I molecules HLA-A, -B, -C, as well as the non-classical molecules HLA-E, -F and –G. Class I genes code for a heavy α chain which becomes linked to a non-polymorphic light chain, ß$_2$-microglobulin, whose gene is on chromosome 15. The class II subregion contains the classical HLA-DR, -DP, and –DQ genes which code for both α and ß chains. Classical HLA class I molecules are expressed on the majority of tissues and blood cells whereas HLA Class II molecules are constitutively expressed on B cells, monocytes and dendritic cells, and can be detected on activated T cells in humans.

Following the identification of the HLA system in 1965 (Dausett and Rapaport, 1965), it became evident that HLA discrepancy between donor and recipient was responsible for graft rejection, increased GvHD and mortality in allogeneic HSCT. A retrospective analysis of 948 unrelated donor allogeneic HSCTs found that a single HLA allele or antigen mismatch was associated with increased mortality compared to no HLA mismatch (Petersdorf *et al*, 2004). HLA typing of both donor and recipient is a prerequisite in the field of transplantation. This was initially carried out using serological methods but has now been replaced with more sensitive DNA sequencing which can detect single nucleotide differences between two unique HLA alleles of the same antigen. Most of the DNA sequencing techniques make use of the polymerase chain reaction (PCR) to amplify the specific genes to be analysed, including PCR-SSP (PCR-sequencing-specific priming) and PCR-SSOP (PCR-sequencing-specific oligonucleotide probing). As one would expect, the outcome after allogeneic HSCT is much improved by fully matching HLA alleles of donor and recipient, but the associated risks when using an unrelated donor compared with a sibling donor have yet to be fully resolved. A retrospective study of 1448 recipients of T-replete HSCT after myeloblative conditioning showed that no statistical differences were found between fully matched unrelated donors (10/10 alleles) compared with HLA-identical siblings in survival, disease-free survival (DFS) and relapse when patients had high-risk disease and bone marrow is the graft source. However, significant differences were observed when patients had intermediate stage diseases and peripheral blood monocyte cells (PBMCs) were the graft source. Recipients of HLA-matched unrelated donors also had a higher incidence of both acute and chronic GvHD (Woolfrey *et al*, 2010). These

results clearly highlight the number of factors that need to be considered when selecting suitable donors, beyond that of HLA typing.

1.5   Human Cytomegalovirus

Cytomegalovirus (CMV) is a member of the family of herpes viruses and infects approximately 60-70% of normal healthy individuals in Western societies and up to 100% in some parts of Africa and Asia (Bego & St, 2006). In almost all cases, CMV infection remains entirely asymptomatic, with the virus persisting in a latent state for the life of the host. The occasional viral reactivations that occur are controlled by the host immune system.

The virion structure of CMV is an icosahedral nucleocapsid, containing a 230-kbp, double-stranded linear DNA genome surrounded by a layer known as the tegument or matrix, which, in turn, is enclosed by a lipid bilayer envelope containing viral glycoproteins. Most of the tegument proteins are phosphorylated and are highly immunogenic, the most abundant of which are pp150 or basic phosphoprotein (UL32) and pp65 (UL83). The virus is usually acquired through the transfer of saliva and also through intrauterine or perinatal infection. In addition, transmission can also occur following solid organ transplantation and through blood transfusion (Roback, 2002). Monocytes and endothelial cells have been implicated as sites of CMV latency and viral replication, contributing extensively to maintaining life-long infection (Jarvis & Nelson, 2002;Taylor-Wiedeman *et al*, 1991).

Whilst CMV seropositive individuals show no morbidity through effective immune control of the virus, in individuals who are immunocompromised, such as in bone marrow transplant recipients, or following HIV infection, CMV can cause life threatening disease. Infectious complications arising from the reactivation of human CMV following allogeneic HSCT continues to be a significant cause of morbidity and mortality (Chakrabarti *et al*, 2002; Gooley *et al*, 2010), with the incidence of reactivation being reported to be as high as 70% (Reusser *et al*, 1991). Untreated, CMV can cause a variety of diseases including pneumonitis, retinitis, gastrointestinal disease and hepatitis. The biology of CMV infection in immunocompetent individuals is one

that is often described as an evolutionary balance between the host cellular immune system and the viral mechanisms of persistence and evasion that results in lifelong maintenance of infection. Remarkably, unlike any other known persistent human virus, the ongoing T cell responses required to control reactivation is so large that CMV-reactive T cells can be directly enumerated in peripheral blood of seropositive individuals in the absence of active disease.

1.5.1   Immune response to CMV

During primary CMV infection, replication is initiated in the mucosal epithelium and subsequently disseminates to monocytic cells of myeloid lineage, where it establishes latent infection. Replication of human CMV leads to the expression of three classes of viral genes known as immediate-early (IE), early (E) and late (L). Each gene plays an important role in the coordination of CMV replication. IE genes are independent of any viral *de novo* synthesis and encode mostly regulatory *trans—*acting factors, a DNA sequence that contains a gene. The expression of E genes is dependent on the presence of viral IE proteins and provides an essential number of factors including viral DNA replication proteins and repair enzymes. L genes contribute to the assembly and morphogenesis of the virion and are expressed after the onset of viral DNA replication (Ma *et al*, 2012). The phosphoprotein pp65 is produced early during viral replication, and regulation of its expression is controlled by the major IE promoter (MIEP), which is thought to be central in the control of the latent state. In healthy individuals, viral replication is mediated by both the innate and adaptive immune systems with independent roles played by NK cells, B cells and both CD4+ and CD8+ T cells. The humoral response to CMV is in the form of neutralising antibodies which are directed towards multiple CMV proteins including structural tegument proteins, envelope glycoproteins and non-structural proteins. Neutralising antibodies directed towards epitopes of the envelope glycoprotein B have been shown to be of particular importance. Studies have shown approximately 40% to 70% of the total serum virus-neutralising antibody activity of individuals with previous infection were directed against this single envelope protein (Britt *et al*, 1990). In murine studies, mice immunised against murine CMV glycoprotein B are protected against a lethal challenge (Rapp *et al*, 1992). More recently it has been found that antibodies directed towards

glycoprotein H/glycoprotein L complexes display an inhibitory activity on human CMV plaque formations and leukocyte transfer of virus from infected cells (Gerna *et al*, 2008).

Early studies demonstrated a protective role for NK cells in murine CMV infection, where NK cells were activated rapidly and the level of activity correlates with the degree of resistance (Bancroft *et al*, 1981). Although NK cells offer an important first line defence against CMV when specific immunity has yet to be fully developed, studies involving the CMV response and NK cells have tended to concentrate more on the evasion mechanisms that the virus has developed. Such mechanisms includes the human CMV UL40 glycoprotein protein that promotes efficient cell surface expression of the non-classical MHC class I molecule HLA-E, which binds the NK cell inhibitory receptor CD94/NKG2A, protecting the cell harbouring the virus from lysis (Tomasec *et al*, 2000). Furthermore in HSCT patients, NK cells appear to engraft rapidly following transplantation, at a time when patients reactivate CMV or become infected, which suggests that they are unlikely to play a key role in susceptibility to viral infection. In contrast, the absence of reconstitution of CMV-T cell immunity following HSCT is strongly associated with reactivation of CMV and subsequent CMV disease (Reusser *et al*, 1991).

1.5.2   T cell immunity to CMV

Studies in both humans and animal models have demonstrated a critical role for both CD4+ and CD8+ T cells in the control of CMV replication and the prevention of CMV disease. In CMV-infected mice, a major fraction of CD8+ cytotoxic T cells (CTL) specific for the IE-1 protein were shown to mediate protection (Koszinowski *et al*, 1990). Extensive research in humans has demonstrated that the CTL response to CMV is dominated by the structural proteins pp65 (UL83) (Wills *et al*, 1996; Laughlin-Taylor *et al*, 1994) and IE-1 (UL123) (Davignon *et al*, 1995). The protective role of CD8+ CMV-reactive T cells was first successfully shown after the adoptive transfer of donor derived T cell clones, in three bone marrow transplant recipients (Riddell *et al*, 1992). Evaluation of CMV-specific CTL responses demonstrated that CD8+ clones persisted for at least a month and none of the three patients developed CMV viraemia providing

evidence of protection from CMV disease. The magnitude of lyic activity of reconstituted CD8+ CMV-specific immunity detected in all recipients after a third cell dose was shown to be equivalent to or greater than that observed in the immunocompetent bone marrow donors. The association of CD8+ CTL with protection against CMV replication and subsequent disease was elegantly demonstrated by using CMVpp65 specific class I HLA-tetramers to show that CD8+ CTL numbers increased significantly after episodes of CMV infection following HSCT (Cwynarski *et al*, 2001). Furthermore, in HSCT recipients undergoing a high-level of CMV replication, frequencies of CD8+ CMV-specific CTL have been shown to be as much as 20% (Gratama *et al*, 2001; Aubert *et al*, 2001). HLA tetramers have helped to define a series of phenotypic markers of CMV-specific CD8+ T cells, characterising them as a late effector, fully differentiated phenotype (CD45RA+, CD27-, CCR7-) (Appay *et al*, 2002a).

Although previous work on CMV-specific T cell immunity has focused primarily on the two principle dominant proteins, pp65 and IE-1, the true breadth of the total T cell response to CMV proteins has been shown using 13,687 overlapping 15mer peptides encompassing all 213 known CMV open reading frames (ORFs) in 33 CMV seropositive healthy subjects (Sylwester *et al*, 2005). In this very substantial publication, Sylwester and colleagues were able to show that at least 151 CMV ORFs are immunogenic for CD4+ and/or CD8+ T cells, with 44 ORFs recognised by CD4+ T cells only and 26 recognised by CD8+ T cells only, as summarised in Figure 1.1. Whilst the majority of CMV seropositive individuals mount T cell responses to epitopes of pp65 and IE-1, it is clear that the anti-CMV response is also directed towards other targets, such as pp28, pp71 and IE-2 (Sylwester *et al*, 2005).

The importance of CD8+ CTL in conferring protection against CMV disease is well described; however, the function of proliferating CD4+ T cells in resistance to CMV is still somewhat unclear and a role for CD4+ T cells with cytotoxic capabilities is still controversial. Identification of a population of CD4+ T cells that can mediate killing of virally infected cells has been successfully shown in both mice depleted of CD8+ T cells (Stuller & Flano, 2009) and deficient in MHC class I expression (Hou *et al*, 1993). These studies provided evidence of cytokine secreting CD4+ T cells with anti-viral

properties which were not seen when CD8+ T cells were present. CD4+ T cells have been shown to be important for maintaining adequate numbers of CD8+ CMV-specific T cells in CMV disorders related to acquired immune deficiency syndrome (AIDS) (Komanduri *et al*, 2001). The importance of CD4+ T cells in maintaining CD8+ numbers with respect to CMV is further supported by evidence that CMV-specific CD8+ T cells increase in number after infusion of CD4+ CMV-specific T cells in vivo (Einsele *et al*, 2002). The minimum repertoire of CMV-reactive T cells needed for containment of CMV remains unresolved.

Although αβ T cells are the principle effector cells in controlling CMV disease, recent interest has focussed on the role of γδ T cells, a minor population of circulating T cells (<5%) in humans which primarily reside in epithelial tissues, where they function as the first line of defence. It has been shown that γδ T cells play an important role in the immune response to CMV, demonstrated by the oligoclonal expansion and cytotoxic function of CMV-reactive Vδ1 and Vδ3 γδ T cell subsets in the peripheral blood of patients receiving renal allografts (Lafarge *et al*, 2001; Dechanet *et al*, 1999). More recently the protective role of Vδ1 and Vδ3 γδ T cells in CMV-seropositive patients with documented CMV reactivation, following allogeneic HSCT has been demonstrated. Isolated γδ T cells subsets from these patients showed significant expansion and potent antiviral capacity *in vivo* (Knight *et al*, 2010)
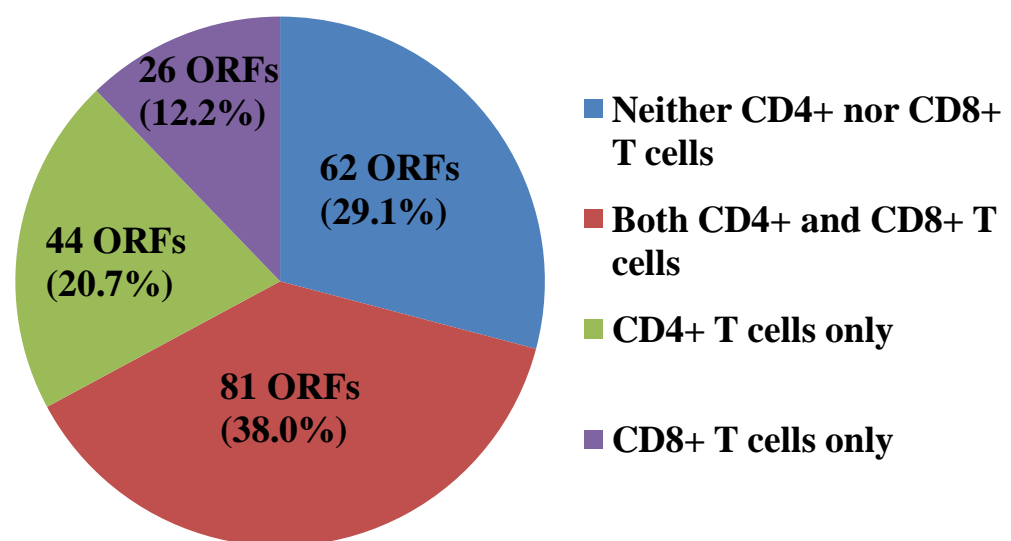
Figure 1.1 Percent of 213 HCMV ORFs recognised by CD4+ and/or CD8+ T cells in 33 CMV seropositive donors. Figure reproduced from (Sylwester *et al*, 2005).

1.5.3   CMV and HSCT

CMV infections continue to prove problematic following allogeneic HSCT and disease can manifest as pneumonitis, gastrointestinal syndromes, hepatitis, retinitis or bone marrow suppression. CMV pneumonitis is one of the major causes of morbidity and mortality following transplantation due to the cytopathic effect of CMV replication in the lungs. The risk of HSCT- associated CMV disease is defined by the CMV status of both donor and recipient. A predictor for a high risk of CMV disease is transplantation of a CMV-seronegative donor into CMV-seropositive recipient and vice versa. The combination of both donor and recipient seropositivity is associated with a lower risk while the combination of donor and recipient seronegativity is associated with no intrinsic CMV risk.

With the incidence of CMV disease following HSCT being reported to be as high as 70% (Reusser *et al*, 1991), a reduction in disease has been achieved through the prophylactic and pre-emptive use of anti-viral drugs including Ganciclovir and Foscarnet (Goodrich *et al*, 1993) coupled with detection and quantification of viral DNA by polymerase chain reaction (PCR) based assays (Einsele *et al*, 1995). Although the prophylactic and pre-emptive administration of anti-viral drugs has helped to reduce CMV disease in the first 100 days following allogeneic HSCT, they have not been effective in improving overall survival in the long term and they are associated with delayed immune reconstitution and late onset CMV disease (Boeckh *et al*, 2003).

The development of a cellular immune response against CMV is the most important factor in conferring protection from CMV infection and this has lead to an increasing number of studies using adoptive immunotherapy to prevent CMV disease in patients at high risk of reactivation. Attempts to restore anti-viral immunity in humans has stemmed from initial observations from preclinical murine models that adoptive transfer of CD8+ virus-specific T cells protects mice against otherwise lethal viral challenge (Reddehase *et al*, 1985).

1.5.4   Adoptive Immunotherapy for CMV

Prior to the transfer of donor T cells with specific anti-viral properties, DLI were used post-HSCT on the premise that these cells contained T cells capable of mediating both anti-tumour and anti-viral activity. DLI has been used extensively to provide anti-tumour immunity for relapsed leukaemia patients (Porter *et al*, 1994; Collins, Jr. *et al*, 1997; Kolb *et al*, 1995) but to a lesser extent for anti-viral immunity. DLI contain high numbers of alloreactive T cells and so dosing is limited due to the potential for GvHD. This in turn limits the dose of virus-specific T cells received, which prompted the investigation of generating a T cell product with the sole role of providing anti-viral immunity without the high risk of GvHD. However, the potential for anti-CMV T cells to cross-react with allogeneic HLA and thus cause GvHD remains a distinct possibility.

The first proof-of-principle studies involving adoptive transfer of donor-derived CMV-specific T cells (CMV-T) involved the use of CD8+ T cell clones expanded by repeated restimulation in long term culture (Riddell *et al*, 1992; Walter *et al*, 1995). Although these studies showed CMV-T reconstitution in all cases, they were poorly maintained due to the absence of CD4+ T cell help. However, these studies were the first clinical evidence for the role of CTLs in controlling CMV infection, and paved the way for several strategies in the field of immunotherapy for CMV disease following HSCT. Strategies for the manufacture of CMV adoptive immunotherapy can be grouped into 4 distinct categories: CMV-specific T cell clones, CMV-specific T cell lines, multi-specific T cell lines and direct selection as outlined in Figure 1.2 (Peggs, 2009). In all instances, the manufacture of CMV-T has involved the use of either donor lymphocytes collected by steady state apheresis or collection of whole blood via a single blood draw as starting material.

1.5.4.1 CMV-specific T cell clones

In the study of Walter *et al.* (Walter *et al*, 1995) CD8+ T cell clones were administered to 14 patients in high cell numbers, the highest dose being 2×10^9 cloned CMV-T in total. Manufacturing such a large number of cloned cells required long culture periods of 8 to 12 weeks. Despite the clinical success of this pioneering study, the translation

into routine clinical practice was limited by the logistics of such long culture periods. Adoptive transfer of CD4+ T cell clones has also been used prophylactically for CMV disease after allogeneic HSCT. In the study of Perruccio and colleagues (Perruccio *et al*, 2005), 25 patients received donor CMV-specific CD4+ T cell clones at a dose range of 10^5 to 10^6 cells/kg body weight. This resulted in the detection of both CD4+ and CD8+ anti-CMV responses after transfer, and control of CMV antigenemia. Just as with the CD8+ T cell clones generated by Walter and colleagues, the generation of CD4+ T cell clones requires a complex logistics of manufacture that has made their widespread clinical application unfeasible.
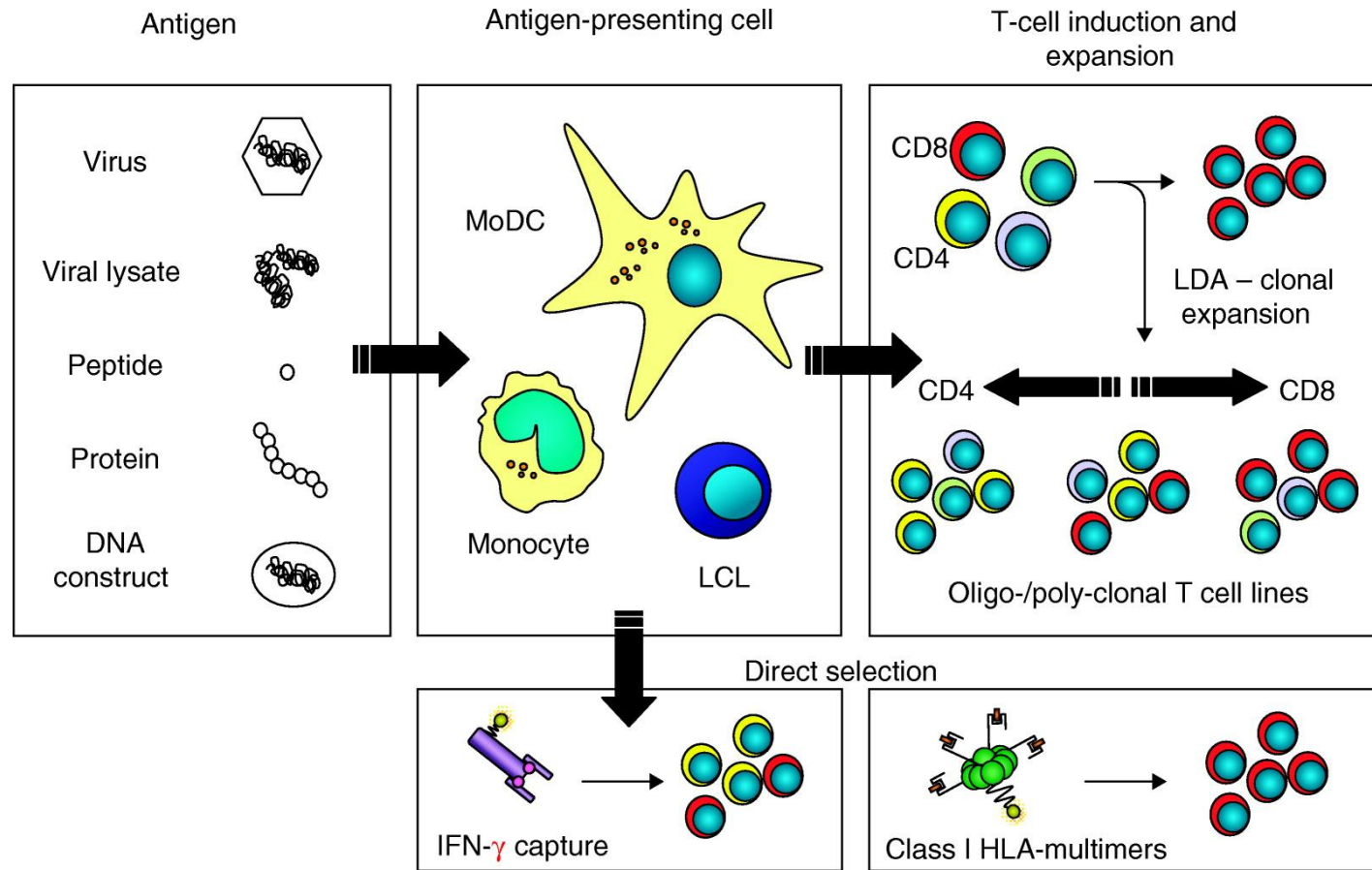
Figure 1.2 CMV adoptive immunotherapy strategies. Figure reproduced from (Peggs, 2009).

1.5.4.2 CMV-specific T cell lines

The adoptive transfer of oligo- or polyclonal T cell lines is an alternative approach to the transfer of T cell clones that has followed on from earlier studies involving the infusion of gene modified T cell lines specific for Epstein Barr virus (EBV) (Heslop *et al*, 1996). Heslop and colleagues showed that cultured cells were associated with the establishment of long-term immunity, as demonstrated by re-challenge up to 18 months post infusion, and were also associated with a limited risk of GvHD. CMV-specific T cell lines for adoptive transfer have been manufactured in culture to contain CD4+, CD8+ and a mixture of both using different strategies. Einsele and colleagues generated a predominantly CD4+ CMV-specific T cell line by pulsing PBMCs with CMV lysate, followed by repetitive stimulation in a 3 to 4 week culture (Einsele *et al*, 2002). Eight patients were treated following HSCT who lacked CMV-specific T cell proliferation, with 5/8 patients achieving viral clearance with associated CMV-specific T cell proliferation after transfer of 10^7 T cells/m^2. A second strategy for manufacture of CMV-specific T cell lines is pulsing monocyte derived dendritic cells (MoDC) with CMV lysate or with a single peptide. Pulsing MoDC with CMV lysate has been demonstrated to result in a mixed CD4+ and CD8+ T cell line, that when transferred to recipients at low doses (1 x 10^5 CD4+ and CD8+ CMV-reactive cells/kg) resulted in massive *in vivo* expansions within days of transfer (Peggs *et al*, 2003). A total of 16 patients were treated after the first episode of CMV viraemia, at a median of 36 days following transplantation, with eight patients clearing viral DNA without the need for antiviral drugs. Three patients developed cutaneous Grade I acute GvHD after adoptive cellular therapy but no clinically relevant GvHD was seen.

The use of MoDC has also been investigated for generation of CMV-specific T cell lines by pulsing with the HLA-A2 restricted peptide NLVPMVATV (NLV) derived from the CMVpp65 protein in a 21 day culture (Micklethwaite *et al*, 2007). Expanded cells were predominantly CD8+ with a median of 62% of final cells NLV-specific. Nine patients were treated prophylactically, with an associated increase in NLV-specific T cells in six patients, although the increase was not maintained beyond several days to weeks. This lack of long term immunity provided by the transferred cells can be explained by the possible lack of CD4+ T cell help much like that observed in the early

studies of Riddell and Walter (Riddell *et al*, 1992; Walter *et al*, 1995). The use of overlapping pools of peptides spanning the entire CMVpp65 protein to pulse MoDC has been investigated, where polyclonal T cell lines containing both CD4+ and CD8+ CMV-T were successfully generated and capable of lysing CMV-infected targets (Trivedi *et al*, 2005).

1.5.4.3 Multi-specific T cell lines

Immunotherapeutic strategies to restore CMV immunity have also been examined through the use of T cells expanded against multiple viruses. In this system, EBV-transformed B cells (EBV-LCLs) were genetically modified with a chimeric adenovirus-CMVpp65 vector (Ad5f35pp65) that generated CTLs specific for EBV, adenovirus and CMV in a single culture. In a proof of concept study (Leen *et al*, 2006), eleven patients received tri-specific CTLs prophylactically at a median of 62 days following HSCT with doses ranging from 5 x 10^6 to 1 x 10^8 cells/m^2. Expansion of multi-specific CTLs was observed *in vivo*, with a concomitant reduction in the levels of all three viruses. Interestingly, the frequency of EBV- and CMV-specific T cells increased whether or not there was evidence of activation of the virus, whereas the expansion of adenovirus-specific T cells was only seen in those patients with a recent or concurrent adenoviral infection. Similar results were also observed after the infusion of T cell lines generated from co-culture with MoDC transduced with the same Ad5f35pp65 vector at a median of 54 days post HSCT at similar doses (Micklethwaite *et al*, 2008). One of the limiting factors in the generation of multi-virus specific T cells has been the complexity of the manufacturing process that requires infectious virus material, production of a clinical grade vector and long term culture (10-12 weeks).

Leen and colleagues have built on these initial experiments to develop a system for the generation of multi-virus specific CTLs that extends the number of pathogens targeted to seven with the addition of specificity directed towards human herpes virus (HHV)-6, respiratory syncytial virus (RSV), influenza and the BK virus (Gerdemann *et al*, 2012). The system also reduces the culture time down to 10 days by a single stimulation of PBMCs with a peptide mixture spanning the target antigens and the addition of the cytokines IL4 and IL7. Two groups have shown that multi-virus specific T cells can be

generated through the activation-dependent expression of both CD154 (Khanna *et al*, 2011) and CD25 (Lugthart *et al*, 2012) after peptide stimulation and magnetic isolation through the associated activation marker. However, none of these protocols has yet to be translated into clinical practice to allow for assessment of their efficacy and safety.

1.5.4.4 Direct Selection

The direct selection of CMV-T is made possible by the high frequency of CMV-reactive T cells in the majority of CMV seropositive individuals. This is an attractive option when considering the reduction in laboratory processing involved compared to the generation of T cell lines and clones. There are two strategies of direct selection that have seen adoptive transfer of CMV-T. The first is the use of HLA-peptide multimers where CMV-specific CD8+ T cells were magnetically enriched from donor PBMCs using varying HLA restriction and peptide specificities (Cobbold *et al*, 2005). Nine patients received cells with doses ranging from 1.2 x 10^4 to 2 x 10^6 cells/kg with a median purity of CMV-specific CTL of >95%. CTLs were shown to be functional after transfer: this was demonstrated most clearly in one patient in whom CMV reactivation was refractory to antiviral drugs but was controlled within eight days of adoptive transfer. One of the limitations of HLA-multimers is that they select only CD8+ T cells, and the lack of CD4+ T cell help might hinder their long term survival. In addition, the restriction of HLA-multimers to only several specificities means that not all patients would be eligible for such treatment.

A second strategy for direct selection of CMV-T is based on the selection of interferon gamma (IFN-γ) secreting cells by magnetic enrichment ('gamma catch') in response to *ex* vivo priming by viral proteins or peptides (Assenmacher *et al*, 2002; Bissinger *et al*, 2002). The adoptive transfer of CMV-reactive IFN-γ secreting cells has been demonstrated in clinical practice (Feuchtinger *et al*, 2010; Peggs *et al*, 2011). We used the 'gamma catch' strategy in the pre-emptive and prophylactic treatment of CMV infection in 18 patients who received a dose of 1 x10^4 CD3+ T cells/kg which contained both CD4+and CD8+ T cells with a median of 43.9% IFN-γ secreting cells (Peggs *et al*, 2011). Expansions in both CD4+ and CD8+ CMV-specific T cells was observed in 17 patients within 2 weeks of transfer and offered protection from re-infection in the

majority of patients. Both strategies of direct selection are considered methodologies that allow widespread application of CMV-T immunotherapy due to their relative simplicity compared to T cell lines and T cell clones.

1.5.4.5 TCR-Transgenic T cells

The genetic modification of T cells using CMV-specific TCRs is an appealing strategy for adoptive transfer, but has yet to be translated into the clinic. TCR gene therapy for CMV is particularly promising when there is not the possibility of manufacturing CMV-specific T cells using the methods already described above. This occurs mainly when the HSCT donor is CMV-seronegative and the recipient CMV-seropositive and therefore at risk to develop severe CMV-related disease. The manufacture of CMV-specific TCRs involves the generation of CMV-specific T cell clones, before cloning retroviral vectors containing the CMV-specific TCR chain genes and subsequent retroviral TCR transfer into TCRαß-deficient cells. Several groups (Heemskerk *et al*, 2007; van Lent *et al*, 2007; Schub *et al*, 2009) have explored this method of generation as a means for adoptive transfer and the first clinical trial is currently underway.

1.5.5   Identification of anti-viral T cells through activation markers

Identification of antigen specific T cells has also been attempted through the use of activation markers that are up-regulated after T cell activation. T cell activation markers offer an increased sensitivity over approaches such as IFN-γ secretion as they are independent of cytokine secretion and therefore could lead to the isolation of antigen-specific T cells with an increased repertoire. Several T cell activation markers have been identified with differing temporal dynamics that allow for simultaneous detection and enrichment.

1.5.5.1 CD25

CD25 is the IL-2α receptor and its expression is a prerequisite for T cell proliferation. Cross linking of the antigen specific TCR is a key signal for CD25 expression, which makes it a potential target for the selection of CMV-T. It has been previously

demonstrated that virus-specific T cells can be purified through CD25 selection after peptide stimulation (Gallot *et al*, 2001) which also make CD25 a promising candidate for the generation of CMV-T. Identification of antigen-specific T cells through CD25 expression can be confounded by the similar CD25 expression pattern exhibited by regulatory T cells (Tregs). Tregs are a subset of CD4+ T cells that are suppressive in nature and regulate responses towards tumour, foreign and allo-antigens that constitutively express CD25 (Sakaguchi *et al*, 1995; Sakaguchi, 2004). The transcription factor forkhead box P3 (FoxP3) has also been identified to play a role in the development and function of Tregs and is also used as a phenotypic marker of Tregs (Fontenot *et al*, 2003). Some groups have explored the removal of CD4+ CD25+ Tregs from starting populations to augment selectivity of antigen-specific T cells for adoptive immunotherapy (Powell, Jr. *et al*, 2005). However, it has also been demonstrated that the removal of this subset has an unpredictable impact on the yield of CMV-T after stimulation (Melenhorst *et al*, 2008a). CD25 has also been used as a target for the generation of multi-virus specific T cells to good manufacturing practice (GMP) grade (Lugthart *et al*, 2012). In this study, a proportion of CD25 enriched cells also expressed FoxP3 and were capable of suppressing T cell proliferation, demonstrating the presence of a functional subset of Tregs.

1.5.5.2 CD69

Although not directly shown to be a marker for the isolation of virus-specific T cells, CD69 offers itself as a prospective target for their enrichment due to its association as an early activation marker on T cells. CD69 is expressed as early as 2 hours post mitogenic stimulation, but is almost undetectable on resting T cells (Craston *et al*, 1997). CD69 has also been used to detect antigen specific T cells following tetanus toxoid stimulation after as much as 48 hours (Mardiney, III *et al*, 1996) and at an optimal time point of 16 hours when stimulating with the superantigen Staphylococcal enterotoxin B (SEB) (Lindsey *et al*, 2007).

1.5.5.3 CD107a

CD107a, also known as lysosomal associated membrane protein 1 (LAMP-1), is a vesicle membrane protein that becomes transiently mobilised to the cell surface during the process of cell killing in effector T cells. CD107a mobilisation has been demonstrated to be a viable marker for identifying antigen specific cells by flow cytometry (Betts *et al*, 2003) and has since been used to enrich a subset of EBV-specific T cells (Moor *et al*, 2007). Moor and colleagues were able to isolate CD8+ EBV-specific T cells using CD107a mobilisation after 5-hour peptide stimulation and subsequent cell sorting. Sorted cells were also capable of in vitro expansion. The CD107a methodology requires stimulation of PBMCs in the presence of monensin to maintain CD107a surface expression. However, one limitation described by Moor and colleagues is that monensin compromises cell viability, which could be a drawback when trying to culture virus-specific T cells at numbers large enough for use as an immunotherapy. CD107a mobilisation has also been used to identify and isolate tumour specific T cells directed towards the melanoma antigen recognized by T cells (MART) or gp100. This allows for the generation of CD8+ T cell clones in short term culture (Rubio *et al*, 2003), demonstrating the potential of CD107a mobilisation in manufacturing cells for adoptive transfer.

1.5.5.4 CD137

CD137, also referred to as 4-1BB, is a member of the tumour necrosis factor (TNF) receptor superfamily and has been shown to have a low baseline expression level on resting T cells, is transiently upregulated on specific CD8+ T cells following alloantigen stimulation and is a target for selective allodepletion (Wehler *et al*, 2007). CD137 was subsequently shown to allow both identification and isolation of CD8+ T cells specific for both the Wilms tumour antigen 1 (WT-1) (Wolfl *et al*, 2007) which is overexpressed in various malignancies and also the HLA-A24-restricted CMVpp65 epitope (Watanabe *et al*, 2008). Both findings highlighted the potential for CD137 to act as a tool for the generation of T cell immunotherapies for adoptive transfer. Wehler and colleagues extended their findings, and also complemented the work of Watanbe and Wolf by demonstrating that CD137 is upregulated on both CD4+ and CD8+ T cells in response

to both CMV and EBV peptide antigens, allowing for the detection and enrichment of virus-specific T cells (Wehler *et al*, 2008).

1.5.5.5 CD154

CD154, otherwise known as CD40 ligand, binds to CD40 on B cells delivering activating signals and is transiently expressed on activated CD4+ T cells and to a lesser extent on CD8+ T cells after antigenic stimulation. CD154 has been shown to identify pathogen-specific T cells because of its high specificity and sensitivity (Chattopadhyay *et al*, 2005; Frentsch *et al*, 2005; Lane *et al*, 1992). Frentsch and colleagues showed that CD154 expression could be preserved at the cell surface after CMVpp65 stimulation through the addition of a CD40-specific blocking monoclonal antibody which allows direct access to CMV-T. More recently CD154 has been used as an activation marker for the generation of multi-pathogen specific T cells, including CMV that were cultured over 14 days and gave rise to both CD4+ and CD8+ T cell lines (Khanna *et al*, 2011).

1.6   Granulocyte colony stimulating factor (G-CSF)

Models of the generation of anti-viral T cell immunotherapies for clinical use have focused primarily on using PBMCs collected by leukapheresis from the original HSCT donor. The prospect of manufacturing anti-viral T cells from an aliquot of the original HSCT graft as an alternative PBMC source has yet to be investigated. HSC are mobilised from the bone marrow into the periphery through the administration of the G-CSF. G-CSF is produced mainly by cells of monocyte/macrophage origin and binds to its receptor on precursor cells in the bone marrow, thereby initiating the proliferation and differentiation of HSCs into mature granulocytes. Thus, recombinant human G-CSF has been used clinically to accelerate neutrophil reconstitution following radiation or chemotherapy where myelosuppression occurs.

1.6.1   G-CSF Mobilisation

The early 1990s saw the first report of HSCs collected after G-CSF administration and used for allogeneic transplantation (Russell *et al*, 1993; Dreger *et al*, 1993). This was

followed by the first detailed publications confirming the feasibility of this approach (Schmitz *et al*, 1998). The use of G-CSF for HSC mobilisation is now widespread and has been adopted as an international standard. The recommended optimal dose of G-CSF is 10µg/kg/day for five to six days followed by apheresis, which enables the collection of large numbers of CD34+ HSCs, significantly more than is achievable when harvesting bone marrow (Russell *et al*, 1996) . G-CSF is also used in combination with cylcophosphamide for the collection of HSCs used for autologous HSCT. The increase in numbers of CD34+ cells in PBSC grafts is considered to be the driving force behind the significantly faster engraftment of both neutrophils and platelets that is observed when using PBSCs compared to bone marrow, as described by Schmitz and colleagues. G-CSF mobilisation is associated with a significantly higher number of circulating lymphocytes (Hartung *et al*, 1999; Rutella *et al*, 1997; Sica *et al*, 1996) when compared to bone marrow harvests, and yet in several randomised studies the risk of acute GvHD grades II-IV were similar between PBSCs and bone marrow.

1.6.2   Haematological effects of G-CSF

G-CSF mobilisation is associated with a significantly higher number of circulating lymphocytes.(Hartung *et al*, 1999; Rutella *et al*, 1997; Sica *et al*, 1996) Murine and human studies have suggested that G-CSF mobilisation induces alterations in the potential for type 1 cytokine production by T cells, through inhibition of secretion at a single cell level as well as reducing the fraction of cytokine secreting cells in the periphery (Tayebi *et al*, 2001; Arpinati *et al*, 2000; Pan *et al*, 1999). In contrast, it has been reported that the use of DLI obtained following G-CSF mobilisation for the treatment of relapse in advance myeloid malignancy compares favourably with other treatment approaches (Levine *et al*, 2002). This argues for maintenance of a GvL effect from within the lymphocyte population. What remains unclear is whether the anti-viral response in G-CSF mobilised PBMCs is retained.

1.6.3   Mechanisms of stem cell mobilisation

Successful mobilisation of HSCs from the bone marrow into the periphery requires the disruption of adhesion interaction in the bone marrow that keeps HSCs anchored to

bone marrow stromal cells. One of the many adhesion interactions that occurs within the bone marrow is the integrin very late antigen-4 (VLA-4) on the surface of HSCs which binds to its ligand vascular cell adhesion molecule-1 (VCAM-1) expressed by stromal cells. The disruption of this interaction through the use of anti-VLA-4 antibodies prevents adhesion and promotes HSCs mobilisation (Papayannopoulou, 2000). Furthermore, mobilised CD34+ HSCs have lower levels of VLA-4 compared with bone marrow HSCs (Graf *et al*, 2001). Another key interaction that influences mobilisation is that of the stromal derived factor-1 (SDF-1) with its receptor the chemokine receptor 4 (CXCR4). Disruption of this chemotactic interaction in the bone marrow during G-CSF mobilisation is induced through the cleavage of the N-terminus of CXCR4 on HSCs which results in the loss of chemotaxis in response to SDF-1, allowing HSC mobilisation into the periphery (Levesque *et al*, 2003). Just as with VLA-4, mobilised CD34+ HSCs also express reduced levels of CXCR4, which correlates with improved mobilisation (Gazitt & Liu, 2001). The result of G-CSF mobilisation is a generalised peripheralisation of many different types of blood cells, including T cells, monocytes and neutrophils.

1.7   Aims of This Thesis

Infectious complications arising from CMV reactivation following HSCT is a significant cause of morbidity and mortality. A therapeutic option has been the manufacture of CMV-T for adoptive immunotherapy and proof of principle has been demonstrated in a small number of Phase I/II studies that vary in the CD4+ and CD8+ T cell content, and has been widely discussed in this chapter. To date, models for manufacturing CMV-T have focused on the use of PBMC collected after steady-state leukapheresis and from a single blood draw. In the case of related donors, procuring a second leukapheresate for CMV-T generation is inconvenient to the donor and is associated with some level of pain and discomfort. Obtaining a second apheresate from unrelated donors has proven more difficult, either due to donor refusal, registry refusal or simply scheduling difficulties. The aims of this thesis are to determine the feasibility of using an aliquot of the original HSCT obtained by leukapheresis after mobilisation by G-CSF as an alternative PBMC source for CMV-T generation. If this approach proves viable, a further aim is to investigate alternative methods of isolation of CMV-T using

activation-dependent expression of cell surface receptors and to determine their specificity and cytotoxicity after expansion in short term culture. The approach of using G-CSF mobilised PBMC for CMV-T generation will be directly compared to conventional non-mobilised PBMC to ascertain the feasibility of this methodology.

1.7.1   Summary of Aims

1. To examine the kinetics of CMV-induced expression of established activation markers and cytokine secretion in G-CSF mobilised PBMCs in response to CMV peptide stimulation compared with non-mobilised PBMCs.

2. To test the hypothesis that CMV-reactive T cells can be isolated from G-CSF mobilised PBMCs through activation-induced antigen expression and compare to conventional methods using non-mobilised PBMCs.

3. To expand CMV-reactive T cells isolated from G-CSF mobilised PBMCs in short term culture and demonstrate specificity through antigenic re-challenge.

4. To demonstrate effective cytotoxicity of expanded CMV-reactive T cells from G-CSF mobilised PBMCs and compare with conventional non-mobilised expanded CMV-reactive T cells

5. To develop a GMP compliant, clinical scale system for the isolation of functional CMV-reactive T cells from G-CSF mobilised PBMCs for the treatment of CMV disease post allogeneic HSCT.

Chapter 2

General Materials and Methods

2.1 Blood Donors

Fresh blood samples were obtained from leukapheresis collections from both G-CSF mobilised and non-mobilised CMV-seropositive healthy donors, after a 3–5 hour collection on the COBE Spectra apheresis system (CardianBCT). This was performed on the Haematology Day Care Unit at The Royal Free Hospital, London. G-CSF (Filgrastim, Amgen) was administered over 4 days at a dose of 10µg/kg prior to HSC donation. Informed consent was obtained in accordance with the Declaration of Helsinki and the study was approved by the Royal Free NHS Trust Research and Development review board. Blood samples (500µl) were taken directly from the collection bags using needle and syringe by staff in the Laboratory of Cellular Therapeutics (LCT) at The Royal Free Hospital, London, and were aliquoted into 5ml tubes (Sterilin). In some cases, blood sample volumes of up to 10ml, obtained from non-mobilised leukapheresis collections that were due for discard, were dispensed into 50ml tubes (Nunc).

2.2 Cell Culture Medium

All cell cultures were performed in complete medium (CM) which consisted of Roswell Park Memorial Institute medium with L-glutamine (RPMI 1640, Life Technologies) supplemented with 10% human AB serum (Biosera) and 1% antibiotic (Life Technologies). For cultures involving the short term expansion of CMV-reactive T cells, complete medium was supplemented with 10ng/ml of IL-7 and 10ng/ml of IL-15 (Cell Genix).

2.3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Samples were transferred to 50ml tubes and diluted in Hank's Balanced Salt Solution (HBSS, Life Technologies) to a volume of 50ml. PBMCs were isolated by

discontinuous density gradient separation. Diluted blood samples were layered onto an equal volume of Lymphoprep (Axis Shield Diagnostics) in 50ml tubes and centrifuged at 400g for 25 minutes at room temperature with no brake. PBMCs recovered at the interface using a sterile Pasteur pipette were transferred into new 50ml tubes, diluted to 50ml with HBSS and centrifuged at room temperature for 10 minutes at 400g. The supernatant was discarded and the cell pellet gently disrupted using a sterile Pasteur pipette in 1-2ml of HBSS. The cell suspension was then diluted to 50ml in HBSS and centrifuged at 200g for 10 minutes at room temperature. The supernatant was discarded and the cell pellet resuspended in complete medium.

2.4   Cell Counting and Viability Assessment

A 10µl aliquot of PBMCs suspension was mixed with 10µl of 0.4% Trypan Blue solution (Sigma-Aldrich) and loaded onto a haemocytometer counting chamber (Hawksley). Cells were counted using a phase contrast microscope and the number of PBMCs determined, excluding non-viable cells that were blue in appearance. The number of cells in the central 25 squares were counted and the PBMCs concentration $(x10^6/ml)$ calculated by multiplying the total number of cells by 10,000.

2.5   Cryopreservation of PBMCs

For cryopreservation of PBMCs, cells were bought to a maximum concentration of 2 x $10^7/ml$ in complete medium and bought to 4°C by leaving on ice. Freezing medium was made up with 80% of 4.5% human serum albumin (HSA), (Bio Products Laboratory) and 20% dimethylsufoxide (DMSO, Wak Chemie). The cryopreserving solution was mixed drop by drop under mild agitation to an equal volume of cells, up to a maximum total volume of 1.5ml in Cryo-vials (Nunc) and placed in isoproponol freezing containers (Mr Frosty, Nalgene) at –80°C for 24 hours. The vials were then transferred for storage in liquid nitrogen at –196°C.

For recovery of cryopreserved PBMCs, samples were removed from liquid nitrogen and transferred on ice to a 37°C waterbath. Vials were immersed halfway and gently

agitated to facilitate rapid thawing. Once the vial was partially thawed, samples were pipetted into a 20ml tube and pre-warmed complete medium at 37°C was added to the cells in a drop wise fashion, whilst gently swirling the tube. Cells were centrifuged at 200g for 10 minutes at room temperature, resuspended in complete medium and centrifuged for a second time at 200g for 10 minutes. The final cell pellet was gently disrupted using a sterile Pasteur pipette in 1–2ml of complete medium for counting and cell viability assessment.

2.6   *In vitro* **Stimulation of PBMCs**

PBMCs isolated from G-CSF mobilised and non-mobilised donors were adjusted to a concentration of 1×10^7/ml in complete medium and stimulations performed in either 6-well plates (Nunc) with 5–6ml per well or 96-well plates (Nunc) with 150–200µl per well for between 1 and 24 hours at 37°C/5% CO_2 dependent upon the experiment. PBMCs were stimulated with a CMVpp65 peptide pool, consisting mainly of 15-mer sequences with an 11 amino acid overlap spanning the entire pp65 protein of the human CMV AD169 strain (CMVpp65 Peptivator, Miltenyi Biotec). The stock concentration of CMVpp65 Peptivator was 30nmol (approximately 50µg) of each peptide per ml, with 20µl added per ml of cell suspension, giving a final concentration in all cultures of 0.6nmol (approximately 1µg) of each peptide per ml. In time course experiments, PBMCs were also stimulated with the superantigen Staphylococcus Enterotoxin B Fragment (SEB, Sigma-Aldrich) as a positive control. The stock concentration of SEB was at 1mg/ml, with 1µl/ml added to the cell suspension, giving a final concentration of 1µg/ml.

2.7   Flow Cytometry

2.7.1   Protocols for Antibody Cell Surface Staining of PBMC

Cell surface staining of PBMCs was carried out in 12x75mm polystyrene tubes (Sarstedt). A total of 10^5 cells were routinely used in a final volume of 100µl of HBSS. Between 2–5µl of fluorochrome conjugated antibodies were added to PBMCs (titration experiments confirmed adequate cell labelling) and incubated in the dark at room temperature for 10 minutes. Cells were then diluted in 2ml of HBSS and centrifuged at 300g for 5 minutes at room temperature, and the cell pellet was resuspended in approximately 300µl of FACS Flow (Becton Dickenson). Samples were kept at 4°C and acquired within 8 hours of staining on a FACScan (Becton Dickenson/Cytek) using CellQuest Pro software for up to 5 parameters or on the MACSQuant (Miltenyi Biotec) for up to 8 parameters. A minimum of 50,000 CD3+ events were acquired after gating of viable cells using forward scatter (FSC) vs. side scatter (SSC) signals and data analysed using FlowJo v7.6 software (TreeStar). Appropriate controls matched for isotype, fluorochrome and manufacturer, as well as single stained and unstained samples were used to set voltages and compensation.

Monoclonal antibodies used were conjugated to the following fluorochromes:
1. Fluorescein isothiocyanate (FITC)
2. Phycoerythrin (PE)
3. Peridinin Chlorophyll Protein (PerCP)
4. Phycoerythrin Cyanine-7 (PE Cy7)
5. Peridinin Chlorophyll Protein Cyanine-5.5 (Percp Cy 5.5)
6. Allophycocyanin (APC)
7. Allophycocyanin Cyanine-7 (APC Cy7)
8. VioBlue

A summary of all monoclonal antibodies and their conjugates used in this study is shown in Table 2.1.

Monoclonal Antibody	Fluorochrome	Clone	Manufacturer	Catalogue Number
CD3	FITC	SK7	BD	345763
CD3	APC	SK7	BD	345767
CD4	PE-Cy7	SK3	BD	557852
CD4	APC-Cy7	RPA-T4	BD	557871
CD4	PerCP	SK3	BD	345770
CD4	FITC	RPA-T4	BD	555346
CD8	PerCP	SK1	BD	345772
CD8	FITC	SK1	BD	345772
CD25	PE	M-A251	BD	555732
CD25	PE	4E3	Miltenyi Biotec	130-091-024
CD25	APC-Cy7	M-A251	BD	560225
CD25	FITC	M-A251	BD	560990
CD28	PerCP-Cy5.5	L293	BD	337181
CD45RA	FITC	L48	BD	335039
CD45RA	V450	H100	BD	560362
CD57	VioBlue	TB03	Miltenyi Biotec	130-096-530
CD69	PE	FN50	BD	555531
CD69	APC-Cy7	FN50	BD	557756
CD137	PE	4B4-1	BD	555956
CD152	PE	BNI3	BD	555853
CD154	PE	TRAP1	BD	555700
CD154	APC	TRAP1	BD	560995
CCR7	PE-Cy7	3d12	BD	557648
FoxP3	Alexa 488	259D/C7	BD	560047
IL-2	PE	M01-17H12	BD	560902
IL-10	PE	JES3-19F1	BD	559330
IFN-γ	PE	25723.11	BD	340452
IFN-γ	PE	45-15	Miltenyi Biotec	130-091-653
TNF-α	PE	6401.111	BD	340512
Granzyme B	PE	GB11	BD	561142
CD178	APC	NOK-1	Miltenyi Biotec	130-096-458
HLA DR	FITC	L243	BD	555811
Mouse IgG1	PE	G18-145	BD	555787
Mouse IgG1	FITC	X40	BD	345815
Mouse IgG1	APC-Cy7	X40	BD	348812

Table 2.1 Summary of monoclonal antibodies

2.7.2   Basic Gating Strategy for Flow Cytometric Analysis

A FSC versus SSC dot plot was always created for each sample and a gate drawn around the area of live lymphocytes. The live lymphocyte gate was projected onto a second dot plot of CD3 versus SSC and a second gate drawn to identify CD3+ T cells. For the assessment of activation markers (e.g. CD25) on CD3+ subsets, a new dot plot was constructed against CD4 or CD8 as illustrated in Figure 2.1. In some experiments a gate was drawn around the population of cells expressing the activation marker to construct a dot plot of CD45RA versus CCR7 for the assessment of memory phenotype using a quadrant gate.
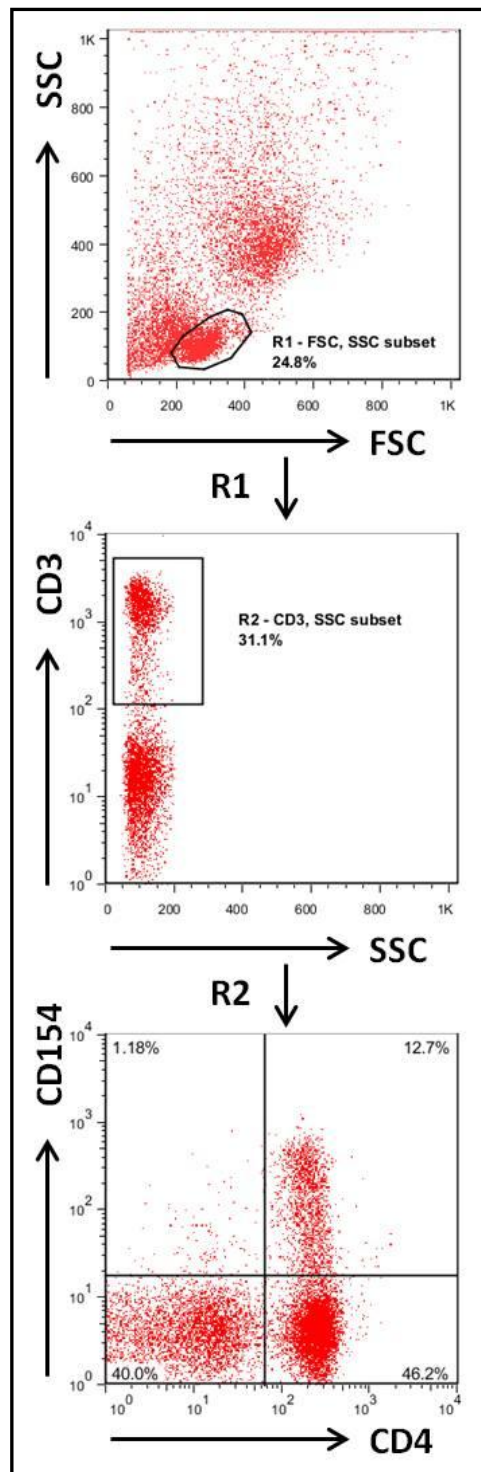
Figure 2.1 Example of gating strategy used to calculate the frequency of antigen specific CD4+ T cells

The frequency of CD154+ CD4+ cells is shown in the upper right quadrant of the bottom dot plot and is derived from the live lymphocyte gate (R1) in the FSC versus SSC dot plot and CD3+ T cells (R2) from the CD3 versus SSC dot plot.

2.8 TH$_1$/TH$_2$ Cytokine Analysis by Cytometric Bead Array (CBA)

The cytometric bead array (CBA, Becton Dickenson) is a flow cytometry based assay that allows the detection of six bead populations with distinct fluorescence intensities that have been coated with capture antibodies specific for IL-2, IL-4, IL-10, TNF and IFN-γ proteins (Figure 2.2). The six bead populations are mixed together to form the CBA that is resolved in the red channel (i.e. FL3 or FL4) of a flow cytometer, after labelling with PE-conjugated detection antibodies and then incubated with test samples or recombinant samples to form sandwich complexes.
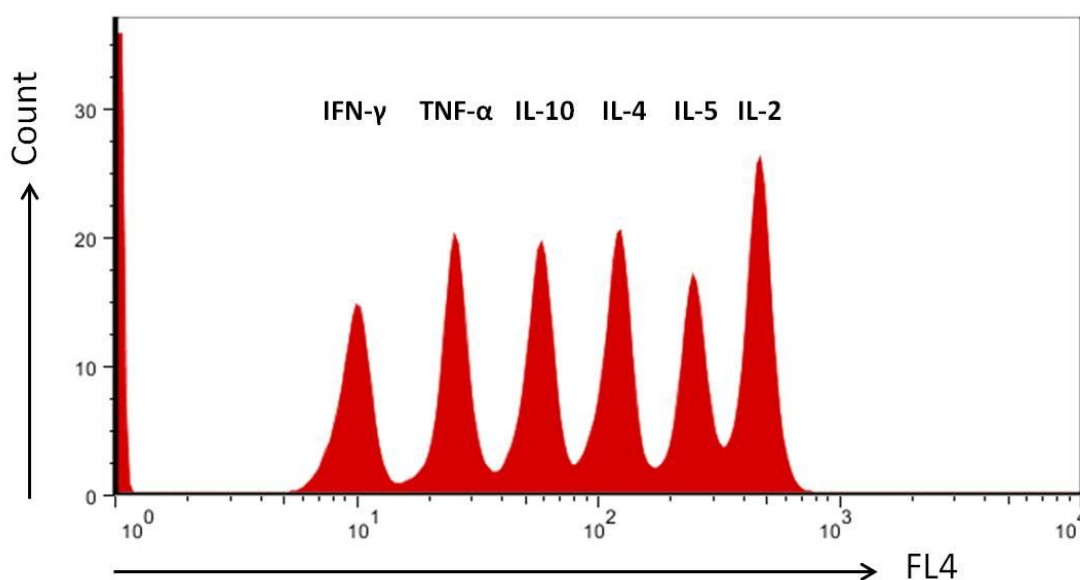
Figure 2.2 Histogram of the cytokine bead populations:

The six cytokine bead populations are resolved in FL4 after gating on the bead population using a FSC versus SSC dot plot.

Supernatants were collected from CMVpp65 stimulated and unstimulated cultures at 16 hours from both G-CSF mobilised and non-mobilised donors and subsequently stored at −80°C. At the time of analysis, the cytokine bead standards were reconstituted with 2ml of assay diluent (top standard) in a 15ml conical tube (Becton Dickenson) and allowed to equilibrate for 15 minutes. Serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:32, 1:128 and 1:256) of the reconstituted bead standard (500 pg/ml) were carried out with assay diluent (Becton Dickinson). A tube with assay diluent served as the negative control. A

10µl aliquot of each of the six capture beads was pooled into a single tube to prepare mixed capture beads. A 50µl aliquot of mixed capture beads was mixed with 50µl of PE detection reagent and either 50µl of thawed supernatant or 50µl of the cytokine standard dilutions. Assay tubes were incubated for 3 hours, washed in 1ml of wash buffer (All Becton Dickinson) and centrifuged at 200g for 5 minutes. The supernatant was removed and the bead pellet resuspended in 300µl of wash buffer. Samples were analysed on a FACS Aria (Becton Dickenson) flow cytometer with 3000 bead events acquired using a FSC versus SSC dot plot and data analysed using FCAP Array version 1.0.1 (SoftFlow, Inc). A standard curve was created for each cytokine using the mean fluorescence intensity (MFI) from each bead standard and the concentration of cytokine (pg/ml) for each test sample calculated (Figure 2.3). Representative FACS plots of cytokine standards used to construct the standard curves are shown in Figure 2.4.
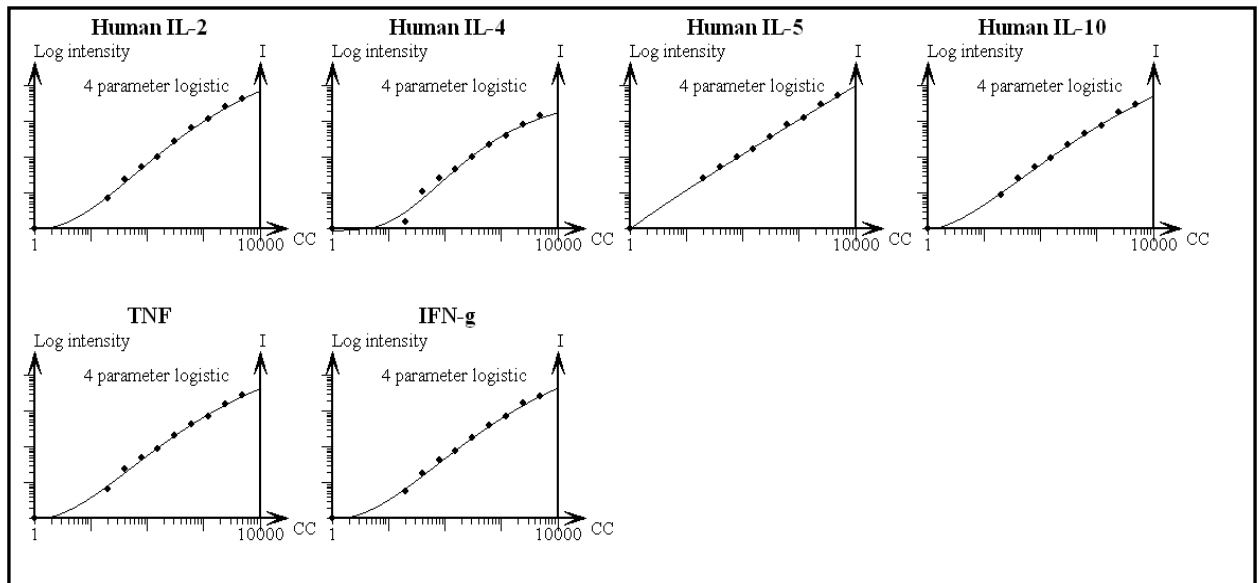
Figure 2.3 Standard Curves constructed to quantify cytokine concentration (pg/ml) in CMVpp65 stimulated G-CSF mobilised and non-mobilised PBMCs
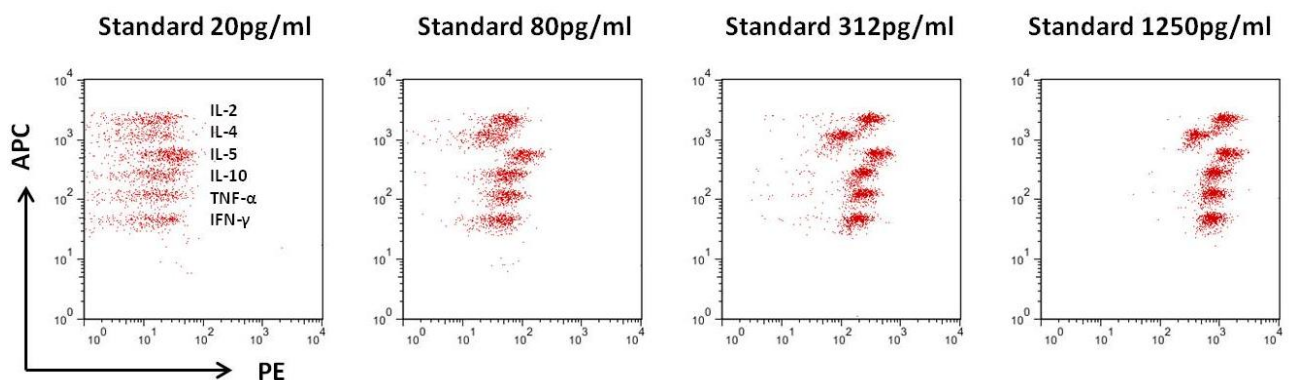
Figure 2.4 Cytometric Bead Array (CBA) Assay Standards

Representative FACS plots of 4 cytokine standards from one experiment used to construct the standard curves.

2.9   Time course Assay

Activation induced expression of cell surface antigen was assessed in a 24 hour time course assay. PBMCs were plated in 96-well flat bottom plates at a concentration of 10 x 10^6/ml (200µl/well) and stimulated with either CMVpp65 peptides, SEB or left untouched. Samples were removed at 1, 4, 6, 16 and 24 hours, centrifuged in 2ml of HBSS at 300g for 5 minutes, the supernatant removed and cell pellets resuspended in 600µl of HBSS. Cells were stained with CD3-APC, CD4-FITC, CD8-PerCP and either CD25-PE, CD69-PE, CD137-PE, CD152-PE, CD154-PE or Mouse IgG-PE (all BD Biosciences) and analysed on the BD/Cytek FACScan flow cytometer.

In some experiments PBMCs were stimulated for 4 and 6 hours in the presence and absence of 1µg/ml of purified anti-CD40 antibody (Clone G28.5; BioLegend) and samples stained as described but with only CD154-PE or Mouse IgG1-PE antibodies. As a control, PBMCs were left untouched and incubated with anti-CD40 alone.

2.10   Magnetic Bead Cell Sorting

Sterile cell sorting was performed using MACS technology (Miltenyi Biotec) that is based on three components. The first component is microbeads which are 50nm superparamagnetic particles conjugated with monoclonal mouse anti-PE antibodies (anti-PE microbeads) or conjugated to a monoclonal antibody against a particular antigen on the cell surface. The second component are columns, which contain a matrix composed of ferromagnetic spheres. The third component, cell separators, which are strong permanent magnets, cause the spheres in the columns to amplify the magnetic field by 10,000-fold and allow for labelled cells to be held in suspension on the column and unlabelled cells to flow through the column.

2.10.1 Enrichment using Anti-PE Microbeads

For positive selection, cells were stained in 15ml conical tubes with 10µl of PE-conjugated monoclonal antibody/10^7 total cells after resuspending the cell pellet at a

concentration of 10^7 cells/100µl of CliniMACS PBS/EDTA buffer (Miltenyi Biotec) and incubating for 10 minutes at 4–8°C in the dark. Cells were diluted to 15ml with CliniMACS buffer and centrifuged at 300g for 10 minutes at room temparature. The supernatant was removed and the cell pellet resuspended at 10^7 cells/80µl in CliniMACS buffer, labelled with 20µl anti-PE Microbeads/10^7 cells and incubated for 15 minutes at 4–8°C in the dark. Cells were diluted to 15ml with CliniMACS buffer, centrifuged at 300g for 10 minutes at room temparature, the supernatant removed and the cell pellet resuspended in 500µl of CliniMACS buffer. MS columns were used for positive selection, which have the capacity for enrichment of 1 x 10^7 labelled cells from up to 2 x 10^8 total cells. The column was placed in the magnetic field of a MiniMACS separator and the column rinsed with 500µl of CliniMACS buffer. The cell suspension was then applied to the column and the unlabelled fraction collected in sterile polystyrene 12x75mm tubes (Becton Dickenson). The column was washed three times with 500µl of CliniMACS buffer. Once the column reservoir was empty the column was removed from the MiniMACS separator and the column flushed with 1ml of CliniMACS buffer and the positive fraction collected in sterile polystyrene 12x75mm tubes.

2.10.2 Separation using CD25-Microbeads

CD25-Microbeads (Miltenyi Biotec) were used for both the isolation and depletion of CD25+ cells dependent upon the experiment being performed. For depletion of CD25 expressing cells, cell pellets were resuspended in 10^7 cells/80µl of CliniMACS buffer, labelled with CD25-Microbeads at 10^7 cells/20µl and incubated at 4–8°C in the dark for 15 minutes. Cells were diluted in 15ml of CliniMACS buffer, centrifuged at 300g for 10 minutes at room temperature, the supernatant removed and the cell pellet resuspended in 500µl of CliniMACS buffer. LD columns were used for depletion, which have the capacity for depletion of up to 1 x 10^8 labelled cells from up to 5 x 10^8 total cells. LD columns were placed in the magnetic field of a VarioMACS separator and the column rinsed with 2ml of CliniMACS buffer. The cell suspension was applied to the column and the unlabelled cells collected into 15ml round bottom tubes. The column was washed twice with 1ml of CliniMACS buffer. In some experiments, the CD25-positive

fraction was retrieved by removing the column from the separator and flushing with 1ml of CliniMACS buffer before collection into 15ml round bottom tubes.

For positive selection of CD25 expressing cells, cell pellets were resuspended in 10^7 cells/90μl of CliniMACS buffer, labelled with CD25-microbeads at 10^7 cells/10μl and incubated at 4–8°C in the dark for 15 minutes. Cells were diluted in 15ml of CliniMACS buffer, centrifuged at 300g for 10 minutes at room temperature, the supernatant removed and the cell pellet resuspended in 500μl of CliniMACS buffer. MS columns were used for positive selection on the MiniMACS separator as previously described.

2.11   IFN-γ Secretion Assay

In principle the IFN-γ secretion assay (Miltenyi Biotec) allows for the detection and isolation of antigen-specific T cells after short term stimulation. PBMCs are labelled with a catch reagent, a bi-specific antibody that binds to CD45 on all leukocytes and is specific for IFN-γ. After a short incubation period, secreted IFN-γ binds to the catch reagent and can be detected with a second IFN-γ specific antibody that is conjugated to PE, allowing detection by flow cytometry (Figure 2.3). IFN-γ secreting cells can then be labelled with using Anti-PE Microbeads and enriched using MS columns placed on a MiniMACS separator.

Up to 50 x 10^6 PBMCs, at a concentration of 10 x 10^6/ml, were stimulated with CMVpp65 peptides or left untouched as a negative control and were incubated in 6-well plates for 16 hours at 37°C/5% CO_2. PBMCs were washed in cold CliniMACS buffer and centrifuged at 300g for 10 minutes at 4–8°C. The supernatant was removed and the cell pellet resuspended in 10^7 cells/80μl of cold complete medium and then incubated with 10^7 cells/20μl of IFN-γ catch reagent on ice for 5 minutes. Cells were diluted to a concentration of 1 x 10^6/ml with culture medium pre-warmed to 37°C and incubated for 45 minutes at 37°C/5% CO_2 under slow rotation using a MACSMix (Miltenyi Biotec) to allow IFN-γ secretion. Cells were then washed with cold CliniMACS buffer and centrifuged at 300g for 10 minutes at 4–8°C, the supernatant removed and the cell pellet resuspended in 10^7 cells/80μl of cold CliniMACS buffer and then incubated with 10^7 cells/20μl of IFN-γ PE Detection Antibody on ice for 10 minutes. Cells were washed

once more with cold CliniMACS buffer and centrifuged at 300g for 10 minutes at 4–8°C, the supernatant removed and the cell pellet resuspended in 10^7 cells/80μl of cold CliniMACS buffer and then incubated with 10^7 cells/20μl of Anti-PE Microbeads at 4–8°C for 15 minutes. Cells were then washed in cold CliniMACS buffer and centrifuged at 300g for 10 minutes at 4–8°C, the supernatant removed and the cell pellet resuspended in 500μl cold CliniMACS buffer. MS columns were used for positive selection on the MiniMACS separator as previously described.

IFN-γ Catch Matrix Reagent

Following CMVpp65 stimulation PBMCs are labelled with the catch reagent, a bi-specific antibody that binds to CD45 and is specific for IFN-γ

Cytokine Secretion and capture

Antigen-specific T cells secrete cytokine during a 45 minute incubation, where IFN-γ is captured on the cell surface

IFN-γ Enrichment Reagent

Cells are labelled with a second antibody specific for IFN-γ and conjugated to PE to allow detection. Labelling with anti-PE microbeads allows for positive selection of IFN-γ secreting cells

Figure 2.5 The principle of the IFN-γ Secretion Assay (reproduced from Miltenyi Biotec)

2.12   Intracellular Antigen Staining

The detection of intracellular antigens, such as cytokines, was performed using fixation and permeabilisation reagents (Intrastain, Dako Cytomation) that leaves the cellular structure, morphology scatter and cell surface immunoreactivity intact. Brefeldin A (Sigma-Aldrich) was added to PBMC cultures at a final concentration of 5µg/ml after 2 hours to inhibit protein transport from the Golgi apparatus to the endoplasmic reticulum, leading to the accumulation of protein that can be detected by flow cytometry.

PBMCs were adjusted to a concentration of 1×10^6/ml and 100µl of the cell suspension was stained with 3-5µl of CD3-APC, CD4 or CD8-PerCP and CD69 APC-Cy7 antibodies (all BD Biosciences) and incubated in the dark for 15 minutes. Samples were fixed with 100µl of Intrastain Reagent A and incubated at room temperature for 15 minutes in the dark, then washed with 2ml of HBSS at 300g for 5 minutes at room temperature and the supernatant removed. The cell pellet was resuspended in the residual volume, 100µl of Intrastain Reagent B added for cell permeabilisation, and 5µl of IL-2 PE, IL-10 PE, TNF-α-PE, Granzyme B-PE (all BD Biosciences) or IFN-γ-PE (Miltenyi Biotec) added for intracellular staining. Cells were incubated for 15 minutes in the dark and washed as previously described. Cell pellets were resuspended in FACSFlow and samples acquired on the FACScan flow cytometer with a minimum of 50,000 CD3+ events recorded.

2.13   Identification of T regulatory cells by FoxP3 Staining

PBMCs were diluted to a concentration of 10×10^6/ml in HBSS for the intracellular staining of the transcription factor FoxP3 using a fixation and permeabilisation assay (BD Biosciences). Buffers were prepared according to manufacturer instructions, but in brief, FoxP3 Buffer A (10X concentrate) was diluted 1:10 with room temperature deionised water and a working solution of Buffer C was prepared by diluting FoxP3 Buffer B into 1X Buffer A at a ratio of 1:50. Working buffers were stored at room temperature in the dark. In polystyrene tubes, 100µl of PBMCs (1×10^6) were stained with 3µl of CD3-APC and 5µl of CD25-PE, CD4-APC Cy7 and CD8-PerCP (all BD

Biosciences) and incubated at room temperature in the dark for 20 minutes. Cells were washed in 2ml HBSS at 250g for 10 minutes at room temperature, the supernatant removed and the cell pellet resuspended in 2ml of the fixation buffer, FoxP3 Buffer A, and incubated at room temperature in the dark for 10 minutes. The cells were centrifuged at 500g for 5 minutes and the supernatant removed by pipette and washed again in 2ml of HBSS at 500g for 5 minutes at room temperature. The supernatant was then removed and the cell pellet resuspended in 0.5ml of permeabilisation buffer (FoxP3 Buffer C) and incubated for 30 minutes at room temperature in the dark. Cells were washed twice as previously described and 5µl of FoxP3-Alexa Fluor 488 (BD Biosciences) antibody added, the cell pellet resuspended and incubated for 30 minutes at room temperature in the dark. In control samples, 5µl of Mouse IgG1-FITC antibody was added. Post incubation, two wash steps were performed as previously described and the final cell pellet resuspended in 300µl of FACSFlow. Samples were acquired on the FACScan flow cytometer with a minimum of 50,000 CD4+ events recorded.

2.14   CFSE Based Suppression Assay

2.14.1 CFSE labelling

The stable incorporation of the intracellular dye carboxyfluorecein diacetate succinimidyl ester (CFSE) into lymphocytes allows the *in vitro* monitoring of cell division, using flow cytometry, through the sequential halving of fluorescence as the dye is inherited by daughter cells after cell division (Figure 2.4). One 50µg vial of CFSE (CellTrace CFSE, Invitrogen) stored at −20°C was reconstituted in 18µl of DMSO (Invitrogen) to give a 5mM stock solution as per the manufacturer's instructions. PBMCs for staining were adjusted to a concentration of 1 x 10^6/ml in RPMI (Invitrogen) without serum, up to a maximum volume of 10ml in 20ml plastic tubes. PBMCs were labelled with 2µl of 5mM stock CFSE for a final working concentration of 1µM and incubated at 37°C for 10 minutes. Following incubation, 1ml of Human AB Serum was added, the cells incubated at 4–8°C for 5 minutes and then topped up with complete medium to 20ml and centrifuged at 300g for 10 minutes at room temperature. The supernatant was removed and the cell pellet resuspended in

20ml of complete medium, and centrifuged at 300g for 10 minutes at room temperature. The supernatant was removed and the cell pellet resuspended in 1ml of complete medium. An aliquot of the cell suspension was analysed using a FACScan flow cytometer for assessment of successful CFSE labelling.

PBMCs are labelled with CFSE and subsequently stimulated with a T cell mitogen such as phytohaemagglutinin (PHA) or with a specific antigen. Cell division results in the halving of fluorescence in daughter cells which is detected by a decrease in signal observed in FL1 by flow cytometry (Figure 2.6).
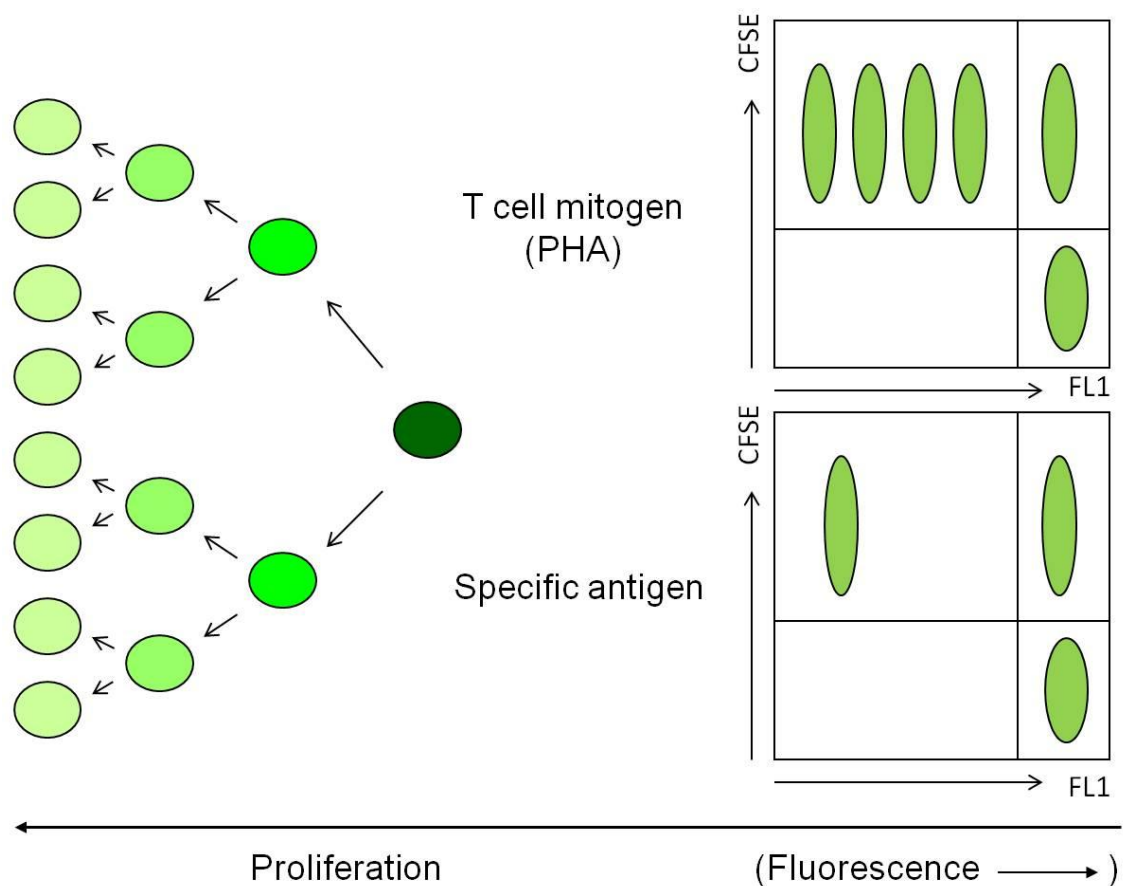
Figure 2.6 Principle of CFSE labelling to monitor cell proliferation

2.14.2 Suppression Assay

The CFSE based suppression assay was used to compare the suppressive capacity of CMV-specific T cells (CMV-T), isolated through the activation marker CD25 after a 16-hour stimulation with CMVpp65 peptides, and compared directly with conventional CD4+ CD25+ T regulatory cells. CFSE labelled responder PBMCs were cultured in round bottom 96-well plates (Nunc) at 2 x 10^4 cells/well with 1 x 10^5 irradiated autologous PBMCs as feeder cells. Depending on the number of effector cells available, CD4+ CD25+ and CD25+ CMV-T were added to the cultures at a ratio of 1:1 (2 x 10^4 cells/well) and 1:2.5 (5 x 10^4 cells/well) of responders to effectors. Cell cultures were stimulated with 2µg/ml of purified anti-CD3 antibody (Clone HIT3a, BioLegend) or 1µg/ml of SEB (Sigma-Aldrich). In control experiments, CFSE labelled and unlabelled PBMCs were cultured alone. Cultures were incubated for 5 days at 37°C/5% CO_2 before harvesting, and cells stained with CD3-APC and CD69-APC Cy7 antibodies (both BD Biosciences). Samples were acquired on the FACScan flow cytometer and a minimum of 5,000 CD3+ CFSE+ events recorded. The frequency of suppression was analysed by gating on the CFSE^{low} population determined by the CFSE labelled and unlabelled PBMC control samples, as shown in Figure 2.5. Suppression was defined as: [100 - (%CFSE^{low} of CD3+ in presence of effectors/%CFSE^{low} of CD3+ in absence of effectors) x 100]
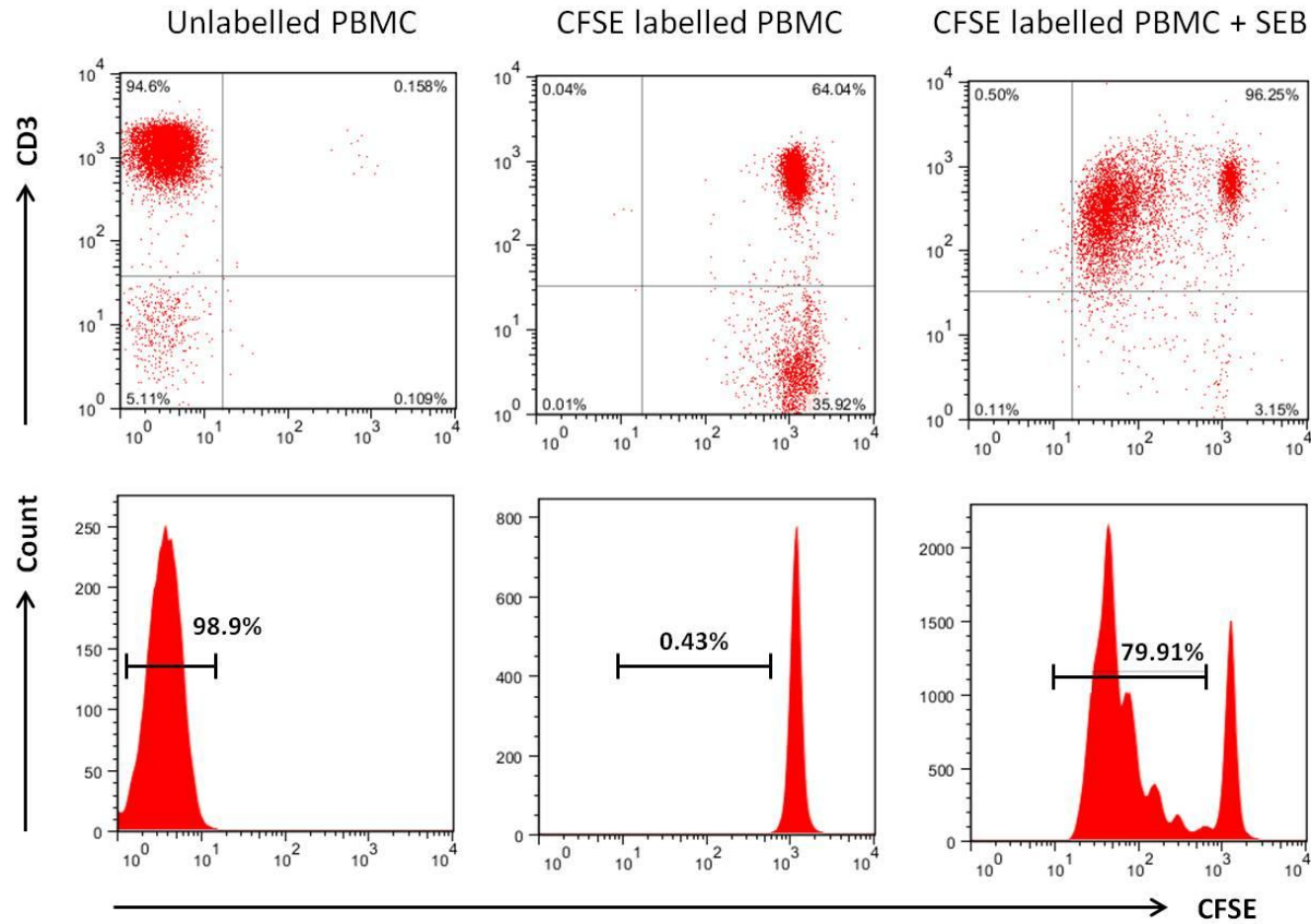
Figure 2.7 CD3+ T cell proliferation using CFSE labelling:

Strategy for gating of CFSE^{low} population to determine proliferation

2.15 γ-irradiation of Feeder Cells

All feeder cells were exposed to a γ-irradiation dose of approximately 50Gy by irradiation for 10 minutes in a Gammacel 3000 ELAN irradiator (Nordion International Inc) which emits approximately 500 rads per minute (5Gy per minute). Cells were irradiated at a concentration of 1–3 x 10^6/ml in 20ml of complete medium in plastic tubes.

2.16 CMV-specific T cell lines

2.16.1 Expansion of CMV-specific T cell lines

CMV-specific T cells isolated by magnetic bead enrichment through activation-dependent expression of both CD25 and CD154 were expanded in short term culture and re-challenged with CMV peptides as outlined in Figure 2.6. Isolated cells were adjusted to a concentration of 0.25 x 10^6/ml in complete medium, and 1ml of the cell suspension was cultured in the presence of 1ml of γ-irradiated autologous PBMCs at a concentration of 12.5 x 10^6/ml (50:1) to act as feeder cells. Expansions were performed in 24-well plates (Nunc) at a final concentration of 6.37 x 10^6/ml. Cultures were supplemented with IL-7 and IL-15 (Cell Genix) to final concentrations of 10ng/ml. Cultures were replenished with IL-7 and IL-15 every 2–3 days and split when cell counts were greater than 3 x 10^6/ml. Every 5–7 days, cultures were phenotyped for CD3-APC, CD4-FITC, CD8-PerCP, CD69 APC-Cy7 and either CD25-PE or CD154-PE (all BD Biosciences) antibodies and samples acquired on the FACScan flow cytometer with a minimum of 30,000 CD3+ events recorded. Cells were expanded up to a maximum of 23 days before harvesting.

2.16.2 Re-stimulation of Expanded CMV-specific T cell lines

Cryopreserved autologous PBMCs were thawed and washed as described in section 2.5 before labelling with CFSE as outlined in section 2.11.1. Cells were resuspended at 3 x 10^6/ml and incubated at 37°C/5% CO_2 in 24-well plates (2ml/well) for 16 hours in the

presence and absence of CMVpp65 peptides and CMV IE-1 peptides (PepMIX HCMV IE1, JPT) at 1µg of each peptide/ml. Following peptide loading, cells were washed in complete medium at 300g for 10 minutes at room temperature and resuspended at 10 x 10^6/ml. Expanded cells were harvested, centrifuged at 300g for 10 minutes at room temperature and resuspended at 10 x 10^6/ml, and incubated with CFSE PBMCs with and without peptides at a ratio of 5:1 (loaded cells: expanded cells) in 48- well plates for 5-6 hours. In some experiments where CFSE PBMCs numbers were low, the ratio was reduced to 2.5:1. For analysis of intracellular cytokines and antigens, cells were incubated in the presence of anti-CD28 antibody (BD Bioscience) for 2 hours, followed by the addition of 1µg/ml of Brefeldin A (Sigma-Aldrich). Cells were stained for intracellular antigens as described in section 2.9 and analysed by flow cytometry. The schematic in Figure 2.8 illustrates the method of expansion and re-challenge of CMV-T. Expanded cells were also stained for CD45RA-Vioblue, CCR7-PE Cy7, CD69 APC Cy7 and either CD154 or CD25 PE (all BD Biosciences) and CD57-Vioblue (Miltenyi Biotec) following re-challenge.
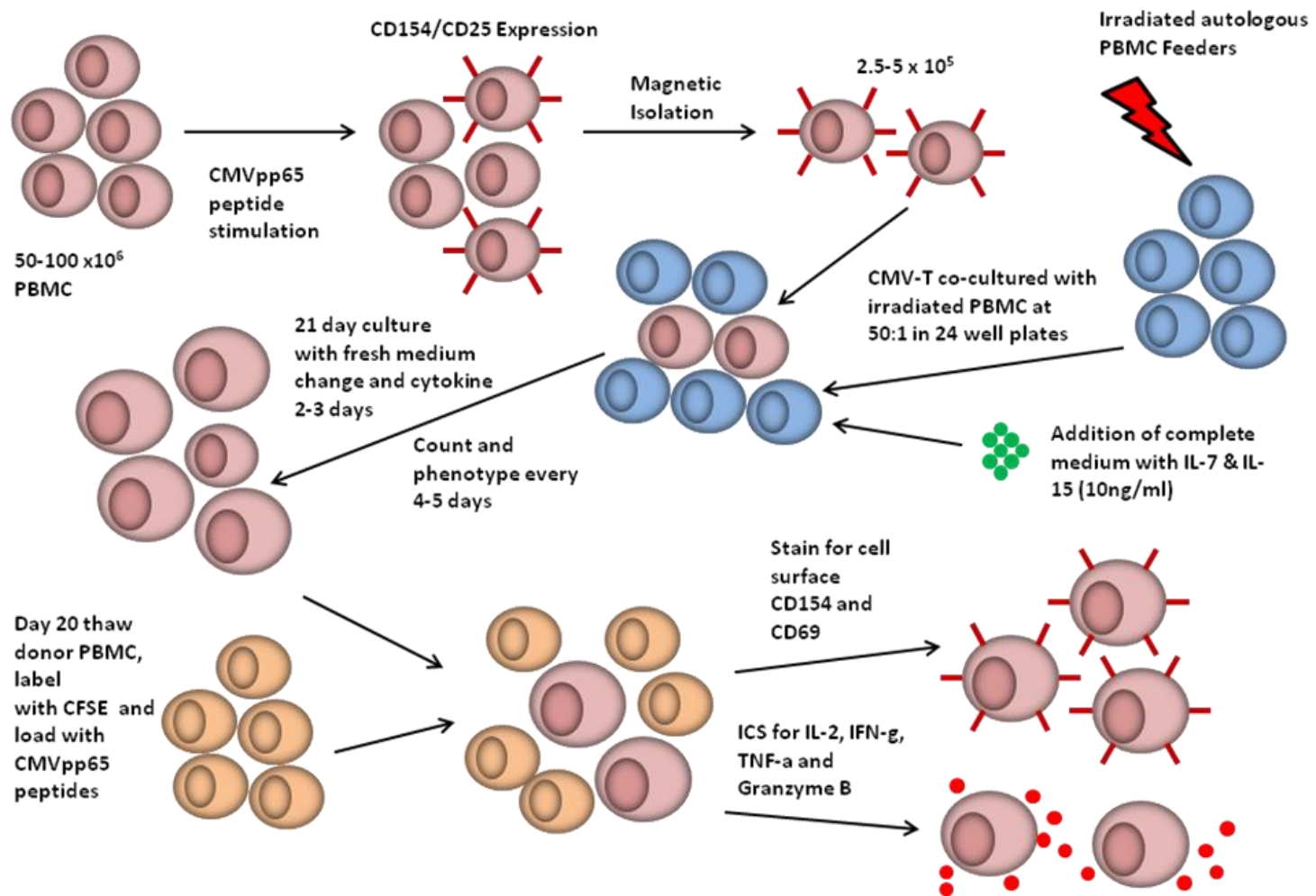
Figure 2.8 Schematic representation of the expansion and re-challenge of expanded CMV-T isolated through CD25 or CD154 activation-dependent expression. ICS indicates intracellular cytokine staining

2.17   Calcein-AM Cytotoxicity Assay

Target cells were labelled with a cell-permeant dye calcein-acetoxymethyl (Calcein-AM, Invitrogen), that is converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis in live cells by intracellular esterases. Release of calcein into the supernatants from cytoxicity assays is measured by fluorimetry.

2.17.1   Calcein-AM labelling of PHA Blasts

Autologous PBMCs were thawed as described in section 2.5 and resuspended at a concentration of 1×10^6/ml in complete medium. Cells were incubated at 37°C/5%CO$_2$ in 24-well plates (2ml/well) in the presence of 3µg/ml of PHA (Sigma) for 24 hours. After 24 hours, 20U/ml of IL-2 (Miltenyi Biotec) was added to each well and cultures incubated for a further 72 hours. PHA blasts were loaded with CMVpp65 peptides for 16 hours or left untouched. Cells were harvested and resuspended at 1×10^6/ml in phenol-red free RPMI (Invitrogen) supplemented with 10% Human AB serum and a maximum of 1×10^6 cells labelled with Calcein-AM to a final concentration of 10µM and incubated for 1 hour at 37°C in a water bath. Cells were centrifuged at 400g for 5 minutes at room temperature, the supernatant removed and the cell pellet resuspended in 2ml of phenol-red free RPMI supplemented with 10% Human AB serum. The wash procedure was repeated 5 times and cells resuspended in phenol-red free RPMI supplemented with 10% Human AB serum at a final concentration of 7×10^4/ml.

2.17.2   Cytotoxicity Assay

Expanded CMV-T cells isolated through both CD25 and CD154 were used as effector cells. After harvesting, cells were washed in 2ml of phenol-red free RPMI supplemented with 10% Human AB serum at 400g for 5 minutes at room temperature and resuspended in medium at a concentration of 1.4×10^6/ml. The Calcein-AM killing assay was set up in triplicate with effector:target ratios of 20:1, 10:1, 5:1, 2.5:1 and 0.5:1 in round bottom 96-well plates (Corning). A minimum of 7×10^3 Calcein-AM-labelled target cells were added to all effector cells, plates centrifuged at 400g for 5 minutes at room temperature and incubated for 4–5 hours at 37°C/5% CO$_2$. In some

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experiments, Calcein-AM-labelled targets loaded with CMVpp65 peptides were incubated with either purified anti-HLA ABC (eBioscience) or purified anti-HLA DP DQ DR antibodies (AbD Serotec). Briefly, 0.5 x 10^6 cells in 100µl of medium were incubated with 10µg/ml of anti-HLA ABC or anti-HLA DP DQ DR for 15 minutes at room temperature in the dark, centrifuged in 2ml of medium, the supernatant removed and cell pellets resuspended at a final concentration of 7 x 10^4/ml. Control plates were also prepared with medium alone, medium plus 2% Triton-X100 (Fisher Scientific), target cells alone (spontaneous release) and target cells plus 2% Triton-X100 (maximal release) incubated under the same conditions as previously described. Following incubation, plates were centrifuged at 400g for 5 minutes at room temperature and 100µl of supernatant removed from each well and transferred into flat bottom 96-well plates (Nunc). Supernatant fluorescence was measured on the FLUOstar Galaxy microplate fluorescence spectrophotometer (MTX Lab Systems) with excitation filter 485 ± 9nm and band-pass filter 530 ± 9nm and data were expressed as arbitrary fluorescent units (AFU). Background fluorescence measured for medium alone was subtracted from all measured effector:target values and from spontaneous release values, and background fluorescence measured for medium plus 2% Triton-X100 was subtracted from maximal release values. Specific target killing was calculated as the [(test release-spontaneous release/maximal release-spontaneous release) x 100] for each ratio.

2.18 Statistics

Statistical analyses were conducted using GraphPad Prism v4.0 (GraphPad Software). The paired and unpaired two-tailed Student's t-test was used for pairwise tests of differences between datasets which have a normal distribution and equal variances. The Mann-Whitney test was used for non-parametric data sets. Differences between data sets were tested for statistical significance at $\alpha = 0.05$.

Chapter 3

Analysis of Activation Markers and Cytokine Profile in CMVpp65-Stimulated G-CSF-Mobilised PBMCs

3.1   Introduction

Several strategies have been employed in the manufacture of CMV-reactive T cells (CMV-T) for clinical use in the treatment of CMV disease in patients at high risk of reactivation, including the use of HLA peptide tetramers, the generation of T cell lines in short term culture and IFN-γ catch technology. To date, models for generating CMV-T have focused primarily on using peripheral blood mononuclear cells (PBMCs) collected by an additional leukapheresis from the original HSCT donor. In the case of related donors, procuring a second apheresate for CMV-T generation is inconvenient to the donor and is associated with some level of pain, discomfort and risk. Obtaining a second apheresate from unrelated donors has proven to be even more problematic, due to donor refusal, registry refusal or simply scheduling difficulties. The prospect of manufacturing antigen-specific T cells from an aliquot of the original HSCT obtained by leukapheresis after mobilisation with recombinant human G-CSF is attractive but has yet to be fully investigated. The successful isolation from G-CSF-mobilised donors of γδ T cells that synthesise immunomodulatory cytokines, such as IFN-γ and TNF-α, and retain a strong tumoricidal activity (Otto *et al*, 2005), effectively demonstrated the potential for the identification and isolation of anti-viral T cells from the original apheresate starting material. G-CSF mobilisation is associated with a significant increase in the numbers of circulating lymphocytes (Hartung *et al*, 1999; Rutella *et al*, 1997; Sica *et al*, 1996). It has been reported that the use of donor lymphocyte collections (DLI) obtained following G-CSF mobilisation for the treatment of relapse in advanced myeloid malignancy compares favourably with other treatment approaches (Levine *et al*, 2002), suggesting maintenance of a graft versus leukaemia (GvL) effect mediated by functional T cells within the lymphocyte population. However, murine and human studies have suggested that G-CSF mobilisation inhibits type 1 cytokine production by T cells, through inhibition of secretion at a single cell level as well as

reducing the fraction of cytokine secreting cells in the periphery, arguing against the use of these cells for adoptive immunotherapy (Tayebi *et al*, 2001; Arpinati *et al*, 2000; Pan *et al*, 1999). What remains unclear is the degree of functionality of the T cell compartment of G-CSF-mobilised PBMCs, in terms of their anti-viral response; and how these CMV-T compare against conventional non-mobilised T cells that are traditionally used for adoptive transfer of CMV immunity post allogeneic HSCT. The objective of the experiments described in this Chapter was to obtain data that enable these questions to be addressed.

3.2   Aims of Experiments Described in this Chapter

The initial aims of this Chapter were to test whether CMV-T could be identified in G-CSF-mobilised PBMCs though IFN-γ secretion, and whether CMV-T could be isolated by magnetic bead enrichment using the IFN-γ catch assay. We began by investigating the cytokine profile of CMVpp65- stimulated G-CSF-mobilised PBMCs using a Cytometric Bead Array (CBA) to establish whether there was polarization of the T cell response towards a TH_2 phenotype, through the inhibition of TH_1 cytokines. Cytokine secretion in G-CSF-mobilised PBMCs was compared directly with non-mobilised PBMCs to ascertain the feasibility of using the IFN-γ secretion assay in future experiments.

CMV-T have also been identified through a number of T cell surface markers that are up-regulated after activation, including CD25, CD69, CD137 and CD154 (Chattopadhyay *et al*, 2005; Frentsch *et al*, 2005; Gallot *et al*, 2001; Watanabe *et al*, 2008; Wehler *et al*, 2008; Wolfl *et al*, 2007). We therefore investigated the feasibility of identifying CMV-T based on activation markers in G-CSF-mobilised PBMCs as an alternative option to identification based on IFN-γ secretion.

Samples were taken from both non-mobilised and G-CSF-mobilised PBMCs apheresis collections and stimulated with CMVpp65 Peptivator for investigating both IFN-γ secretion and activation-induced expression of surface markers. CMVpp65 Peptivator is a peptide pool consisting mainly of 15-mer sequences with 11 amino acid overlap

spanning the entire pp65 protein. Experiments focused on the T cell response to CMVpp65, as it is generally accepted that the cytotoxic T lymphocyte (CTL) response is dominated by this protein (Harcourt *et al*, 2006;Wills *et al*, 1996).

The experimental aims of the Chapter were as follows:

1. To compare the profile of cytokines secreted in response to CMVpp65 stimulation between G-CSF-mobilised and non-mobilised PBMCs.

2. To compare the efficacy of IFN-γ-based identification and enrichment of CMV-reactive T cells in G-CSF-mobilised and non-mobilised PBMCs.

3. To examine the temporal kinetics of activation-induced antigen expression in G-CSF-mobilised PBMCs after CMVpp65 peptide stimulation over 24 hours.

4. To compare the temporal kinetics of activation-induced antigen expression with conventional non-mobilised PBMCs.

3.3   Materials and Methods

Fresh samples were obtained from the leukapheresis collections from both G-CSF-mobilised and non-mobilised consenting CMV-seropositive healthy donors, and PBMCs were isolated by density gradient centrifugation. Cultures from both CMVpp65-stimulated (1μg/peptide/ml) and non-stimulated PBMCs from G-CSF-mobilised and non-mobilised donors (n=6) were incubated at a concentration of 10^7/ml in 6-well plates, with 50 x 10^6 cells added to each well. Supernatants were subsequently collected and stored at −80°C. The assessment of cytokine secretion was performed using the stored supernatants which were thawed at room temperature before incubation of 50μl for 3 hours with 50μl of mixed capture beads capable of identifying the cytokines IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α, together with 50μl of PE-conjugated detection antibody. Following incubation, samples were centrifuged at 200g for 10 minutes at room temperature and resuspended in 300μl of FACS Flow for flow cytometric analysis. Known quantities of cytokine were used as standards for the construction of a standard curve that was used to quantify concentration (pg/ml) of each cytokine in the supernatant samples. Samples were analysed using FCAP Array v1.0.1

For the identification and enrichment of CMV-reactive T cells in G-CSF-mobilised and non-mobilised PBMCs, an IFN-γ-secretion assay was performed. PBMCs were stimulated with CMVpp65 peptides in a 16 hour culture, and labelled with an IFN-γ catch reagent before an IFN-γ secretion step of 45 minutes at 37°C/5%CO_2, as described in section 2.11. Cells were then labelled with an IFN-γ PE detection antibody prior to labelling with anti-PE microbeads before enrichment on a MiniMACS using MS columns. Counts were performed pre- and post-enrichment and analysis of IFN-γ+ CD4+ and IFN-γ+ CD8+ subsets performed by flow cytometry to assess both yield and purity of CMV-reactive T cells between G-CSF-mobilised and non-mobilised PBMCs.

To analyse activation marker expression, PBMCs were isolated from G-CSF-mobilised and non-mobilised apheresis, prior to the incubation of 2 x 10^6 cells per well in 96-well flat bottom plates at a concentration of 10^7/ml in complete medium. Cells were stimulated with either 1μg/ml SEB (positive control) or 1μg/peptide/ml CMVpp65

peptide pool or left untouched to act as a negative control. Cultures were then incubated at 37°C/5% CO_2 and samples taken at 1, 4, 6, 16 and 24 hour time points for the assessment of cell surface expression of CD25, CD69, CD137, CD152 and CD154 amongst CD3+ T cells. After the culture periods, cells were incubated with the following panels of monoclonal antibodies and analysed by flow cytometry (Table 3.1) using the staining procedure described in Chapter 2, section 2.7.1.

Panel/Fluorochrome	FITC	PE	PerCP	APC
1	CD4	IgG1	CD8	CD3
2	CD4	CD25	CD8	CD3
3	CD4	CD69	CD8	CD3
4	CD4	CD137	CD8	CD3
5	CD4	CD152	CD8	CD3
6	CD4	CD154	CD8	CD3

Table 3.1 Antibody panels used to assess temporal dynamics of activator marker expression in CMVpp65 stimulated PBMC isolated from G-CSF mobilised and non-mobilised PBMC

3.4 Results

3.4.1 Cytokine profile of CMVpp65 stimulated G-CSF-mobilised and non-mobilised PBMCs

Supernatants from G-CSF mobilised and non-mobilised PBMC cultures with and without CMVpp65 peptide stimulation were assessed for the secretion of the cytokines IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ after 16 hours. Following acquisition of a minimum of 3,000 events on a FACS Aria flow cytometer, a region was drawn on a FSC vs. SSC dot plot around the bead population. The bead populations coated with capture antibodies specific for the cytokines are resolved in the FL3 channel based on their distinct fluorescence intensity and then reported in the FL2 channel using a PE-conjugated detection antibody. Figure 3.1 illustrates a representative result from non-stimulated (negative control) and CMVpp65 stimulated G-CSF mobilised PBMC sample.
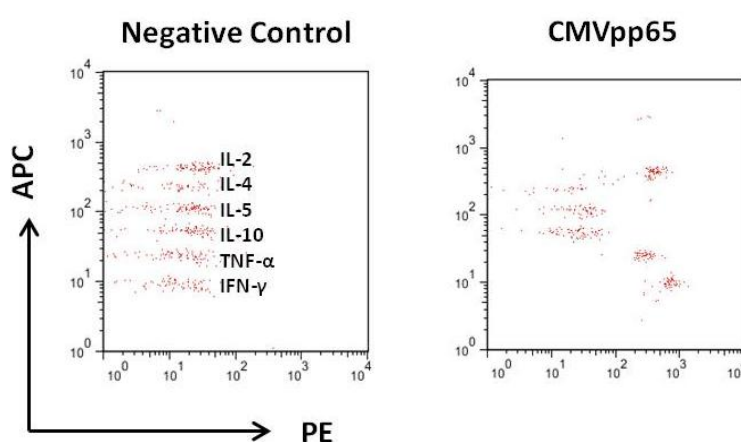
Figure 3.1 Cytometric bead array (CBA) Assay – G-CSF Mobilised PBMC:
Representative FACS plots from one experiment using supernatant from G-CSF mobilised PBMC stimulated with CMVpp65 or left untouched (negative control).

When comparing CMVpp65 stimulated with non-stimulated cultures, significant levels of IFN-γ, TNF-α and IL-2 were seen in both G-CSF mobilised and non-mobilised donors ($p<0.005$). On analysis of IL-10, IL-4 and IL-5 secretion we observed low to undetectable levels in the supernatants of both G-CSF mobilised and non-mobilised cultures, which after statistical analysis were shown to be not significant when

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compared to non-stimulated cultures. Table 3.2 shows the calculated concentration of cytokine (pg/ml) secreted after flow cytometric analysis in PBMC from a G-CSF mobilised and non-mobilised donor which is representative of 6 donors that were analysed in both settings.

Comparison of the cytokine profile of G-CSF-mobilised and non-mobilised PBMCs after CMVpp65 stimulation is shown in Figure 3.2. There was no significant difference between the donor groups in the concentration of the pro-inflammatory cytokines IL-2, IFN-γ and TNF-α, although a trend towards an increase in TNF-α and IFN-γ levels was observed in non-mobilised PBMC cultures. We observed a significant increase in IL-10 secretion (p=0.01) and a trend towards an increase in levels of IL-4 and IL-5 from non-mobilised PBMC cultures. It must be noted, however, that no significant increase in IL-4, IL-5 or IL-10 levels was seen in CMVpp65-stimulated vs. non-stimulated PBMC cultures for both the G-CSF-mobilised and non-mobilised groups. Therefore, the significant increase in IL-10 secretion in non-mobilised PBMCs is probably not due to CMVpp65 stimulation, but to higher baseline levels of IL-10.

G-CSF Mobilised

	IL-2		TNF-α		IFN-γ		IL-10		IL-4		IL-5	
	CMV	Neg	CMV	Neg	CMV	Neg	CMV	Neg	CMV	Neg	CMV	Neg
Event Number	394	449	540	494	438	423	518	496	455	537	558	478
MFI	834.4	2.7	49.9	1.8	473	0.43	7.3	9.7	3.2	1.3	1.9	1.0
CV% (MFI)	30.8	86.7	26.7	112.8	24.4	426.2	36.2	36.7	59.0	136.1	85.8	183.8
Calculated CC (pg/ml)	1328	0.0	118.1	3.0	1196	0.0	11.7	15.7	3.2	0.0	1.8	0.0

Non- Mobilised

	IL-2		TNF-α		IFN-γ		IL-10		IL-4		IL-5	
	CMV	Neg	CMV	Neg	CMV	Neg	CMV	Neg	CMV	Neg	CMV	Neg
Event Number	483	440	462	485	502	461	472	503	528	549	459	437
MFI	309.6	4.2	541.5	54.2	963.7	5.6	10.8	3.4	5.8	1.8	3.3	0.7
CV% (MFI)	27.4	57.6	25.9	31.0	26.6	49.3	33.8	68.9	128.3	107.0	85.9	239.5
Calculated CC (pg/ml)	453	2.9	1507	129	2645	9.5	17.5	5.2	7.1	0.0	3.1	0.0

Table 3.2 Example of cytokine secretion (pg/ml) from G-CSF-mobilised and non-mobilised PBMCs after CMVpp65 stimulation by cytometric bead array:

Cytokine secretion was evaluated in the supernatant of cultures stimulated with CMVpp65 peptide pools or left untouched (negative control) by flow cytometry using a cytometric bead array assay from G-CSF-mobilised (top table) and non-mobilised (bottom table) PBMCs. Cultures were incubated for 16 hours at a concentration of 10^7/ml. MFI: median of fluorescence intensity, CV: coefficient of variance, CC: concentration calculated from the standard curve
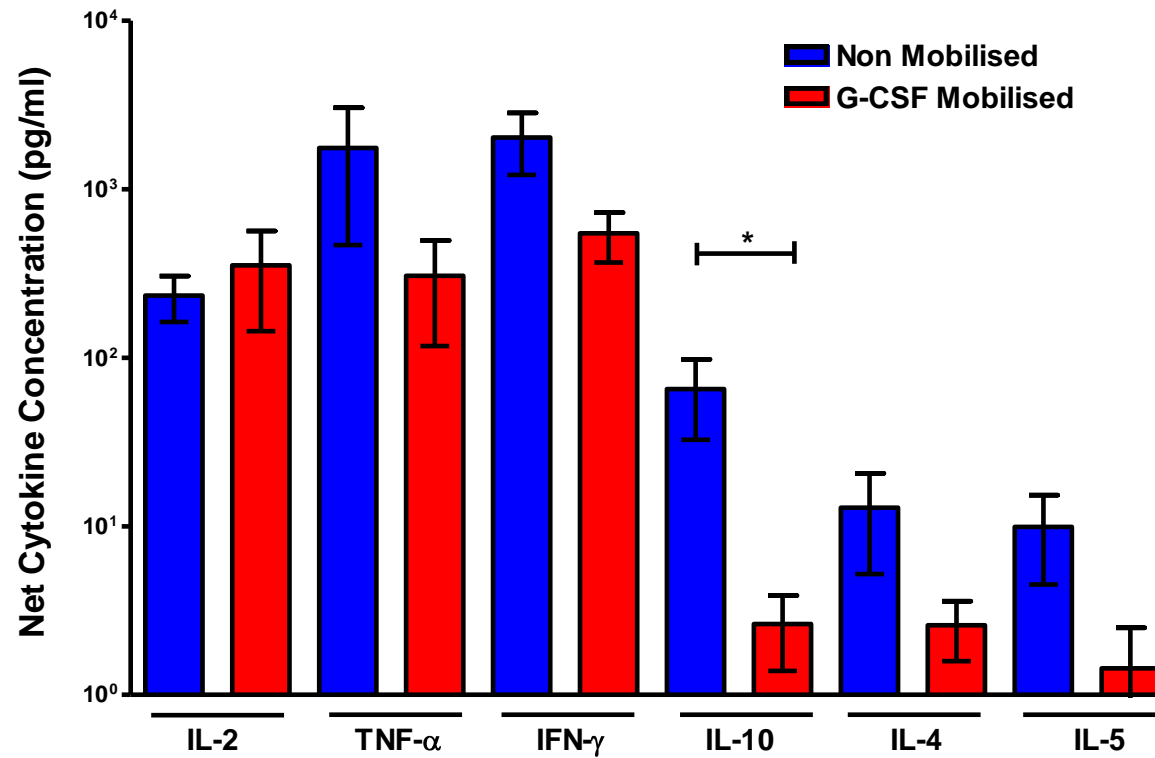
Figure 3.2 Cytokine profile in G-CSF-mobilised (n=6) and non-mobilised (n=6) PBMCs after CMVpp65 stimulation:

Quantitative assessment of IL-2, TNF-α, IFN-γ, IL-10, IL-4 and IL-5 in the supernatant of cultures after 16 hour stimulation with CMVpp65 peptide pools. Bars represent the mean (± SD) cytokine concentration (pg/ml) from non-mobilised (blue bars) and G-CSF-mobilised (red bars) PBMCs after subtraction of the negative control (no peptide). *$p<0.05$ in a non-paired *t* test.

3.4.2   Identification of IFN-γ-secreting CMV-specific T cells in G-CSF-mobilised PBMCs

In the previous section, significant levels of IFN-γ were detected in the supernatant of CMVpp65-stimulated G-CSF-mobilised PBMC cultures. This prompted the question of whether CMV-T could be identified and isolated from the supernatant of PBMC cultures. The identification and isolation of CMV-T utilised the IFN-γ catch assay and magnetic bead enrichment, as this system had been used previously for the manufacture of clinically efficacious CMV-T from non-mobilised PBMCs (Harcourt *et al*, 2006; Peggs *et al*, 2011; Wills *et al*, 1996). IFN-γ was measured before and after magnetic enrichment to assess purity and yield in mobilised and non-mobilised PBMCs. As illustrated in Figure 3.3, the mean proportion of IFN-γ-secreting cells was reduced in G-CSF-mobilised PBMCs (1.42% ± 0.35) compared with non-mobilised PBMCs (2.50% ± 0.63). Analysis of the CD3+ T cell compartment of IFN-γ-secreting cells showed that in non-mobilised donors the ratio of CD4+ to CD8+ T cells was evenly split (44% vs. 56%) whereas in G-CSF-mobilised donors the ratio was shifted towards a more predominantly CD4+ T cell response (69% vs. 31%) which was statistically significant (p=0.04).
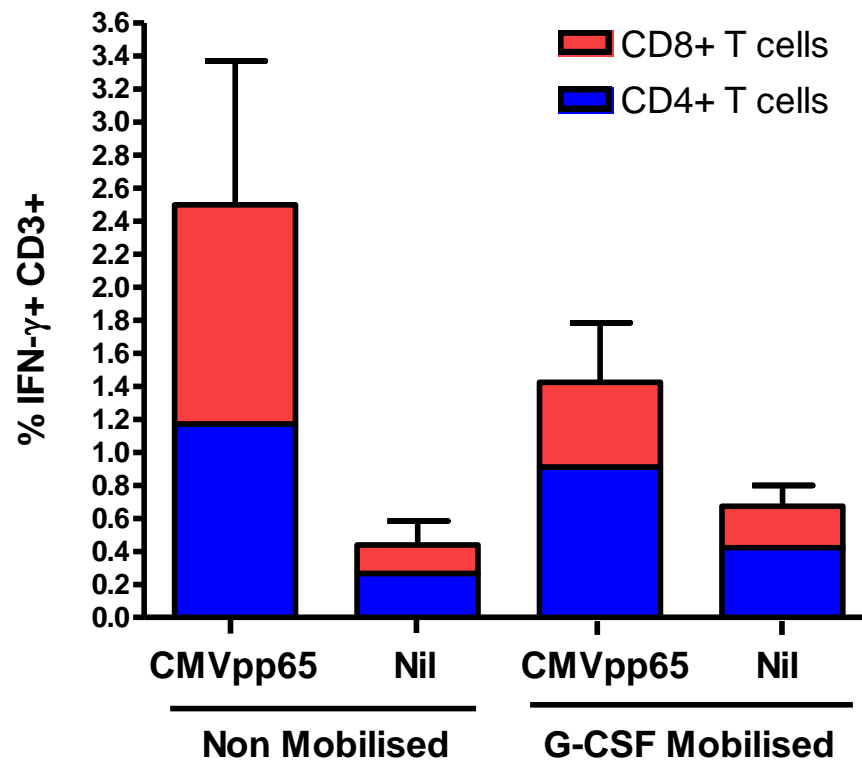
Figure 3.3 Identification of IFN-γ secreting antigen-specific T cells in unpaired G-CSF-mobilised (n=6) and non-mobilised (n=6) donors:

Samples from G-CSF-mobilised and non-mobilised apheresis were stimulated with CMVpp65 peptide pools or left untouched (Nil) for 16 hours and the frequency of IFN-γ-secreting cells analysed amongst CD3+ T cells. CD3+ IFN-γ+ populations were further sub-divided into CD8+ (red bars) and CD4+ (blue bars). Bars represent mean (± SD) percentage of CD3+ IFN-γ+ cells.

3.4.3 Enrichment of IFN-γ-secreting CMV-reactive T cells in G-CSF-mobilised PBMCs

Following stimulation with CMVpp65 peptides, IFN-γ-secreting cells were isolated by magnetic bead enrichment as previously described. Enrichment of IFN-γ+ CMV-T was performed from G-CSF-mobilised (n=6) and non-mobilised (n=6) PBMC donors and the experimental results are summarised in Table 3.3. The mean lymphocyte percentage of PBMCs was significantly decreased in G-CSF-mobilised vs. non-mobilised PBMCs both pre- (14.04% vs. 53.96%, respectively; $p<0.0001$) and post-enrichment (4.24% vs. 19.95%, respectively; $p=0.04$) but the percentage of CD3+ T cells within the lymphocyte population was comparable between the two donor groups pre- (69.1% vs. 59.0%, respectively; $p=0.24$) and post-enrichment (43.1% vs. 63.1%, respectively; $p=0.24$). Table 3.3 also illustrates a significant reduction in the total number of IFN-γ secreting CD3+ T cells in G-CSF-mobilised PBMCs ($p=0.04$) as discussed in section 3.4.2.

The mean purity of IFN-γ+ CMV-T after magnetic enrichment was shown to be decreased in G-CSF-mobilised PBMCs (35.68% ± 13.18) compared with non-mobilised PBMCs (51.82% ± 10.66). However this observation proved not to be statistically significant ($p=0.21$).The yield of IFN-γ+ CMV-T was calculated by dividing the absolute number of IFN-γ+ CD3+ in the IFN-γ positive fractions after enrichment by the absolute number of IFN-γ+ CD3+ cells before enrichment, then multiplied by 100 to give a percentage. Combined results from 6 separate enrichments showed that the mean yield of IFN-γ+ CMV-T was decreased in G-CSF-mobilised PBMCs (6.61% ± 2.22) compared with non-mobilised PBMCs (18.07% ± 11.09), although no statistically significance was observed This result is reflected in the marked reduction seen in the absolute number of cells in the positive fractions of G-CSF-mobilised (0.23×10^6) relative to non-mobilised (1.62×10^6) PBMCs after enrichment, even though the absolute number of cells prior to enrichment was equivalent (135.6 vs. 132.8×10^6) between the two groups. The ratio of CD4+ to CD8+ IFN-γ+ secreting cells post-enrichment was comparable between G-CSF-mobilised (44.3% vs. 55.7%) and non-mobilised (43.6% vs. 56.4%) PBMCs.

Representative staining for IFN-γ secretion from G-CSF-mobilised and non-mobilised PBMCs is shown in Figure 3.4 which highlights the reduced proportion of IFN-γ+ CD3+ T cells in G-CSF-mobilised vs. non-mobilised PBMCs before and after enrichment. The efficiency of isolation of IFN-γ-secreting cells is summarised in Figure 3.5, which illustrates a trend towards a reduction in G-CSF-mobilised PBMCs vs. non-mobilised PBMCs in both purity and yield of IFN-γ-secreting CMV-T.

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 as shown in section 3.4.1, 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 (Tayebi *et al*, 2001) which could result in reduced detection of CMV-T using this method and ultimately in the efficiency of magnetic bead enrichment.

	Pre Enrichment		Post Enrichment	
	Non-Mobilised	**G-CSF Mobilised**	**Non-Mobilised**	**G-CSF Mobilised**
Cell Number (x10^5)	132.8 (105-177)	135.6 (44-186)	1.6 (0.3-3.9)	0.2 (0.04-0.6)
Lymphocyte (% of PBMC)	54.0 (45.0-63.3)	14.0 (9.0-19.6)	20.0 (1.2-32.8)	4.2 (1.5-6.3)
CD3 (% of lymphocytes)	59.0 (49.2-63.2)	69.1 (43.5-84.6)	56.1 (29.1-80.7)	43.1 (29.8-66.2)
CD3+ IFN-γ+ (% of lymphocytes)	2.50 (0.8-3.4)	1.4 (0.8-2.7)	51.8 (12.7-76.0)	35.7 (12.1-86.3)
CD4+ (% of CD3+ IFN-γ+)	42.6 (35.0-57.0)	59.5 (50.7-63.7)	42.7 (39.6-52.8)	44.3 (12.5-80.2)
CD8+ (% of CD3+ IFN-γ+)	55.4 (45.7-65.0)	40.5 (36.6 – 49.3)	55.1 (46.0-64.0)	55.7 (19.8-87.5)
CD3+ IFN-γ+ Cell Number (x10^5)	1.0 (0.5-1.7)	0.2 (0.1-0.9)	0.2 (0.01-0.8)	0.01 (0.001-0.04)

Table 3.3 Summary of enrichment of CMV-reactive T cells through IFN-γ secretion in non-mobilised (n=6) and G-CSF-mobilised (n=6) PBMCs:

The mean and range of G-CSF-mobilised and non-mobilised IFN-γ enrichment experiments (n=5, both) using PBMCs from CMV seropositive donors after 16 hour culture with CMVpp65 peptide pools as determined by flow cytometry. Magnetic enrichments were performed on the MiniMACS using MS columns after labelling of IFN-γ+ cells using PE-microbeads
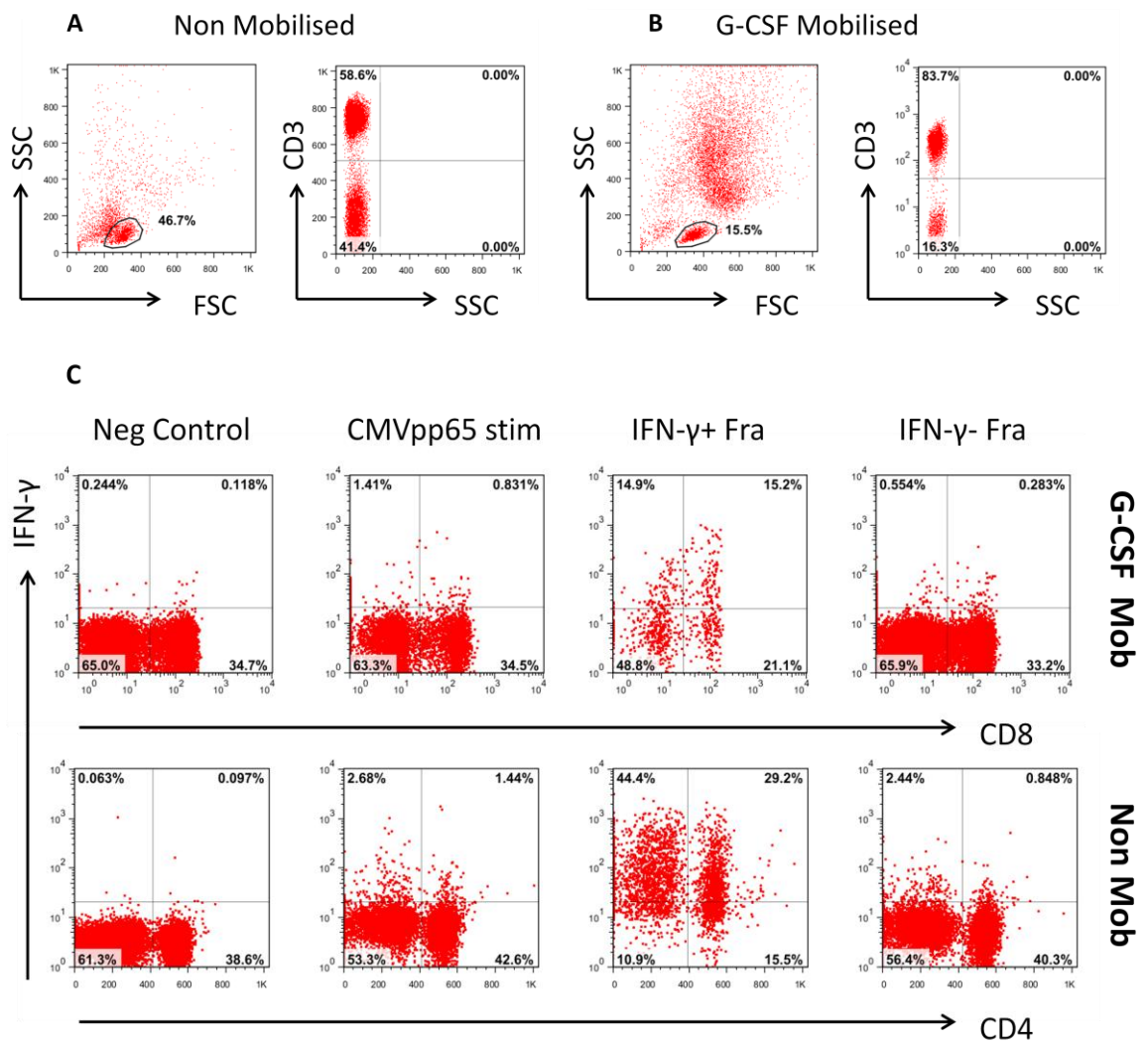
Figure 3.4 Comparison of IFN-γ secretion and enrichment after CMVpp65 stimulation between a G-CSF-mobilised and non-mobilised donor:

PBMCs were stained with CD3-APC, IFN-γ-PE and either CD4-FITC or CD8-FITC conjugated antibodies and analysed by flow cytometry. Samples were gated on live lymphocytes as side scatter (SSC) versus forward scatter (FSC) followed by gating on SSC^{low} CD3+ T cells in both (A) non-mobilised and (B) G-CSF-mobilised PBMCs. (C) IFN-γ+ cells were analysed in the negative control (no peptide), CMVpp65-stimulated and IFN-γ positive and negative fractions after magnetic enrichment.

(Fra, fraction; Mob, mobilised; Neg, negative; Stim, stimulated)
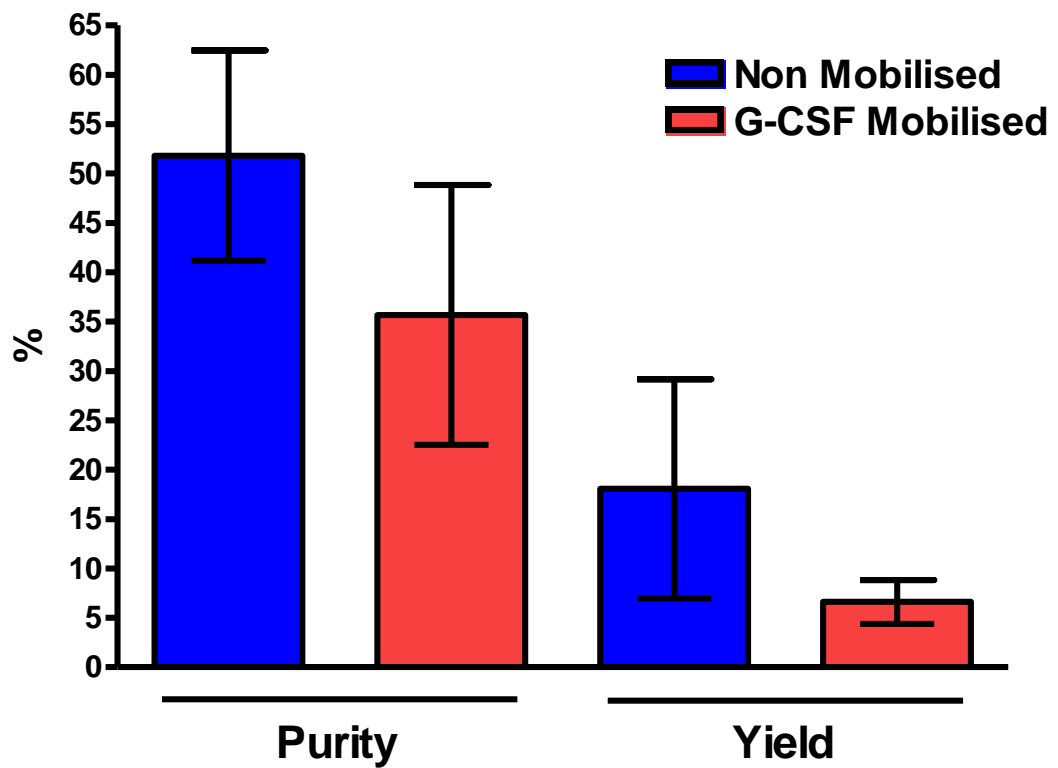
Figure 3.5 Isolation of IFN-γ-secreting antigen-specific T cells in unpaired G-CSF-mobilised (n=6) and non-mobilised (n=6) donors:

G-CSF-mobilised (red bars) and non-mobilised (blue bars) PBMCs were stimulated with CMVpp65 peptide pools for 16 hours and IFN-γ-secreting cells isolated by magnetic bead enrichment on a MiniMACS with MS columns after labelling using the IFN-γ catch assay. Bars represent mean (± SD) percentage of purity and yield of IFN-γ+ cells from within the CD3+ population.

3.4.4 Temporal dynamics of activation marker expression on CD3+ T cells after CMVpp65 peptide stimulation in G-CSF-mobilised and non-mobilised PBMCs

The kinetics of activation-induced CD25, CD69, CD137, CD152 and CD154 expression on CMV-reactive T cells in G-CSF-mobilised PBMCs was investigated over a 24 hour period to determine the optimal duration of stimulation and compared directly to that of non-mobilised PBMCs. PBMCs were stimulated with CMVpp65 peptides and the superantigen SEB as a positive control. Expression of activation markers were quantified as the percentage of positive cells within the CD3+ population after subtraction of the negative control (non-stimulated PBMCs). Results show experiments from 5 G-CSF-mobilised and 5 non-mobilised PBMCs donors.

3.4.4.1 Baseline expression

Baseline expression of all activation markers, assessed in resting PBMCs prior to stimulation, was comparable between G-CSF-mobilised and non-mobilised PBMCs (Figure 3.6). Mean baseline CD25 expression was shown to be elevated in both G-CSF-mobilised ($4.5\% \pm 0.59$) and non-mobilised ($4.3\% \pm 0.85$) PBMCs when compared with CD69, CD137, CD152 and CD154, which showed mean expression levels of 0.73%-1.19% (G-CSF-mobilised) and 0.38%-1.59% (non-mobilised)]. The elevation in CD25 expression in comparison with other activation markers can be explained by the presence of naturally occurring T regulatory cells (Tregs) that also express the marker CD25, and is explored further in Chapter 4.
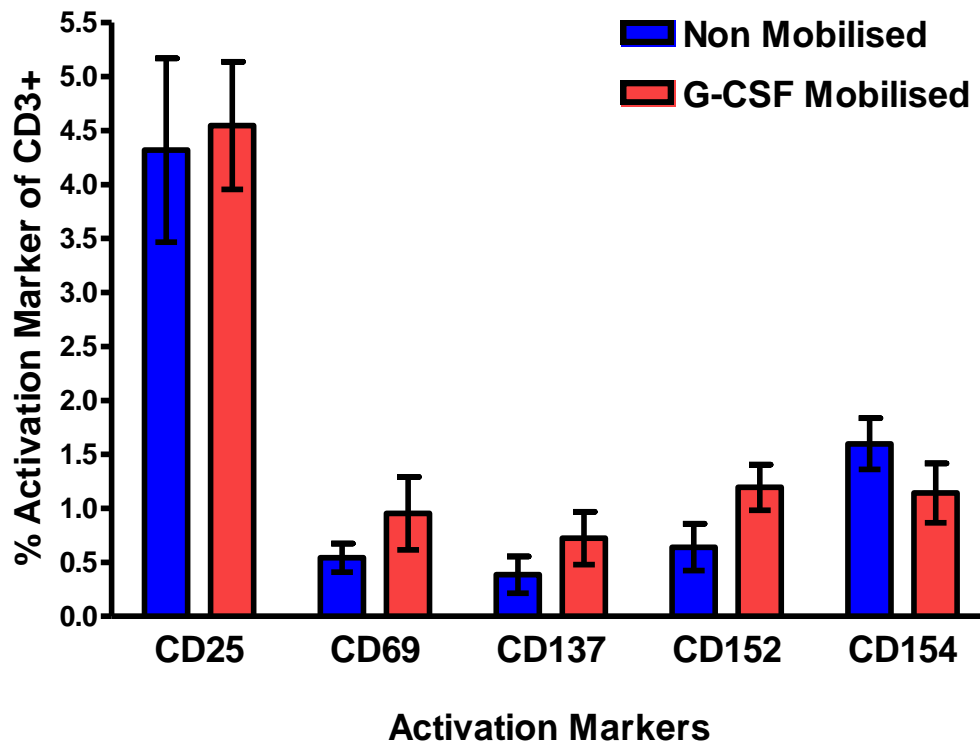
Figure 3.6 Comparison of T cell activation markers between G-CSF-mobilised (n=5) and non-mobilised (n=5) PBMCs at rest:

Freshly isolated PBMCs were stained for activation markers CD25, CD69, CD137, CD152 and CD154 in resting G-CSF-mobilised (red bars) and non-mobilised (blue bars) PBMCs to assess baseline expression. Bars represent the mean (+SD) percentage of the CD3+ T cell population after gating on live lymphocytes by flow cytometry.

3.4.4.2 Activation marker expression after SEB stimulation

Activation marker expression on CD3+ T cells were analysed at 1, 4, 6, 16 and 24 hours after SEB stimulation by flow cytometry. PBMCs from G-CSF-mobilised donors were stimulated with the superantigen SEB to compare the activation status with non-mobilised PBMCs and to assess whether the kinetics of antigen expression were comparable between the two donor groups. As summarised in Table 3.4, SEB induced a similar expression profile over 24 hours in all activation markers with a trend towards higher expression in G-CSF-mobilised PBMCs after 24 hours.

	CD25%		CD69%		CD137%		CD152%		CD154%	
	NM	**MOB**	**NM**	**MOB**	**NM**	**MOB**	**NM**	**MOB**	**NM**	**MOB**
1hr	4.14	3.05	0.83	6.26	0.92	1.45	0.46	4.17	1.22	2.37
4hr	2.67	3.39	8.71	9.73	0.93	4.40	0.82	5.72	4.70	6.27
6hr	4.09	5.95	10.4	11.4	2.84	5.07	0.73	2.44	4.49	3.68
16hr	12.2	11.5	11.4	9.92	4.98	4.42	0.84	1.72	1.91	2.55
24hr	11.7	18.3	10.3	11.9	4.00	6.12	0.93	3.57	1.45	3.96

Table 3.4 Activation marker expression after SEB stimulation
SEB stimulation at 1, 4, 6, 16 and 24 hours in G-CSF-mobilised (MOB; n=3) and non-mobilised (NM; n=5) PBMCs within the CD3+ T cell population. Data are mean percentage of the CD3+ T cell population after subtraction of the negative control

Figure 3.7 illustrates a representative staining pattern of the activation markers CD25, CD69, CD37, CD152 and CD154 after SEB stimulation on CD3+ CD8+ in a G-CSF-mobilised donor at all time points.
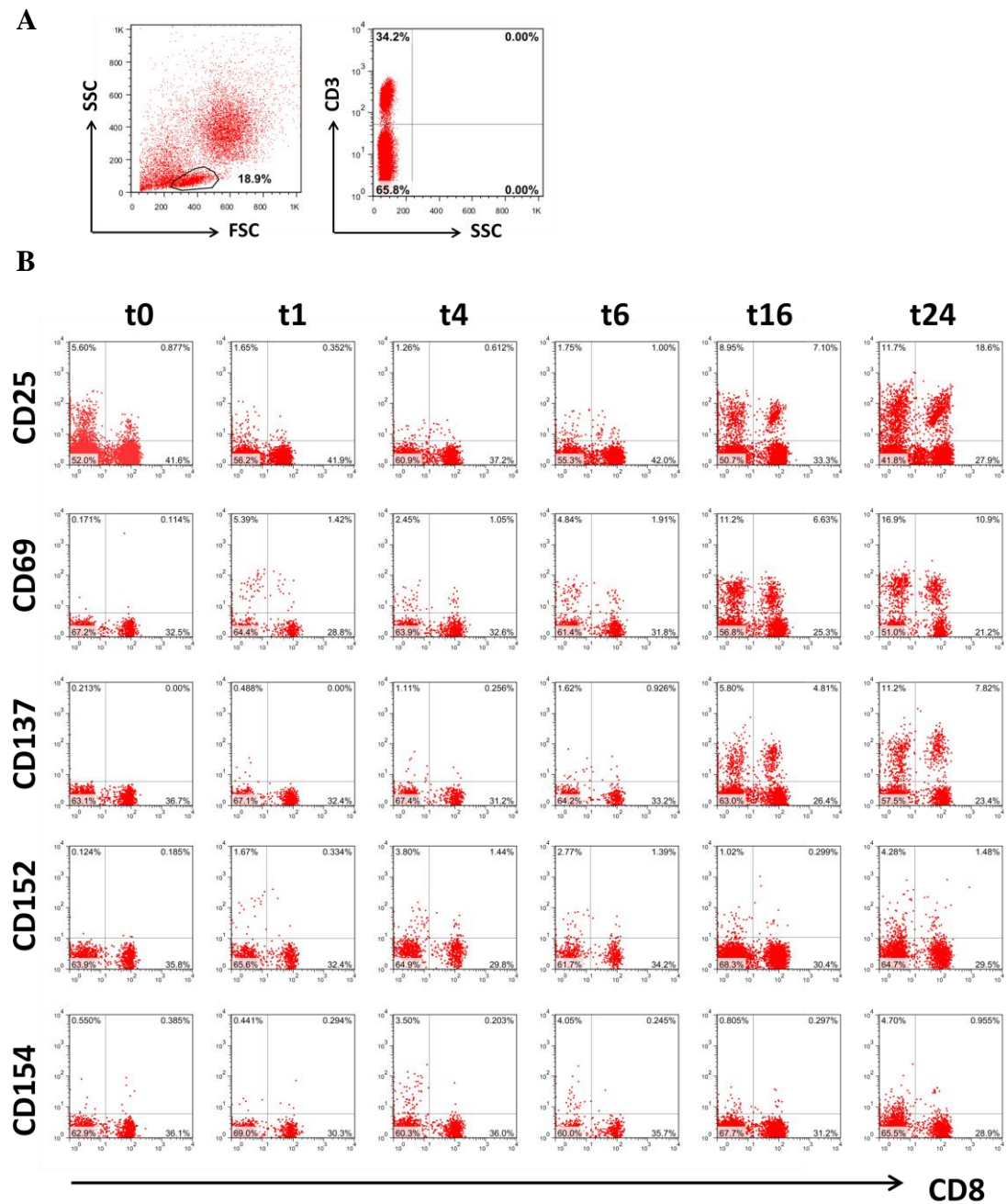
Figure 3.7 Analysis of activation markers after SEB stimulation at 1, 4, 6, 16 and 24 hours in G-CSF-mobilised PBMCs:

Freshly isolated PBMCs from a G-CSF-mobilised donor were stimulated in 96-well plates with SEB and samples removed at 1, 4, 6, 16 and 24 hours for the assessment of the expression of activation markers CD25, CD69, CD137, CD152 and CD154. Samples were gated on live lymphocytes as side scatter (SSC) versus forward scatter (FSC) followed by gating on SSC[low] CD3+ T cells (A) CD3+ populations when then analysed or activation marker expression versus CD8 (B) Antigen dependent expression of CD25 was maximal at 16 hours

3.4.4.3 Activation marker expression after CMVpp65 stimulation

The kinetics of activation-induced CD25, CD69, CD154 and CD137 expression on CMVpp65 specific T cells in G-CSF-mobilised PBMCs was investigated to determine both the optimal target and the optimal time for maximal expression and to compare these results directly with non-mobilised PBMCs. PBMCs were stimulated over a 24 hour period with CMVpp65 peptides, removed from cultures at 1, 6, 16 and 24 hours, and analysed for surface expression of activation markers by flow cytometry. Figure 3.8 shows activation marker expression against time in a G-CSF-mobilised donor in CMVpp65-stimulated, SEB-stimulated and non-stimulated PBMCs and illustrates the optimal time point for maximal expression. The results comparing mean expression of the activation markers CD25, CD69, CD137, CD152 and CD154 in G-CSF-mobilised (n=5) and non-mobilised donors (n=5) are shown in Figure 3.10. The mean antigen-dependent expression of CD25 was maximal at 16 hours in G-CSF-mobilised PBMCs (3.19% $\pm$ 1.43) and was increased compared with non-mobilised PBMCs (1.73% $\pm$ 0.63). Mean CD69 expression in G-CSF-mobilised PBMCs (1.32% $\pm$ 0.51) peaked at 16 hours and was higher than that seen in non-mobilised PBMCs (0.33% $\pm$ 0.21) which peaked at 6 hours. CD137 reached peak expression at 24 hours after CMVpp65 stimulation and was comparable in intensity between G-CSF-mobilised (0.88% $\pm$ 0.45) and non-mobilised (0.56% $\pm$ 0.16) PBMCs. CD154 expression in G-CSF-mobilised PBMCs was maximal at 6 hours (0.95% $\pm$ 0.44) and was increased compared with non-mobilised PBMCs (0.16% $\pm$ 0.15). Analysis of CD152 expression showed a maximal time of expression at 16 hours with a trend (*p*=0.05) towards increased expression in G-CSF-mobilised PBMCs (1.37% $\pm$ 0.46) compared with non-mobilised PBMCs (0.18% $\pm$ 0.07). The results comparing mean expression of CD25, CD69, CD137, CD152 and CD154 in five G-CSF-mobilised and five non-mobilised donors are illustrated in Figure 3.9, indicating the time point of maximal expression for each activation marker.
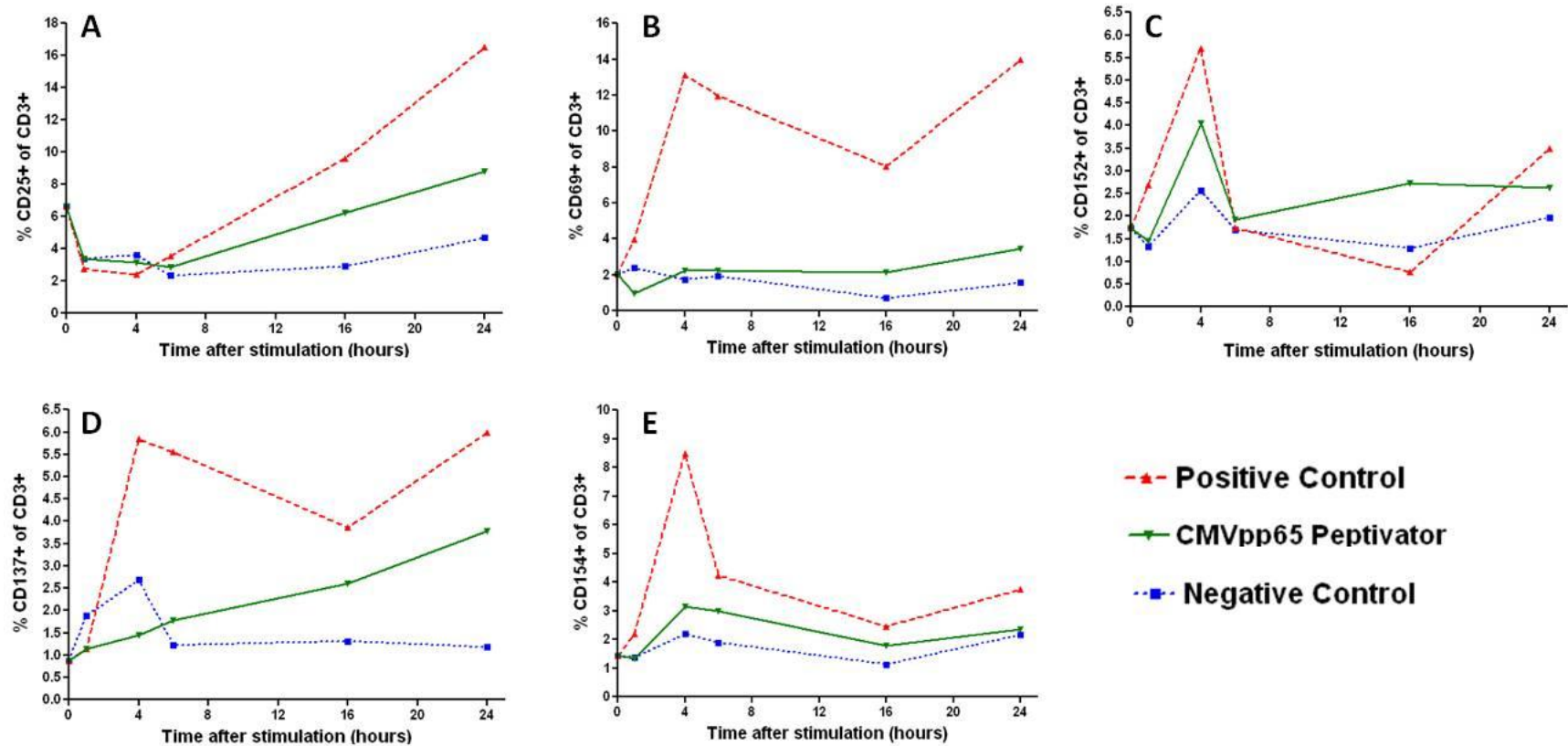
Figure 3.8 Analysis of activation markers after CMVpp65 stimulation at 1, 4, 6, 16 and 24 hours in G-CSF-mobilised PBMCs:
PBMCs isolated from G-CSF-mobilised apheresis were stimulated with a CMVpp65 peptide pool and samples taken at 1, 4, 6, 16 and 24 hours for the assessment of activation dependent expression of the cell surface markers (A) CD25, (B) CD69, (C) CD152, (D) CD137 and (E) CD154 from within the CD3+ T cell population. Cells were also stimulated with SEB (1µg/ml) as a positive control and left untouched as a negative control.
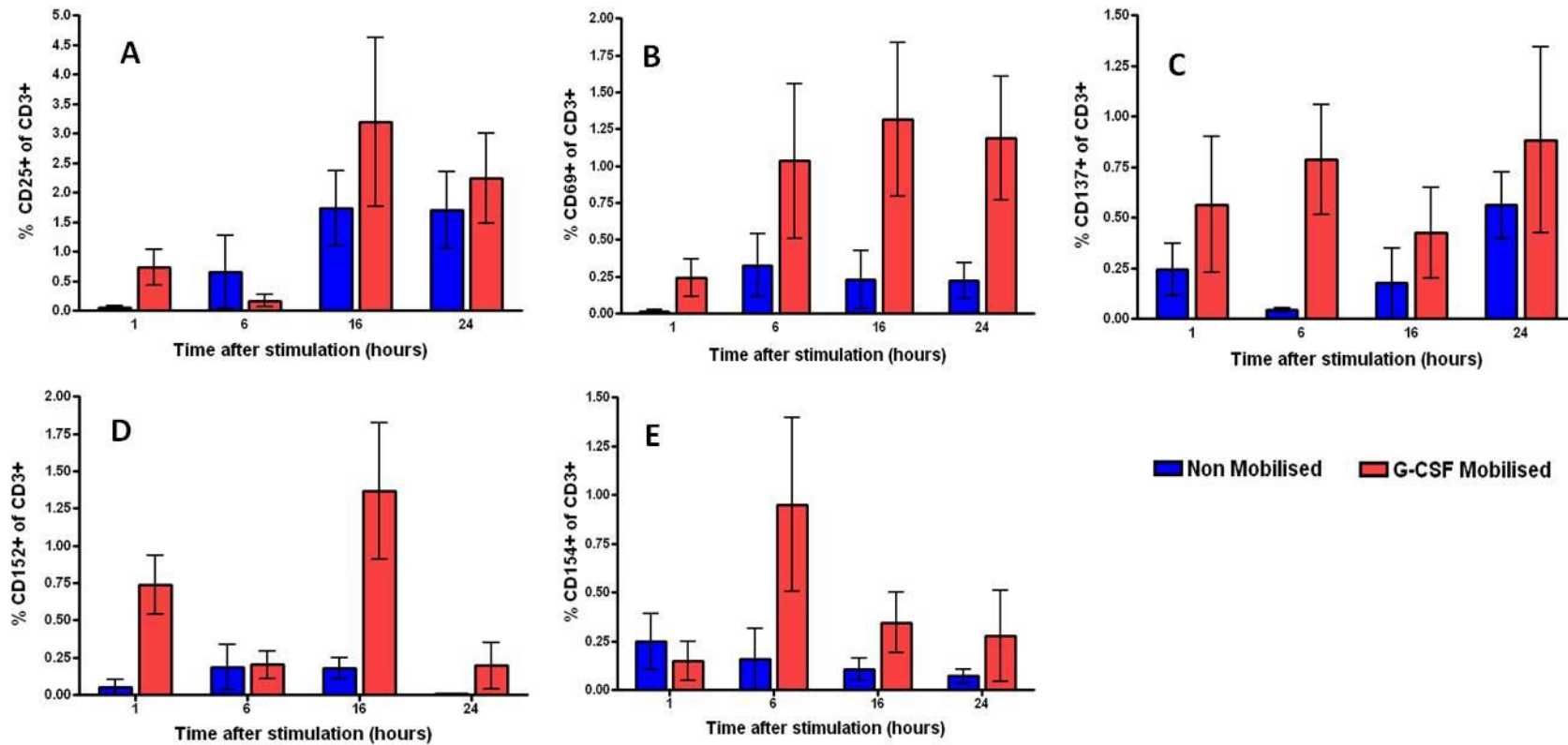
Figure 3.9 Temporal dynamics of activation marker expression after CMVpp65 peptide stimulation in G-CSF-mobilised (n=5) and non-mobilised (n=5) PBMCs:

PBMCs isolated from G-CSF-mobilised (red bars) and non-mobilised (blue bars) apheresis were stimulated with a CMVpp65 peptide pool and analysed for the expression of (A) CD25, (B) CD69, (C) CD137, (D) CD152 and (E) CD154 at 1, 6, 16 and 24 hours. Bars represent the mean percentage (+SD) in the CD3+ T cell population after subtraction of the negative control (no peptide).

3.4.5 Assessment of CD154 activation-induced expression in G-CSF-Mobilised PBMCs after blocking CD40-CD154 interaction

Previously published data have demonstrated that CD154 is a suitable marker for the detection and isolation of CMV-specific T cells (Chattopadhyay *et al*, 2005; Frentsch *et al*, 2005; Khanna *et al*, 2011) from conventional non-mobilised PBMCs in the presence of CD40 blockade. Effective blocking of CD40 and CD154 ligation leads to the preservation of CD154 expression at the cell surface (Frentsch *et al*, 2005), although the effect of G-CSF mobilisation on this procedure was unknown. PBMCs were stimulated with either SEB or CMVpp65 peptides for 4 and 6 hours in the presence or absence of CD40-specific antibody, and then analysed for CD154 expression amongst the CD4+ T cell population. Figure 3.10 shows representative FACS plots from a G-CSF-mobilised and non-mobilised donor, illustrating the effect of CD40 blockade on CD154 expression after CMVpp65 stimulation. Results from G-CSF-mobilised and non-mobilised PBMCs donors (n=5, both) are shown in Figure 3.12. We found low background CD154 expression in resting CD4+ T cells that was comparable between the two donor groups (G-CSF-mobilised: 0.30% ± 0.14; non-mobilised: 0.22% ± 0.06). Mean CD154 expression in the presence of CD40-specific antibody at the optimal time point of 6 hours, showed no significant difference between G-CSF-mobilised PBMCs (1.86% ± 0.59) and non-mobilised PBMCs (1.22% ± 0.13). CD40 blocking significantly increased CD154 expression in CMVpp65 stimulated G-CSF-mobilised (*p*=0.0033) and non-mobilised (*p*=0.0008) PBMCs. In both donor groups, CD40 blocking antibody alone showed no significant effect on CD154 expression. The results from 5 G-CSF mobilised and 5 non-mobilised PBMC donors are summarised in Figure 3.11.
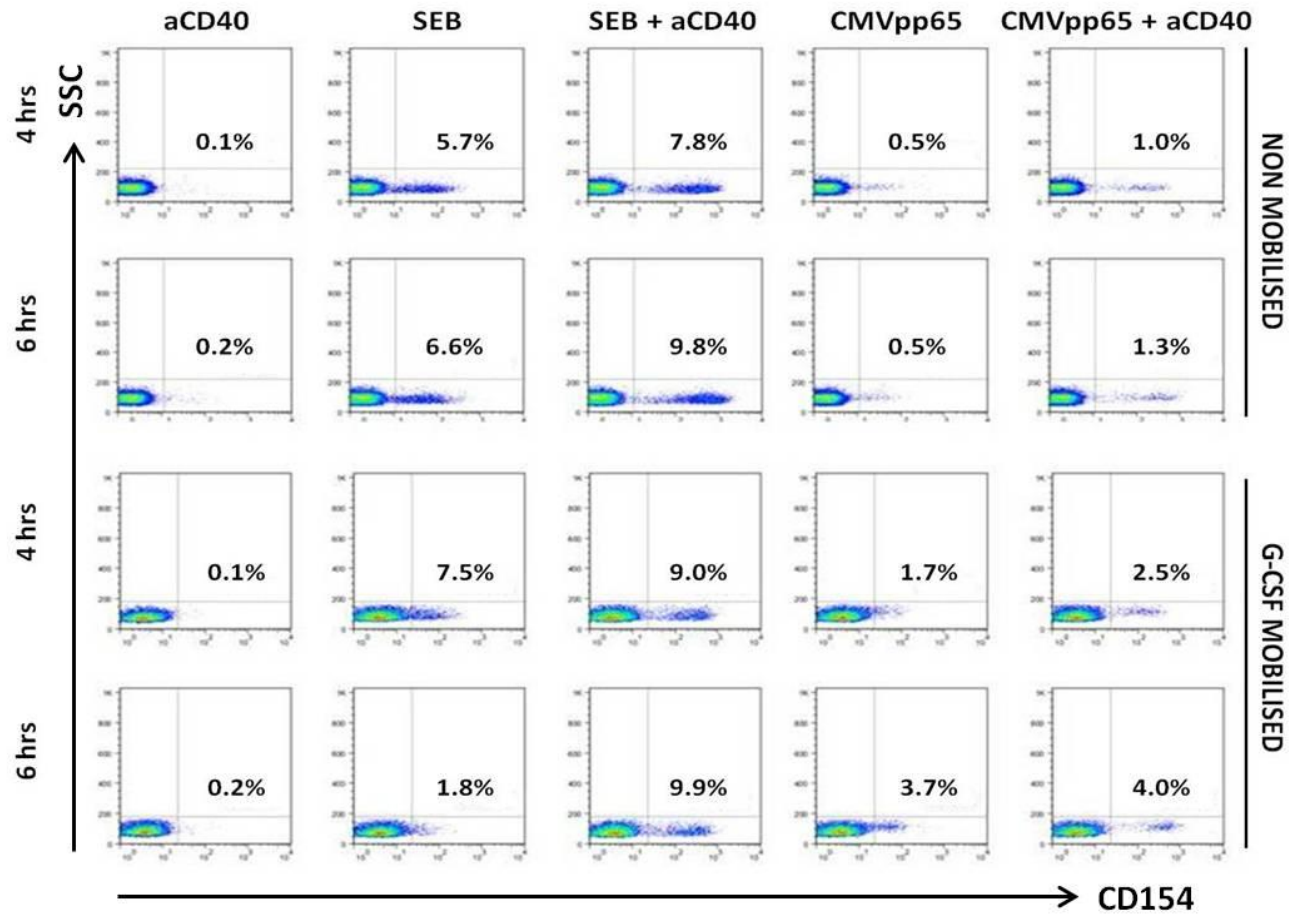
Figure 3.10 Direct comparison of CD154 surface expression at 4 and 6 hours between a G-CSF-mobilised and non-mobilised donor after CD40 blocking:

PBMCs were stimulated with either CMVpp65 peptides or SEB in the presence or absence of CD40-specific antibody (1μg/ml) and with CD40-specific antibody alone. Cells are gated on CD3+ CD4+ T cells. (aCD40, CD40 antibody; SEB, staphylococcal enterotoxin B)

NON MOBILISED
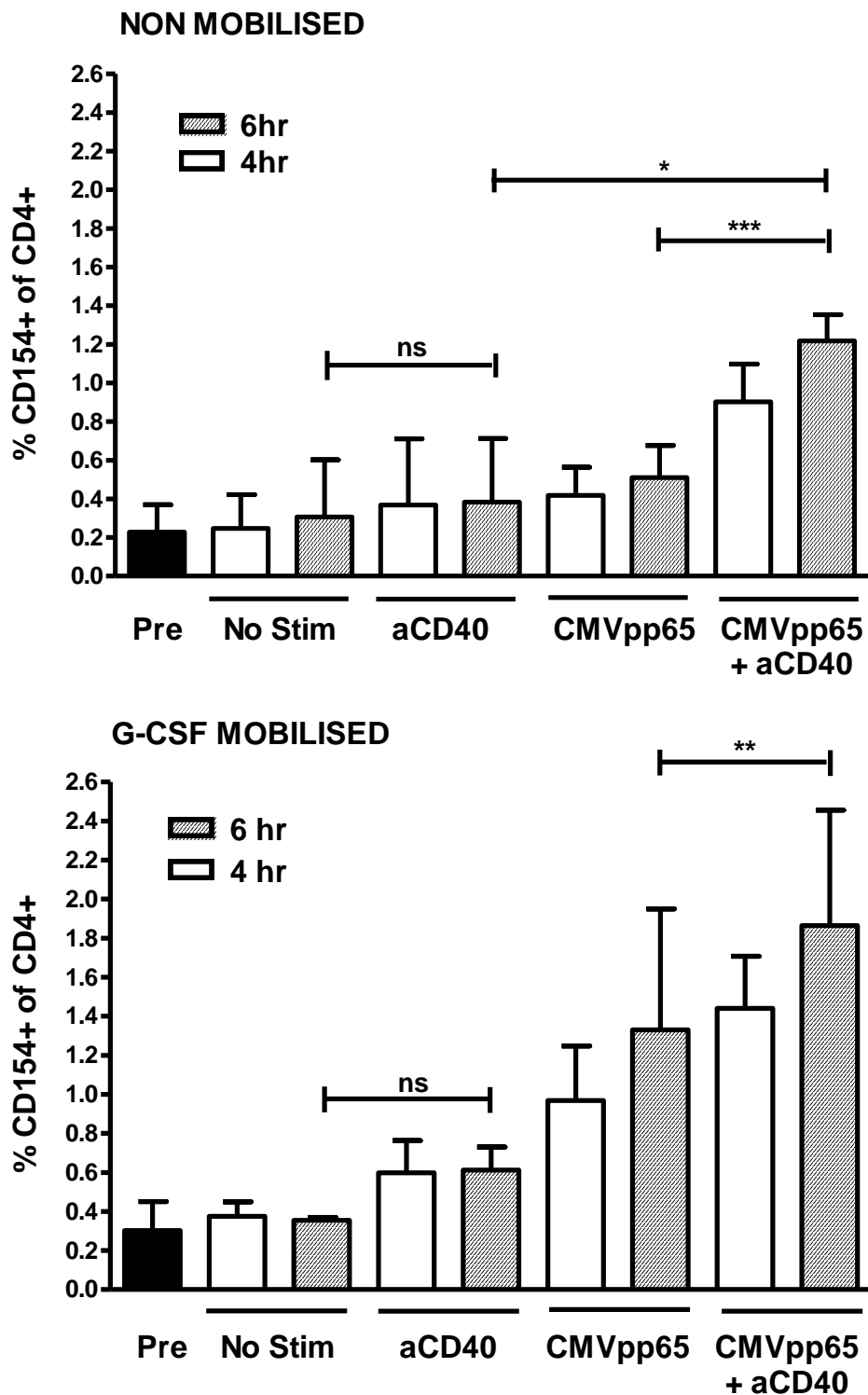
G-CSF MOBILISED

Figure 3.11 Comparison of CD154 expression in non-mobilised (n=5) and G-CSF-mobilised (n=5) donors at 4 and 6 hours.

*P<0.05, ** P<0.01, *** P<0.001 in a paired *t* test.

(Pre, pre stimulation; No stim, no stimulation; aCD40, CD40 antibody)

3.5   Discussion

The manufacture of clinical grade CMV-T for adoptive immunotherapy has been successfully demonstrated in conventional non-mobilised through IFN-γ secretion (Peggs *et al*, 2011). This has been achieved through a short culture period in which cells were stimulated with either the CMVpp65 protein or overlapping CMVpp65 peptides covering the whole of the CMVpp65 protein. This method of direct selection is consistent with clinical grade manufacture due to the short stimulation period of 6-16 hours without the necessity for long term culture. In this Chapter, we investigated the feasibility of using G-CSF-mobilised PBMCs rather than conventional non-mobilised PBMCs to identify and isolate CMV-T. Initial experiments assessed the cytokine profile of G-CSF-mobilised PBMCs after stimulation with CMVpp65 peptides using a cytokine bead array (CBA) assay and subsequently to compare cytokine secretion directly against non-mobilised PBMCs. Following cytokine profiling, we identified CMV-T in G-CSF-mobilised PBMCs based on IFN-γ secretion and subsequently isolated these cells using magnetic bead enrichment. In the final section of this Chapter, we investigated a panel of activation markers that have previously been shown to be upregulated after CMVpp65 stimulation in non-mobilised PBMCs (Chattopadhyay *et al*, 2005; Frentsch *et al*, 2005; Gallot *et al*, 2001; Peggs *et al*, 2011; Watanabe *et al*, 2008; Wehler *et al*, 2008; Wolfl *et al*, 2007) and assessed their candidacy for targeting the identification of CMV-T in G-CSF-mobilised PBMCs.

Cytokine profiling by CBA showed that G-CSF mobilisation was not associated with a significant decrease in secretion of the inflammatory or anti-viral cytokines IL-2, TNF-α and IFN-γ in response to CMVpp65 stimulation compared with conventional non-mobilised PBMCs. Similar low levels of secretion of IL-4 and IL-5 were observed in G-CSF-mobilised and non-mobilised PBMCs, both of which are associated with a TH$_2$ phenotype. This result suggests that G-CSF mobilisation does not appear to significantly alter the potential for type 1 cytokine production observed when the whole PBMC population is studied. However, previous studies have reported inhibition of type 1 cytokine production by T cells at a single cell level in G-CSF-mobilised PBMCs (Arpinati *et al*, 2000; Pan *et al*, 1999; Tayebi *et al*, 2001). The finding that IL-10

secretion was significantly decreased in G-CSF-mobilised PBMCs (*p*=0.01*)* is an interesting one, as it argues against evidence from previous studies that G-CSF augments the generation of IL-10-producing Tregs (Morris *et al*, 2004; Rutella *et al*, 2002; Tayebi *et al*, 2001). However, these findings were observed in an early immune response whereas the administration of G-CSF to HSCT donors occurs every day for 5 days leading up to the collection of HSCs. The reduction in IL-10 secretion seen in G-CSF-mobilised PBMCs could be explained by the potential exhausting effect that administration of G-CSF over a five day period could have on the level of IL-10 secretion. Furthermore IL-10 release has been shown to be induced in antigen presenting cells (APCs) after lipopolysaccharide (LPS) stimulation (Byrne & Reen, 2002). If antigen uptake by APCs is less efficient in G-CSF-mobilised PBMCs, this could result in a decrease in IL-10 secretion when compared with non-mobilised PBMCs, although I have no data to support this hypothesis.

Having observed that the level of IFN-γ secretion was equivalent between G-CSF-mobilised and non-mobilised PBMCs after CMVpp65 stimulation determined by CBA, we evaluated the suitability of using IFN-γ secretion to both identify and isolate CMV-T. Although not significant, the mean proportion of IFN-γ-secreting cells was found to be reduced in G-CSF-mobilised PBMCs compared with non-mobilised PBMCs (1.42% vs. 2.50%), which was reflected in a decrease in both purity and yield after isolation. The reduction in identification and isolation of CMV-T using the IFN-γ catch method from G-CSF-mobilised PBMCs suggests that G-CSF mobilisation does have a inhibitory effect upon IFN-γ production, in line with previous findings (Arpinati *et al*, 2000; Pan *et al*, 1999; Tayebi *et al*, 2001). It must also be noted that the reduction in absolute IFN-γ secretion by CD3+ T cells observed here might be attributed to the significant reduction in the percentage of lymphocytes from within the PBMC pool in G-CSF-mobilised vs. non-mobilised donors (*p*<0.0001). G-CSF mobilisation is associated with a significant increase in the number of circulating lymphocytes (Hartung *et al*, 1999; Rutella *et al*, 1997; Sica *et al*, 1996). However, the reduction in lymphocyte percentage, partly due to the high percentage of granulocytes mobilised into the periphery, could result in a reduction in the absolute number of T cells capable of secreting IFN-γ after CMVpp65 stimulation from within a fixed number of PBMCs. Another hypothesis to explain the decrease in IFN-γ secretion seen from the T cell pool,

is that G-CSF mobilises a population of immature or non-functional T cells that are not capable of responding to antigenic stimulation, which would also dilute the pool of IFN-γ-secreting CMV-T.

It remains unclear whether the use of individual CMVpp65 peptides based upon donor HLA type or the use of CMV IE-1 peptides would result in a more efficient system for the identification and isolation of CMV-T (Gratama *et al*, 2008; Zandvliet *et al*, 2010; Slezak *et al*, 2007) as the kinetics for IFN-γ secretion in G-CSF-mobilised PBMCs may differ between epitopes at different peptide concentrations. This provides the basis for possible future experiments to ascertain the nature of IFN-γ secretion to individual HLA-matched CMV peptides in G-CSF-mobilised PBMCs.

The next aim of this chapter was to investigate the temporal dynamics of a panel of activation markers after both SEB and CMVpp65 stimulation in GSF-mobilised and non-mobilised PBMCs. Samples from 5 non-mobilised and 5 G-CSF mobilised apheresis collections were stimulated over a 24 hour time period to assess activation induced expression of CD25, CD69, CD137, CD152 and CD154. No significant difference was observed in the baseline expression levels of CD25, CD69, CD137, CD152 and CD154 between G-CSF-mobilised and non-mobilised PBMCs, although CD25 expression was consistently 2- to 3-fold higher than the other makers, due to the pool of naturally circulating Tregs that also express CD25. SEB stimulation of G-CSF-mobilised PBMCs provided the basis to assess whether T cells maintained the same level of maximal activation as non-mobilised PBMCs, which would establish evidence for continuing experiments with CMVpp65 stimulation. SEB-stimulated G-CSF-mobilised PBMCs maintained similar expression levels of CD25, CD69, CD137 and CD154 as in non-mobilised PBMCs but showed an increase in expression of CD152. Stimulation with CMVpp65 showed no significant differences in the level of expression of CD25, CD69, CD137, CD152 and CD154 between non-mobilised and G-CSF-mobilised PBMCs, although we did identify a trend towards increased expression of activation markers in G-CSF-mobilised PBMCs. CD25 was upregulated on a larger proportion of T cells after CMVpp65 stimulation, after the subtraction of CD25 expression in non-stimulated T cells, when compared with other activation markers. Previous studies have explored the benefit of depleting naturally expressing CD25+

cells from the PBMC pool prior to CMVpp65 stimulation (Gallot *et al*, 2001; Melenhorst *et al*, 2008b), which suggests the idea of using CD25 as a potential marker for the isolation of CMV-T from G-CSF-mobilised PBMCs. It is tempting to observe that the optimal time of CD25 expression, at 16 hours, fits within a convenient, logistical time frame for the clinical grade manufacture of CMV-T.

Analysis of CD154 as a target antigen for isolation of CMV-T from G-CSF-mobilised PBMCs was optimal at a time point of 6 hours, which like the 16 hour time point for optimal CD25 expression, makes it a suitable marker for clinical grade manufacture. The short period of stimulation needed for CD154 activated expression together with the low to undetectable levels of background CD154 expression also make this antigen an optimal target. In line with published results in non-mobilised PBMCs, (Chattopadhyay *et al*, 2005; Frentsch *et al*, 2005) we have shown that after CMVpp65 peptide stimulation, CD154 expression in G-CSF-mobilised PBMCs was preserved at the cell surface through CD40 blockade which prevented ligation of CD154. We also observed a trend towards greater mean expression of CD154 in G-CSF-mobilised PBMCs (1.86% ± 0.59) compared with non-mobilised PBMCs (1.22 ± 0.13%).

In terms of their potential utility as activation markers for the isolation of CMV-T, CD25 and CD154 show equivalent expression kinetics in G-CSF-mobilised and non-mobilised PBMCs, and both CD25 (Lugthart *et al*, 2012) and CD154 (Khanna *et al*, 2011) have previously been demonstrated to be viable markers for the clinical scale production of virus-specific T cells.

In conclusion, the results presented in this Chapter provide supporting evidence for the further investigation of CD25, at an optimal time of 16 hours and CD154 at an optimal time of 6 hours, as activation markers for the isolation of CMV-T from G-CSF-mobilised PBMCs.

Chapter 4

Isolation of CMV-T from G-CSF-Mobilised PBMCs Through CD25 and CD154 Activation-Induced Expression

4.1   Introduction

Data presented in Chapter 3 indicated that both CD25 and CD154 are promising activation markers for the isolation of CMV-T in G-CSF-mobilised PBMCs. Furthermore CD25 and CD154 reveal maximal expression after CMVpp65 stimulation at 16 and 6 hours respectively, which has the potential to translate into clinical grade manufacture. CD25 is up-regulated after CMVpp65 peptide stimulation in conventional non-mobilised PBMCs (Gallot *et al*, 2001) and allows for CMV-T isolation using magnetic enrichment. Lugthart and colleagues (2012) (Lugthart *et al*, 2012) recently demonstrated that CD25 can be used as a target for isolating multivirus-specific T cells to clinical scale. In this study, normal donor PBMCs were stimulated with CMVpp65, EBV and AdV peptides, cultured for 3 days, and then CD25+ cells were enriched using anti-CD25 magnetic beads sorted on the CliniMACS plus system. This method for generating CMV-T is made attractive by the commercial availability of clinical grade CD25 antibodies and a manufacturing system that allows for their magnetic enrichment or depletion (CliniMACS, Miltenyi Biotec). Indeed the feasibility of using magnetic enrichment with CD25 GMP grade antibodies to clinical scale in non-mobilised has been demonstrated (Powell *et al*, 2005).

Two groups have examined the depletion of contaminating expressing cells already present in PBMCs prior to CMV stimulation (Gallot *et al*, 2001;Melenhorst *et al*, 2008a) in order to remove T regulatory cells (Tregs) which constitutively express CD25. However, in both studies, the purity and yield of CMV-T after CD25 enrichment were found to be highly variable between donors.

CD4+CD25+ Tregs, which occur naturally in the peripheral blood and originate from the thymus (Jordan *et al*, 2001), have been the subject of intensive research over the last

three decades. Tregs express the transcription factor FoxP3, which is generally accepted as being the hallmark for the identification of Tregs. They have been show to contribute to peripheral self-tolerance in humans (Dieckmann *et al*, 2001; Jonuleit *et al*, 2001). The importance of naturally occurring CD4+CD25+FoxP3+ Tregs as mediators of self-tolerance, through suppression of T cell function, is highlighted by humans who lack a functional FoxP3 gene. This results in a deficiency of Tregs and patients suffer from immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), an often fatal disorder of early childhood characterised by an overactive immune system caused by the proliferation of autoaggressive T cells and autoantibody-producing B cells (Bennett *et al*, 2001).

Curiel and colleagues have shown that Tregs can contribute to the growth of human tumours in vivo by suppressing tumour-specific T cell immunity in ovarian cancer (Curiel *et al*, 2004). Thus, the removal of Tregs may be important for maximising the virus-specific content of CMV-T isolated through CD25. However, as discussed by Lugthart and colleagues (2012), there may be some benefit in a virus-specific T cell immunotherapy containing a population of Tregs, especially in the HSCT setting where patients are also susceptible to some degree of GvHD concomitant with CMV reactivation. GvHD can lead to skin, gut and liver disease that can be fatal, and Tregs have been successfully demonstrated to prevent GvHD when administered at the time of HSCT (Di *et al*, 2011). Therefore, a population of Tregs contained within a formulated dose of CMV-T could suppress the alloreactivity associated with GvHD.. However, it should be noted that this theory has yet to be fully elucidated and, furthermore, the alloreactivity of virus-specific T cells with the capability of inducing GvHD is an intense area of debate. Alloreactivity of virus-specific T cells was first illustrated by demonstrating that EBV-specific HLA-B8 restricted T cells cross-react with HLA-B44 (Burrows *et al*, 1994), and it has also been suggested in the studies of Amir and colleagues that CMV-T cells have the ability to exert allo-HLA reactivity (Amir *et al*, 2010). The true risk of GvHD associated with CMV-T immunotherapy is not yet clearly defined, but of the studies published to date there has been no excess incidence of GvHD

.

4.2   Aims of Experiments Described in this Chapter

In this Chapter, the level of contaminating CD25+ Tregs in G-CSF-mobilised PBMCs is investigated pre- and post-CMVpp65 stimulation, and following CD25 enrichment through assay of FoxP3 expression. The suppressive capacity of CD25–enriched CMV-T is also investigated using a CFSE-based assay measuring proliferation of PBMCs co-incubated with CD25+ CMV-T. Tregs have been shown to suppress proliferation of CD4+ CD25– (Shevach *et al*, 2001) and CD8+ T cells (Camara *et al*, 2003) as well as suppressing proliferation of virus-specific CD8+ T cells during chronic hepatitis C infection (Boettler *et al*, 2005). Therefore, the isolation of CMV-T from G-CSF-mobilised PBMCs through CD25 expression requires the investigation of a possible suppressive capacity to determine whether CMV-T manufactured in this way are suitable for use in CMV immunotherapy.

In addition to CD25, CD154 has also been shown to be a promising candidate for the identification and isolation of virus-specific T cells (Frentsch *et al*, 2005; Cohen *et al*, 2005) and has recently been used as a marker to generate a functional population of multivirus-specific T cells (Khanna *et al*, 2011). Therefore, CMV-T isolation through CD154 activation-induced expression is also assessed in this Chapter. CD154+ CMV-T from G-CSF-mobilised PBMCs was isolated with magnetic-bead enrichment and compared to CD154+ CMV-T from conventional non-mobilised PBMCs to determine whether this method is a viable option for clinical grade manufacture of CMV-T. Furthermore, comparison with CD25–based isolation will reveal which of the two activation markers is the more promising candidate for the identification of CMV-T in G-CSF-mobilised PBMCs.

The experimental aims of the Chapter were as follows:

1. To examine the efficiency of magnetic based enrichment of CMV-T from G-CSF-mobilised PBMCs through CD25 and CD154 and compare directly with non-mobilised PBMCs.

2. To identify the level of Treg enrichment and suppressive activity in CMV-T isolated through CD25 from G-CSF-mobilised and non-mobilised PBMCs.

3. To investigate the impact of depletion of naturally occurring CD25+ T cells prior to CMVpp65 stimulation on the generation of CMV-T through CD25 expression in both G-CSF-mobilised and non-mobilised PBMCs.

4.3   Materials and Methods

PBMCs isolated from G-CSF-mobilised and non-mobilised CMV-seropositive healthy donors were stimulated with CMVpp65 peptides as outlined in Section 2.6. Cultures were stimulated for either 16 hours for the isolation of CMV-T through CD25 expression or 6 hours for the isolation of CMV-T through CD154 expression. For the isolation of CMV-T through CD154, cultures were incubated in the presence of anti-CD40 antibody (1µg/ml) to prevent ligation of CD154 and preserve its expression at the cell surface. Following CMVpp65 stimulation, CD25 or CD154 expressing cells were magnetically sorted with anti-PE microbeads conjugated to either CD25– PE or CD154-PE using MS columns on a MiniMACS device. Counts were performed pre- and post-enrichment and analysis of CD25+ or CD154+ T cell subsets performed by flow cytometry to assess both yield and purity of CMV-T in G-CSF-mobilised and non-mobilised PBMCs. In some experiments, co-expression of CD25, CD154 and CD69 was investigated both pre- and post-magnetic enrichment.

CMV-T identified and isolated through CD25 expression were stained for the transcription factor FoxP3, which, along with CD4+CD25+ expression, is associated with a Treg phenotype. Intracellular staining of FoxP3 was performed using a fixation and permeabilisation protocol as described in Section 2.3. FoxP3 expression was investigated pre-stimulation, post-stimulation and post-enrichment in G-CSF-mobilised (n = 5) and non-mobilised (n = 5) PBMC donors. The suppressive nature of CMV-T isolated through CD25 in both G-CSF-mobilised and non-mobilised PBMCs was also investigated using a CFSE-based suppression assay. The suppression assay was performed as described in Section 2.14.2 by labelling autologous PBMCs with CFSE and stimulating them in culture with either SEB or anti-CD3 antibody in the presence of either CD25+ or CD25– cells obtained following magnetic enrichment after CMVpp65 stimulation. The negative controls were unstimulated CFSE-labelled PBMCs and unlabelled autologous PBMCs. Conventional CD25+ Tregs isolated from resting G-CSF-mobilised and non-mobilised PBMCs were also used as a positive control to demonstrate effective suppression. Cells were incubated for five days in 96-well round bottom plates before harvesting, staining with anti-CD3 APC and acquiring on a

FACScan flow cytometer. A minimum of 5,000 CD3+ CFSE+ events were recorded and the frequency of expression determined by the percentage of CFSE^{high} CD3+ T cells of all CFSE+ CD3+ T cells. Suppression experiments with CD25+ CMV-T were performed with cells isolated from G-CSF-mobilised (n = 4) and non-mobilised (n = 4) PBMC donors.

The effect of depleting contaminating CD25-expressing cells prior to antigenic stimulation was investigated by comparing non-depleted PBMCs with CD25- depleted PBMCs in G-CSF-mobilised (n = 3) and non-mobilised (n = 3) donors. For the depletion of CD25-expressing cells, PBMCs were incubated with anti-CD25 microbeads and passed through LD columns placed in the magnetic field of a VarioMACS separator (Section 2.10.2). Unlabelled (CD25-depleted) cells were collected and stimulated for 16 hours with CMVpp65 peptides along with non-depleted PBMCs from the same donor. CD25 expression, following CMV stimulation, was assessed by flow cytometry, before enrichment of CD25+ CMV-T as previously described. Counts were performed pre- and post- enrichment and analysis of CD25+ T cell subsets performed by flow cytometry to assess both the yield and purity of CMV-T in non-depleted and CD25-depleted PBMCs.

4.4 Results

4.4.1 Isolation of CMV-T through CD25 and CD154 activation-induced expression

The efficiency of magnetic enrichment of CMV-T through CD25 and CD154 activation-induced expression in G-CSF-mobilised PBMCs was compared with non-mobilised PBMCs (Table 4.1). No significant differences were observed in purity and yield of CMV-T between G-CSF-mobilised and non-mobilised PBMCs, although the data did show a trend (p=0.11) towards a reduction in CD25 yield with non-mobilised PBMCs. Mean purity was shown to be significantly higher in CD25+ compared with CD154+ cells (89.9% vs. 48.9%, respectively; p=0.01) in G-CSF-mobilised PBMCs. A similar pattern was observed in non-mobilised PBMCs but the difference was not significant (82.5% vs. 64.7%; p=0.38). Analysis of CD25+ and CD154+ enriched cells also showed that in both G-CSF-mobilised and non-mobilised PBMCs, CD3+ T cells were predominantly (>90%) CD4+ (Figure 4.1).

Representative staining for CD25 and CD154 expression from a G-CSF-mobilised and non-mobilised PBMC donor is illustrated in Figure 4.2. This highlights the low background level of CD154 expression seen in both G-CSF-mobilised (0.31% ± 0.11) and non-mobilised PBMCs (0.29% ± 0.11) prior to CMVpp65 stimulation. In comparison CD25 expression at rest was significantly elevated (p=0.03) when compared with CD154 expression, in both G-CSF mobilised (mean, 3.39% ± 1.34) and non-mobilised (mean, 4.69% ± 1.71) PBMCs, thus confirming previous results from section 3.4.4.1. Following CMVpp65 stimulation, CD25 expression was significantly elevated compared with CD154 expression in both G-CSF-mobilised (6.41% ± 0.63 vs. 1.97% ± 0.45, respectively; p=0.0004) and non-mobilised (7.50% ± 1.70 vs. 1.35% ± 0.33, respectively; p=0.007) PBMCs, which could explain the improved purity observed in CD25-enriched CMV-T compared to CD154-based enrichment.

Co-staining for CD25 and CD154 after CMVpp65 stimulation and CD154 enrichment showed that expression of both activation markers was mutually exclusive, with low co-expression observed. This was not the case when looking at co-expression of CD69 and

CD154. FACS plots from a G-CSF-mobilised donor (Figure 4.3) show that CD25 was minimal following CD154 enrichment (3% of CD154+) at 6 hours, whereas the majority of CD154+ cells co-expressed CD69 (>99%). In comparison co-expression of CD25 and CD69 was minimal following CD25 enrichment (9.3% of CD25+) at 16 hours (Figure 4.4). Given that CD69 is a marker of T-cell activation (Craston *et al*, 1997) this suggested that CD154 might be a more reliable marker of functional CMV-T than CD25.

	Non Mobilised	G-CSF Mobilised
CD25 Yield (%)	11.3% (0.1-34.75)	26.12% (3.3-52.7)
CD25 Purity (%CD25+CD3+)	82.5% (43.0-95.9)	89.9% (85.1-96.5)
Proportion of CD25+CD4+ (% of CD3+)	75.9% (91.8)	87.7% (97.5)
Proportion of CD25+CD8+ (% of CD3+)	6.6% (8.2)	2.2% (2.5)
CD154 Yield (%)	28.4% (5.9-44.5)	30.8% (4.0-48.2)
CD154 Purity (%CD154+CD3+)	64.7% (10.6-91.2)	48.9% (9.6-78.7)
Proportion of CD154+CD4+ (% of CD3+)	59.9% (92.6)	45.3% (92.6)
Proportion of CD154+CD8+ (% of CD3+)	4.8% (7.4)	3.6% (7.4)

Table 4.1 Comparison of CMV-T cell enrichment through CD25 and CD154 activation-induced expression in G-CSF-mobilised and non-mobilised PBMCs: The mean and range of yield and purity of CD154+ (n = 5) and CD25+ (n = 5) enrichments in both G-CSF-mobilised and non-mobilised PBMCs after stimulation with CMVpp65 peptides. The mean purity is further dissected to show CD4+ and CD8+ frequencies within the CD3+ populations of positively enriched fractions.
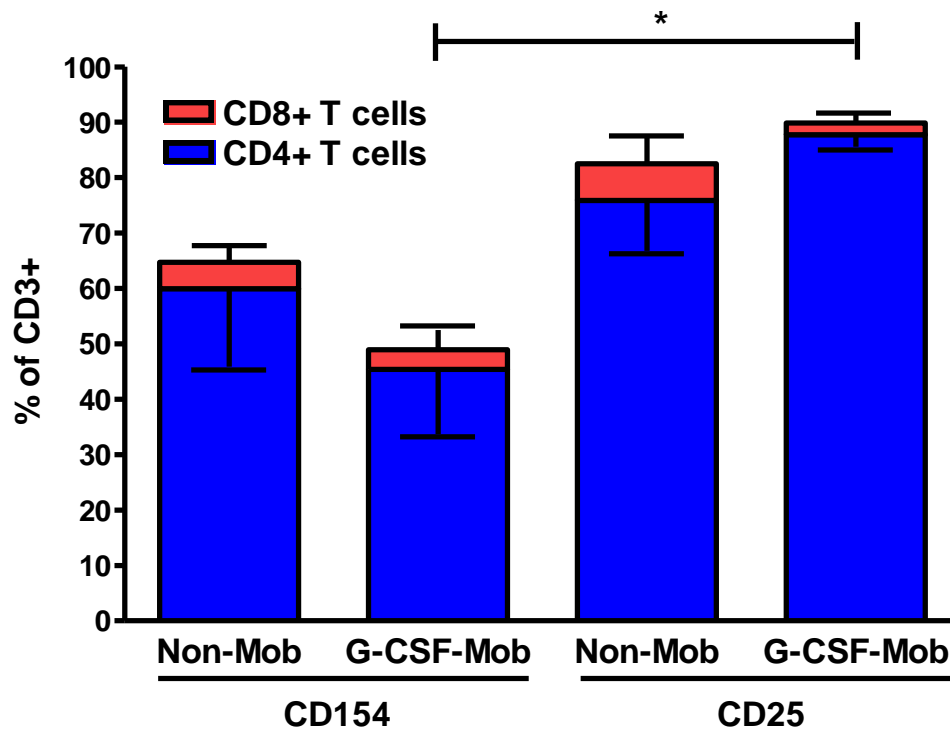
Figure 4.1 Summary of isolation of CMV-T through CD25 and CD154 in non-mobilised (n=5) and G-CSF-mobilised (n=5) donors:

CD154+ and CD25+ fractions after magnetic enrichment were measured for purity and the CD8+ and CD4+ T cell populations assessed.

*P<0.05

Mob, mobilised
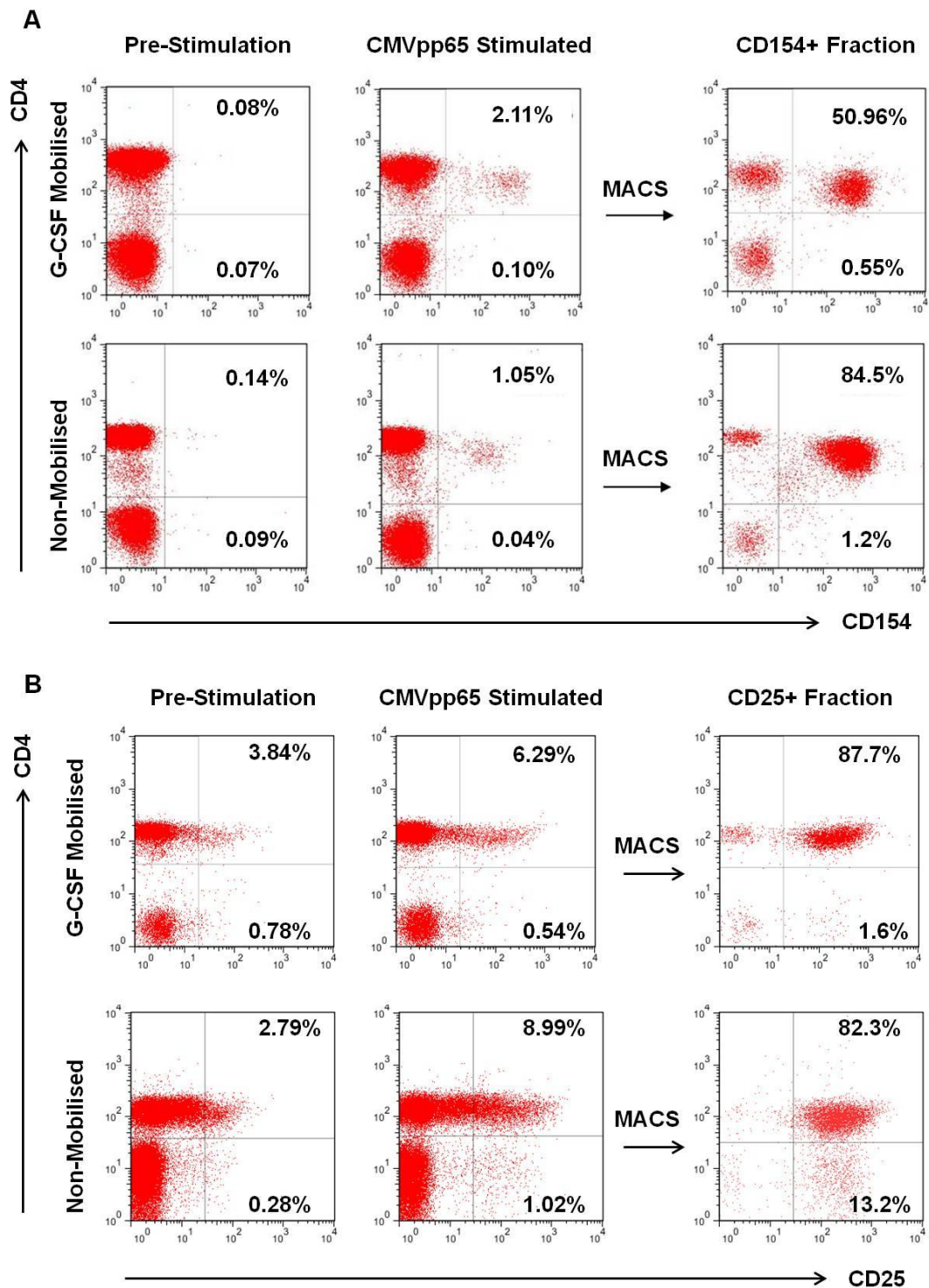
Figure 4.2 Isolation of CMV-T through CD154 and CD25 activation-induced expression.

PBMCs isolated from non-mobilised and G-CSF-mobilised donors were stimulated with CMVpp65 peptides for either 6 hours for CD154 isolation (A) or 16 hours for CD25 isolation (B) and antigen expression measured amongst CD3+ lymphocytes, before stimulation, after stimulation and after magnetic enrichment.
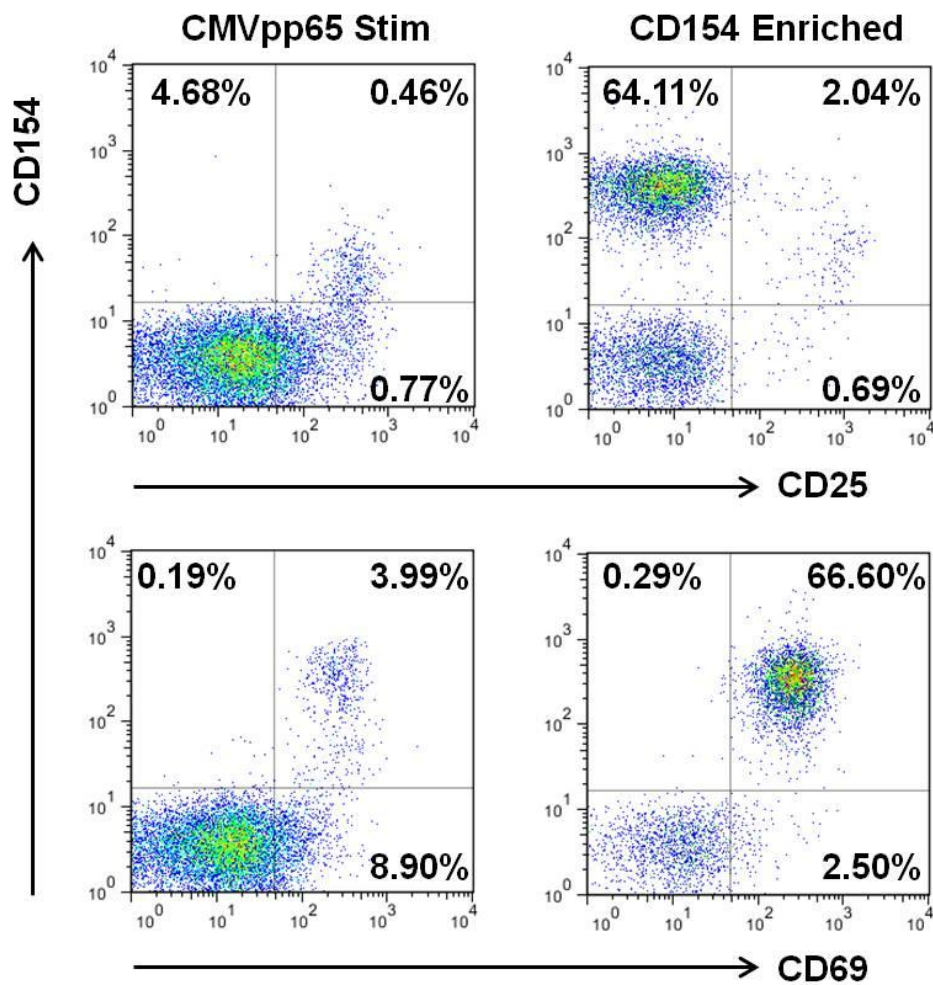
Figure 4.3 Co-expression of activation markers in CD154 CMV-T in G-CSF-mobilised PBMCs:

After a 6-hour CMVpp65 peptide stimulation and subsequent CD154 isolation by magnetic bead enrichment, CD3+ T cells were analysed for CD154, CD25 and CD69 co-expression for phenotypic assessment.
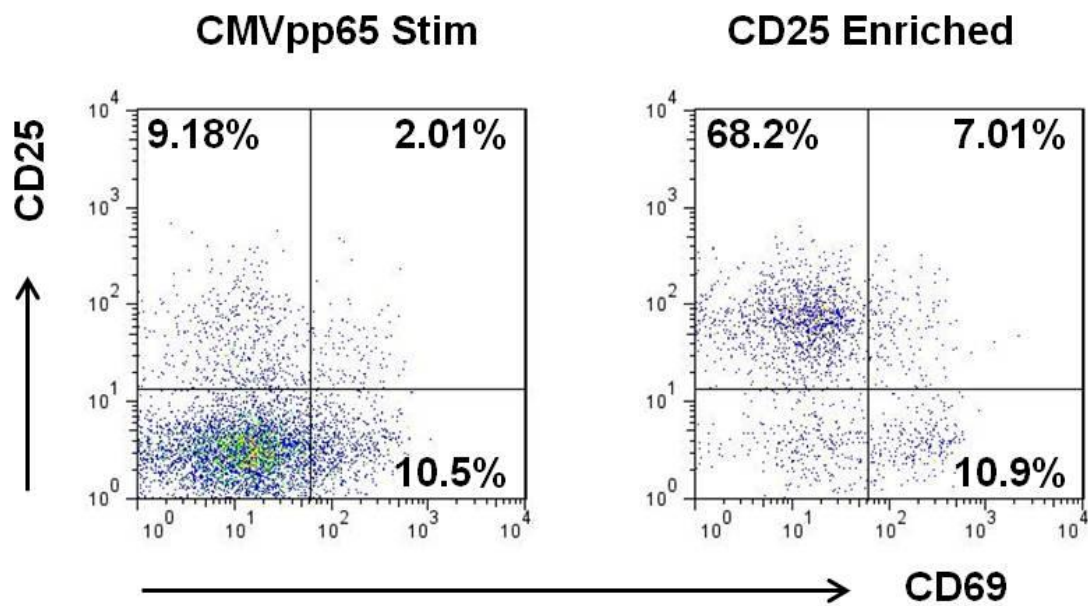
Figure 4.4 Co-expression of activation markers in CD25 CMV-T in G-CSF-mobilised PBMCs:

After a 16-hour CMVpp65 peptide stimulation and subsequent CD25 isolation by magnetic bead enrichment, CD3+ T cells were analysed for CD25 and CD69 co-expression for phenotypic assessment.

4.4.2 FoxP3 expression in CD25+ CMV-T

To evaluate whether CD25 is a suitable target for the isolation of CMV-T, the level of Treg enrichment was determined by analysing the proportion of CD25+FoxP3+ cells amongst CD4+ T cells at three time points: (1) pre-CMVpp65 stimulation; (2) post-CMVpp65 stimulation; and (3) post-CD25 enrichment. Figure 4.5 illustrates a representative experiment in both a G-CSF-mobilised and non-mobilised donor and shows two main findings; first that CMVpp65 stimulated G-CSF-mobilised PBMCs contain a higher proportion of CD25+ cells expressing FoxP3 and second that this trend was continued after enrichment.

Figure 4.6 summarises experiments from G-CSF-mobilised and non-mobilised donors (n=5, both). In pre-stimulated cells, a trend towards a significant increase in the mean proportion of CD25+FoxP3+ cells was observed in G-CSF-mobilised (1.80% ± 0.32) vs. non-mobilised (0.94% ± 0.29) PBMCs (p=0.07), shown in Figure 4.6A. This was consistent with a significant decrease of CD25+FoxP3– cells (Figure 4.6B) in G-CSF-mobilised (1.31% ± 0.42) vs. non-mobilised (4.82% ± 1.35) pre-stimulated PBMCs (p=0.03). Interestingly, no significant difference in CD25+FoxP3+ expression was observed post-CMVpp65 stimulation in G-CSF-mobilised (3.67% ± 0.62) and non-mobilised (3.16% ± 1.09) PBMCs (p=0.69). However, the proportion of CD25+FoxP3+ cells after CD25 enrichment was shown to be significantly increased in G-CSF-mobilised (64.24% ± 4.88) vs. non-mobilised (33.84% ± 7.17) PBMCs (p=0.008), and conversely with CD25-FoxP3- cells (21.19% ± 4.03 vs. 38.72% ± 2.70 respectively; p=0.006).

Experiments were carried out to determine the absolute number of CD25+ and FoxP3+ cells per 10,000 CD4+ T cells. Figure 4.6 presents the data from CD25+ CMV-T isolation in G-CSF-mobilised and non-mobilised PBMCs. Analysis of mean FoxP3 expression amongst CD25+CD4+ T cells highlights the increased FoxP3 expression in G-CSF-mobilised (41.35% ± 8.41) compared with non-mobilised (17.5% ± 6.5) PBMCs at rest (p=0.05). After CMVpp65 stimulation, a significant increase in mean FoxP3 expression amongst CD25+CD4+ was observed in G-CSF-mobilised (42.79% ± 4.07) compared with non-mobilised (20.84% ± 5.46) PBMCs (p=0.012). Figure 4.7 illustrates the significantly increased expression of FoxP3 amongst CD25+CD4+ after CD25

enrichment in G-CSF-mobilised (73.64% ± 4.88) compared to non-mobilised (43.82% ± 5.64) PBMCs (*p*=0.004).
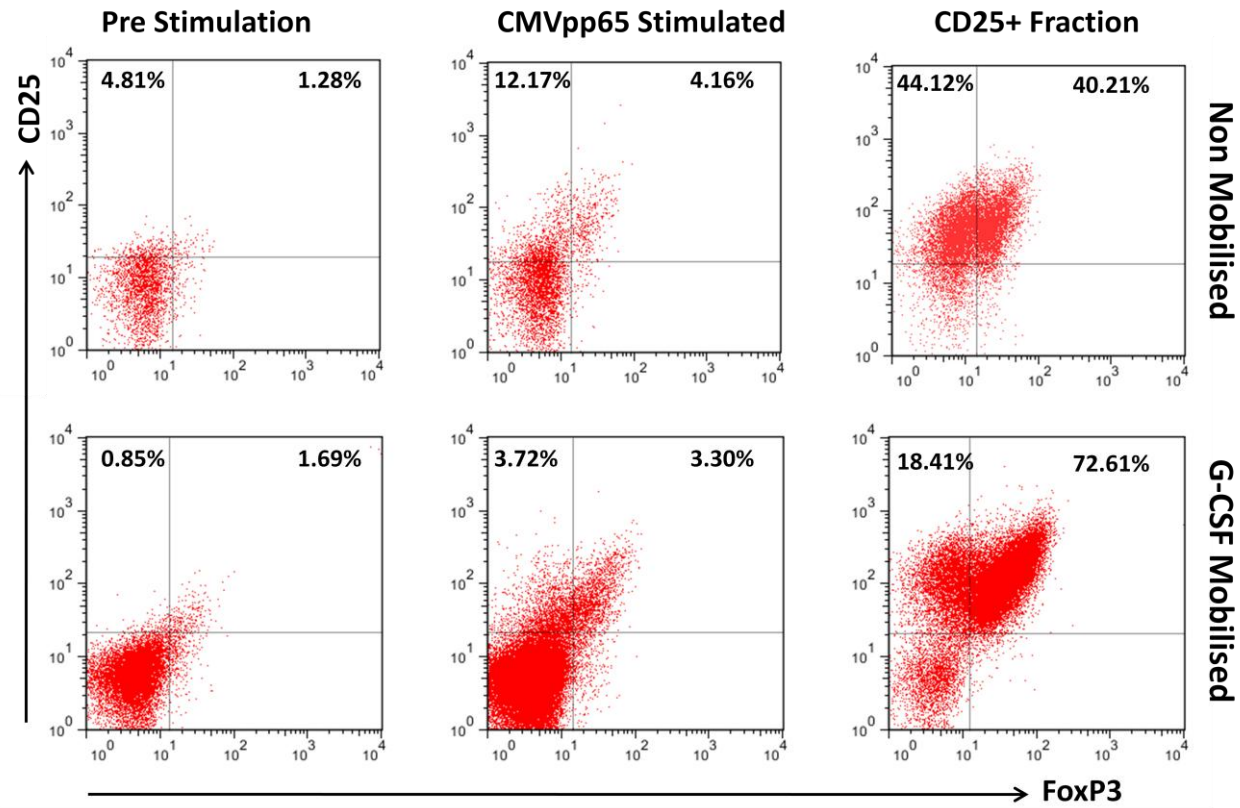
Figure 4.5 Comparison of FoxP3 expression in CD4+ CD25+ T cells between a non-mobilised and a G-CSF-mobilised PBMC donor:
FoxP3 expression was assessed in pre-stimulated cells, CMVpp65-stimulated cells, and in CD25+ fractions post enrichment. Plots are derived from gating on CD3+ CD4+ cells.
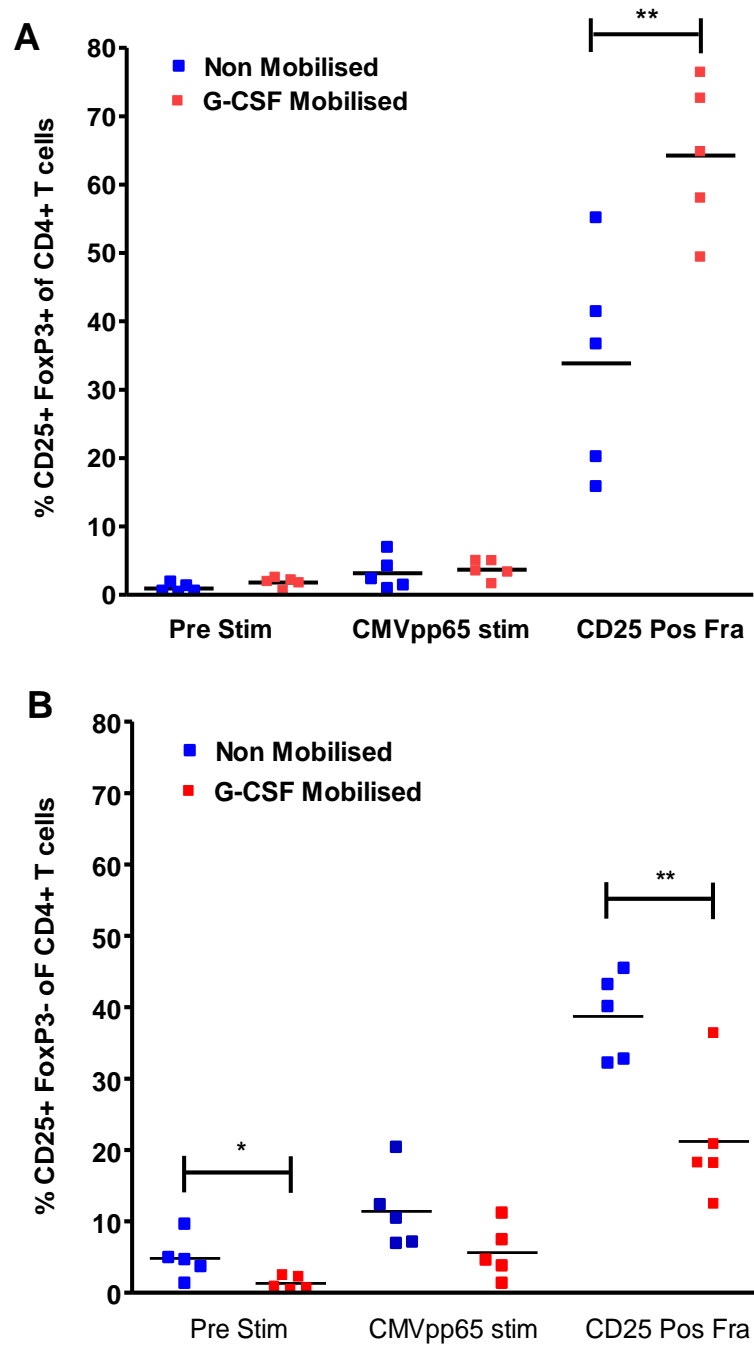
Figure 4.6 Evaluation of FoxP3 expression in G-CSF-mobilised and non-mobilised PBMCs:

FoxP3 expression was analysed in CD25-expressing (A) and CD25 negative (B) CD4+ T cells in G-CSF-mobilised (n=5) and non-mobilised (n=5) donors pre-CMVpp65 stimulation, post-CMVpp65 stimulation and post-CD25 enrichment.

*$P<0.05$ and **$P<0.01$, in an unpaired *t* test

Stim, stimulated; Fra, fraction;
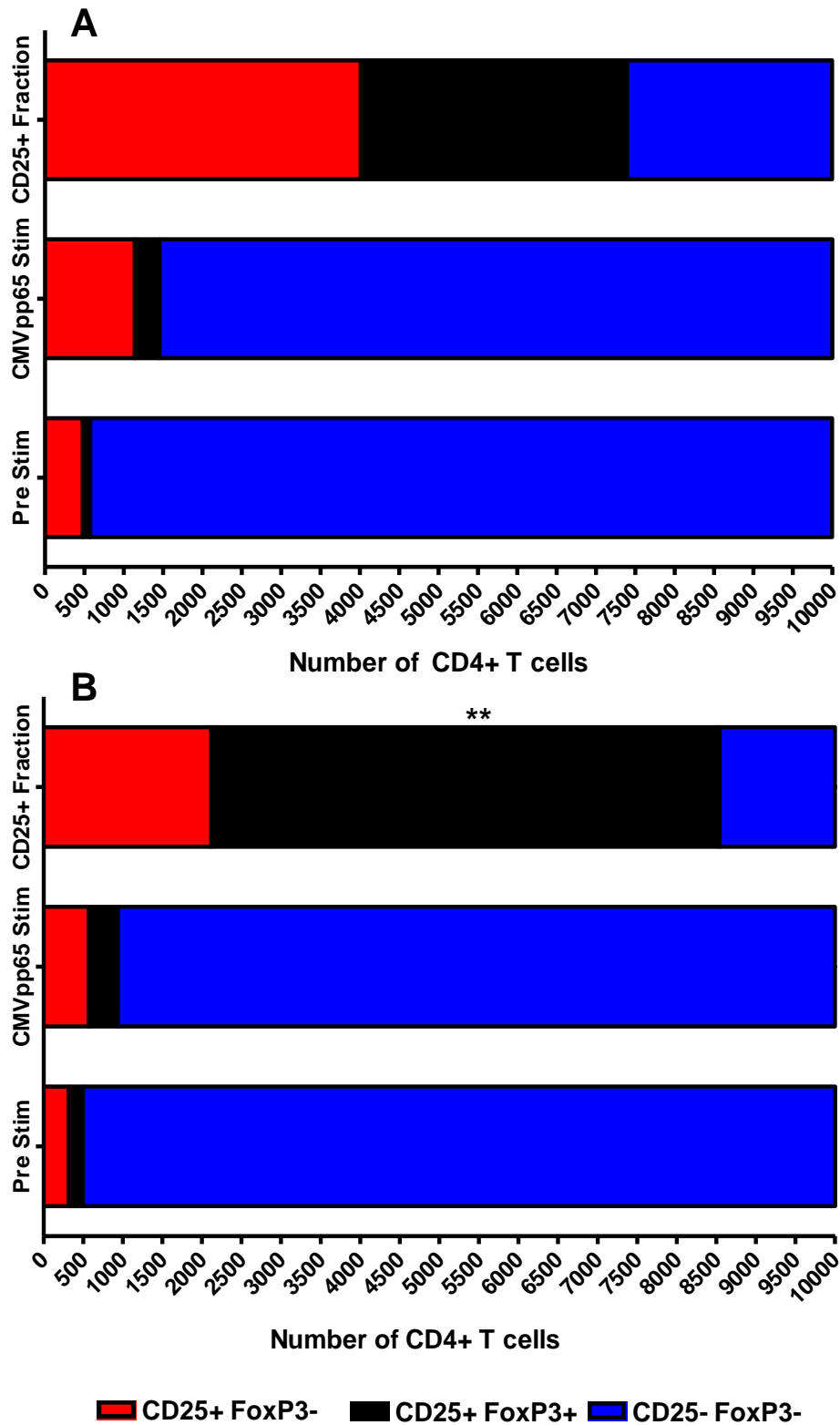
Figure 4.7 Assessment of FoxP3 expression in non-mobilised (A) and G-CSF-mobilised (B) PBMCs:

CD25 and FoxP3 expression was assessed per 10,000 CD4+ T cells pre-CMVpp65 stimulation, post-CMVpp65 stimulation and post-CD25 enrichment. $**P<0.01$ in an unpaired *t* test comparing CD25+FoxP3+ expression in the CD25+ fraction between G-CSF-mobilised and non-mobilised PBMCs

4.4.3 Assessment of suppressive nature of CD25+ enriched CMV-T in G-CSF-mobilised PBMCs

Having demonstrated that CD25+ cells enriched after CMVpp65 stimulation from G-CSF-mobilised PBMCs contain a significant proportion of FoxP3-expressing cells, their suppressive activity was assessed using a CFSE-based proliferation assay. Initial experiments aimed to establish whether conventional CD25+FoxP3+ Tregs isolated from G-CSF-mobilised PBMCs were capable of suppressing T cell proliferation in response to SEB stimulation. As illustrated in Figure 4.8 in a representative experiment, CD25+ conventional Tregs were able to suppress proliferation of CFSE-labelled PBMCs when cultured at a ratio of 1:1 (38% of undivided CD3+ T cells) compared with PBMCs alone (22.3% undivided CD3+ T cells). When CFSE-labelled PBMCs were cultured in the presence of CD25− cells, no suppression was evident (13.9% of undivided CD3+ T cells) compared to PBMCs alone. The elevated T cell proliferation compared with PBMCs alone could be explained by an improvement in culture conditions with CD25− cells acting as feeder cells for the CFSE-labelled PBMCs.

Next we evaluated the suppressive capacity of CD25+ T cells enriched after CMVpp65 stimulation in G-CSF-mobilised PBMCs and compared this with the CD25− fraction after magnetic enrichment. Both fractions were cultured at ratios of 1:1 and 2:1 and in some experiments at 5:1, with CFSE-labelled PBMCs. Suppression experiments were carried out with CD25+ enriched cells from G-CSF-mobilised and non-mobilised donors (n=4, both). Figure 4.8 illustrates two representative experiments from the four G-CSF-mobilised donors. T cell proliferation in the presence of CD25− cells was similar to PBMCs alone (18.4% undivided CD3+ T cells) at both 1:1 (25.6% undivided CD3+ T cells) and 2:1 (24.3% undivided CD3+ T cells) illustrated in Figure 4.9A. In contrast, T cell proliferation was greatly reduced when CFSE-labelled PBMCs were cultured in the presence of CD25+ CMV-T at both 1:1 (95% undivided CD3+ T cells) and 2:1 (96.3% undivided CD3+ T cells), highlighting a strong suppressive capacity. Figure 4.9B shows a similar result in terms of strong T cell suppression exerted by CD25+ CMV-T and is achieved at a ratio of 1:1 (28.5% CD3+ T cells undivided) and at

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5:1 (34.6% CD3+ T cells undivided) compared to PBMCs alone after SEB stimulation (5.69% CD3+ T cells undivided).

Suppression of T cell proliferation by CD25+ CMV-T from non-mobilised PBMCs was lower than that from G-CSF-mobilised PBMCs. Figure 4.10 shows representative experimental results from two donors. Proliferation of CFSE-labelled PBMCs in response to SEB was lower when cultured with CD25+ cells at both 1:1 (19.9% undivided CD3+ T cells) and 5:1 (27.7% undivided CD3+ T cells) ratios than with CD25− cells at ratios of both 1:1 (15.3% undivided CD3+ T cells) and 5:1 (14.1% undivided CD3+ T cells) (Fig 4.10A). However, when PBMCs were stimulated alone, 27.9% of CD3+ T cells remained undivided, suggesting that no suppression occurred with CD25+ CMV-T from non-mobilised PBMCs. A probable explanation for this observation is the significant reduction in the proportion of FoxP3+ cells seen in the CD4+ CD25+ fraction from non-mobilised PBMCs compared with G-CSF-mobilised PBMCs after CMVpp65 stimulation. The reduction in the proportion of Tregs would be expected to reduce the suppressive activity of non-mobilised CD25+ CMV-T. Figure 4.10B illustrates suppression of T cell proliferation by CD25+ CMV-T at a ratio of 1:1 with CFSE-labelled PBMCs (39.2% CD3+ T cells undivided) compared with CD25− cells (14.7% CD3+ T cells undivided), but also at a reduced level than that seen with CD25+ CMV-T isolated from G-CSF-mobilised PBMCs.
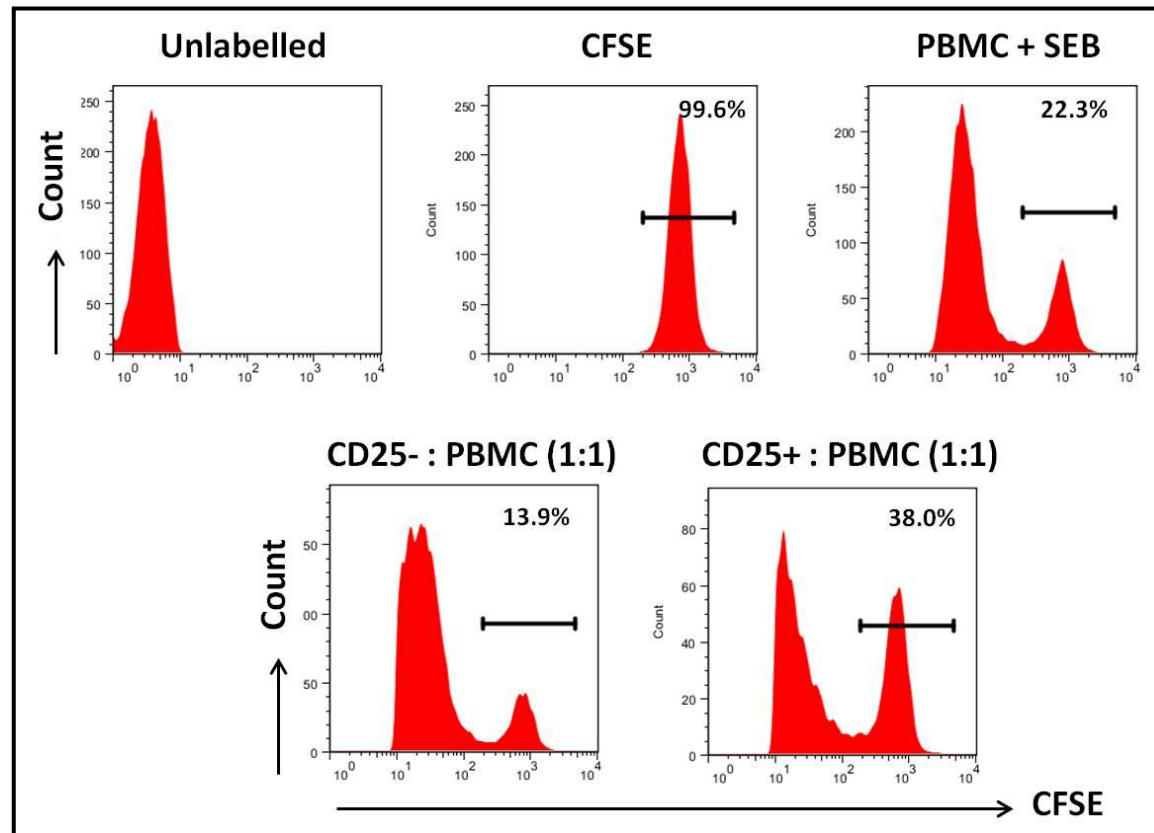
Figure 4.8 Dose-dependent suppression of T cell proliferation by CD25+ T cells in G-CSF-mobilised PBMCs: CD25– and CD25+ T cells isolated by magnetic enrichment were cultured at a 1:1 ratio with autologous PBMCs in the presence of SEB. Unlabelled PBMCs, CFSE-labelled PBMCs alone (CFSE), and CFSE-labelled PBMCs in the presence of SEB were cultured as experimental controls.
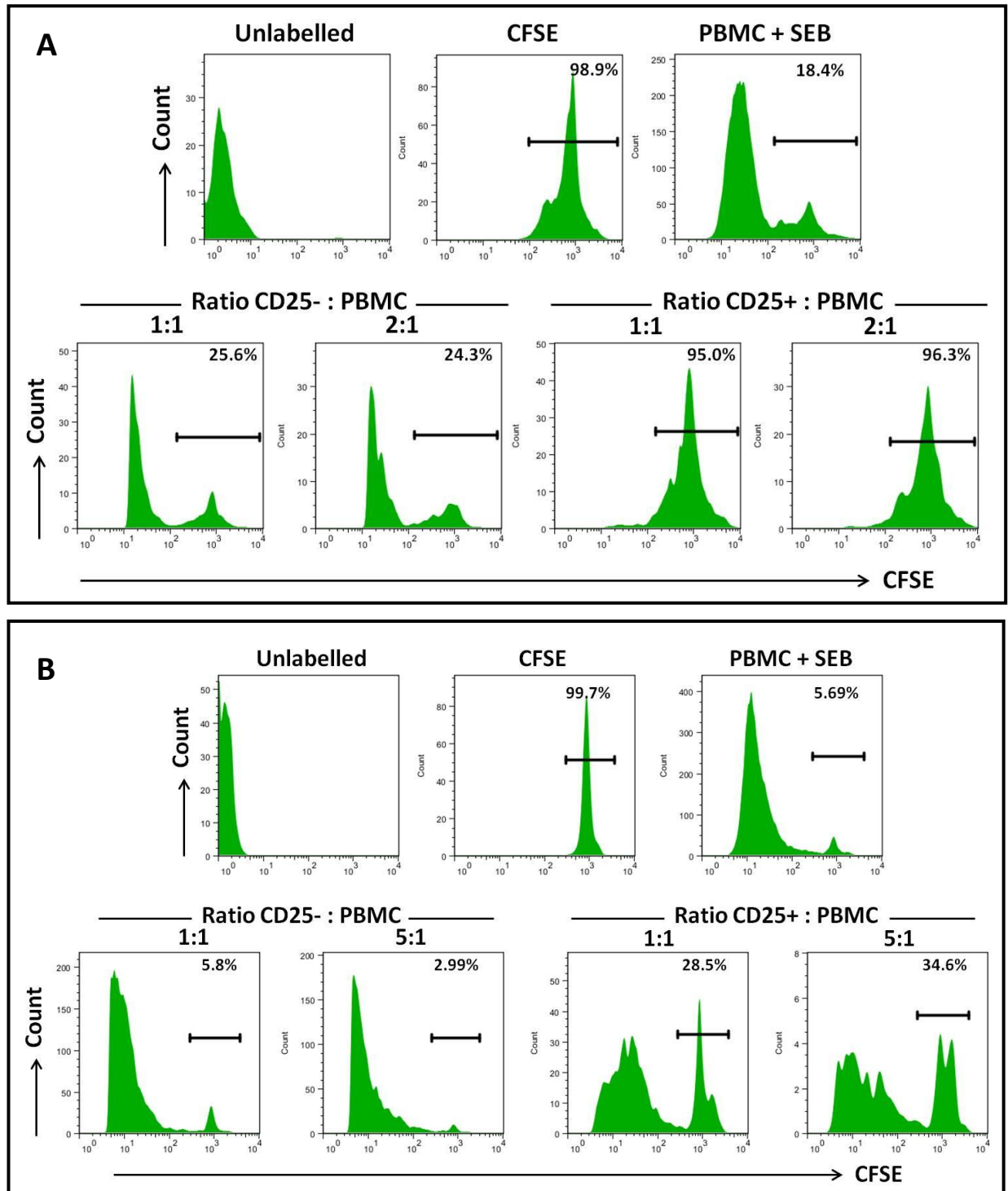
Figure 4.9 CFSE Suppression assay in G-CSF-mobilised PBMCs in two donors (A-B): Dose dependent suppression of T cell proliferation was assessed by CD25 positive and CD25 negative fractions after CMVpp65 stimulation and subsequent CD25 enrichment. CFSE-labelled PBMCs were cultured at two ratios in the presence of SEB for 5 days. Unlabelled, PBMCs alone (CFSE) and PBMCs with SEB were cultured as experimental controls.
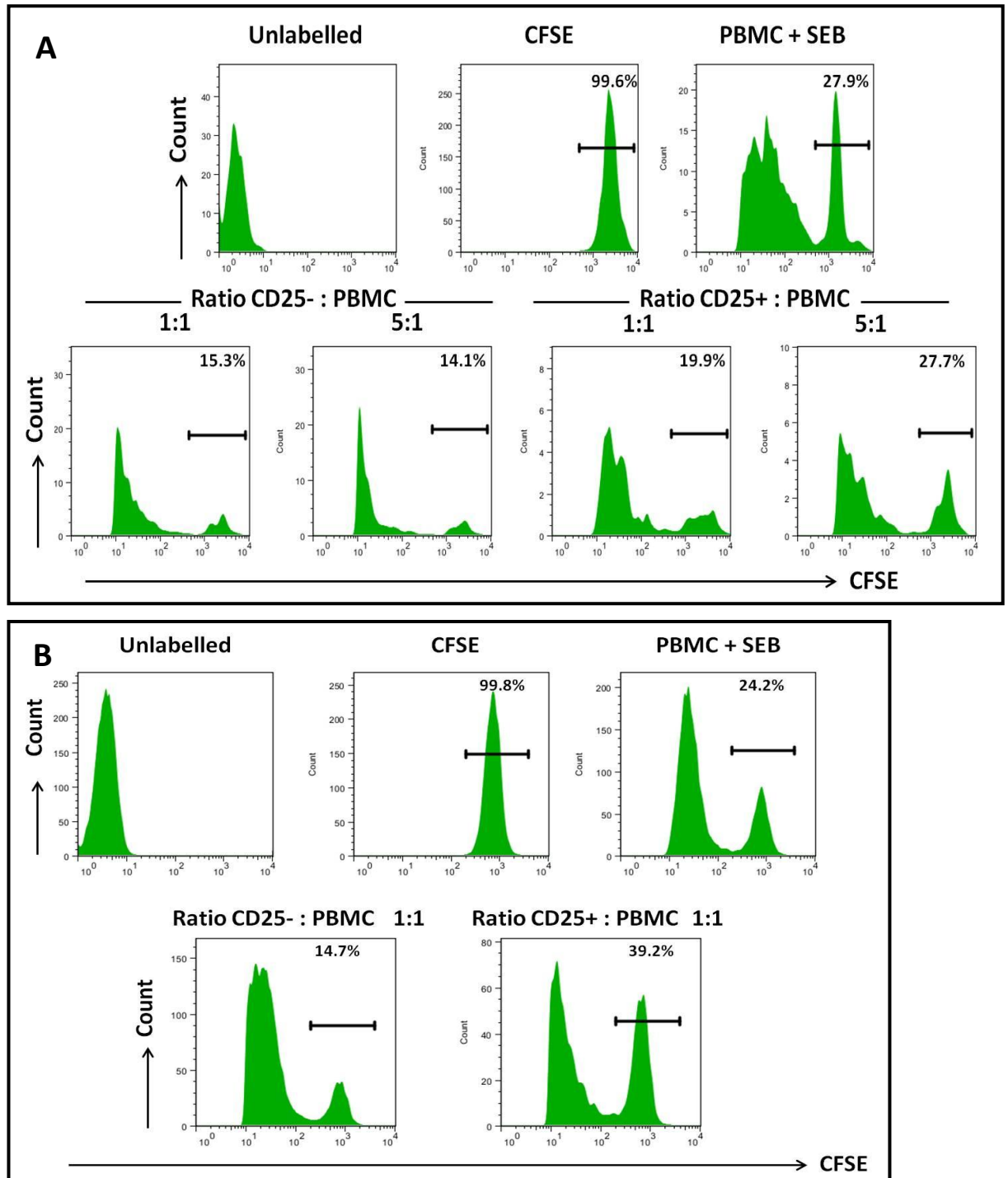
Figure 4.10 CFSE Suppression assay in Non-Mobilised PBMCs in two donors (A-B):

Dose dependent suppression of T cell proliferation was assessed by CD25 positive and CD25 negative fractions after CMVpp65 stimulation and subsequent CD25 enrichment. CFSE-labelled PBMCs were cultured at two ratios in the presence of SEB for 5 days. Unlabelled, PBMCs alone (CFSE) and PBMCs with SEB were cultured as experimental controls.

4.4.4 Depletion of CD25-expressing cells prior to CMV stimulation

4.4.4.1 Impact on CD25 activation-induced expression and isolation

Having found that CMV-T isolated from G- CSF-mobilised PBMCs- through CD25 expression contain a significant proportion of FoxP3 expressing cells and exert a strong suppressive capacity, the aim of the next series of experiments was to determine the effect of removal of naturally expressing CD25 cells, prior to CMVpp65 stimulation, on the Treg content of isolated CD25+ CMV-T. Figure 4.11 shows the effect of CD25 depletion compared with non-depleted PBMCs in a representative G-CSF-mobilised donor. CD25 expression post-CMVpp65 stimulation was dramatically reduced in 'CD25-depleted' PBMCs as was background CD25 expression in the negative control (unstimulated) sample. CD25 depletion prior to CMVpp65 stimulation had a detrimental effect on the purity of the CD25+ fraction after magnetic enrichment compared with the non-depleted sample (17.92% vs. 74.24% respectively). Figure 4.12 summarises the effect of CD25 depletion in G-CSF-mobilised (n=3) and non-mobilised (n=4) donors. Although not significant, results showed a trend towards reduced mean CD25+ expression amongst CD3+ T cells in both G-CSF-mobilised (2.87% ± 0.22 vs. 1.58% ± 0.59; *p*=0.14) and non-mobilised (3.92% ± 1.39 vs. 1.13% ± 0.61; *p*=0.16) PBMCs after CD25 depletion prior to CMVpp65 stimulation.

The effect of CD25 depletion on the purity and yield of CD25+ cells is shown in Figure 4.13. In G-CSF-mobilised PBMCs, purity (88.91% ± 0.58 vs. 17.08% ± 12.78; *p*=0.03) was significantly reduced and a trend towards reduced yield (30.68% ± 0.88 vs. 11.26% ± 7.80; *p*=0.11) was observed, whereas in non-mobilised PBMCs, both yield (28.27% ± 9.69 vs. 19.01% ± 10.94; *p*=0.02) and purity (69.16% ± 11.29 vs. 25.68% ± 3.37; *p*=0.03) were significantly reduced in CD25-depleted PBMCs.
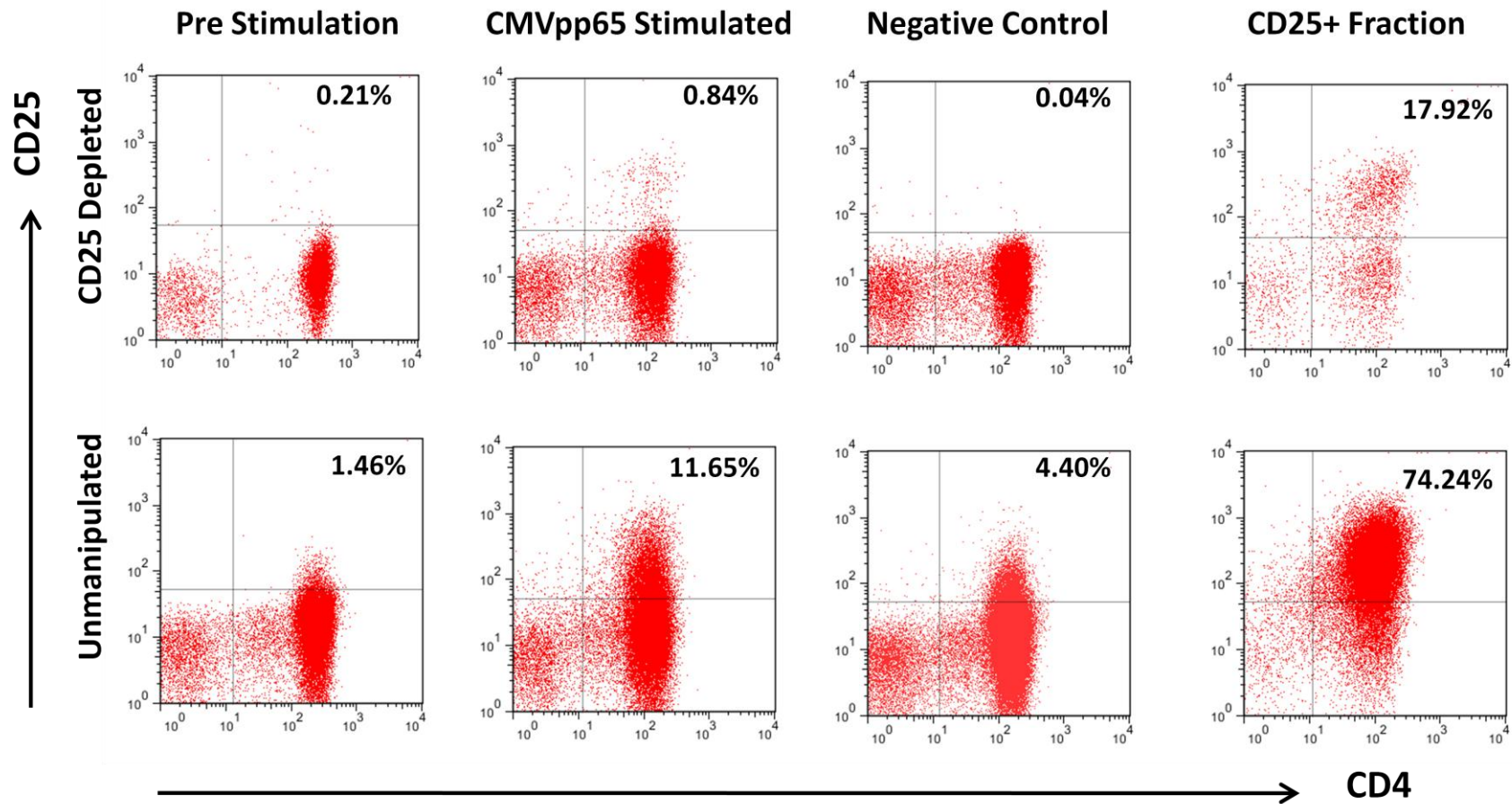
Figure 4.11 Effect of depletion of naturally occurring CD25+ cells in G-CSF-mobilised PBMCs on CD25 expression post-CMVpp65 stimulation and post-CD25 enrichment: A Paired sample either non-depleted or CD25-depleted are shown from a G-CSF-mobilised donor. Plots are derived from CD3+ gated cells after gating on the live lymphocyte population using FSC vs. SSC
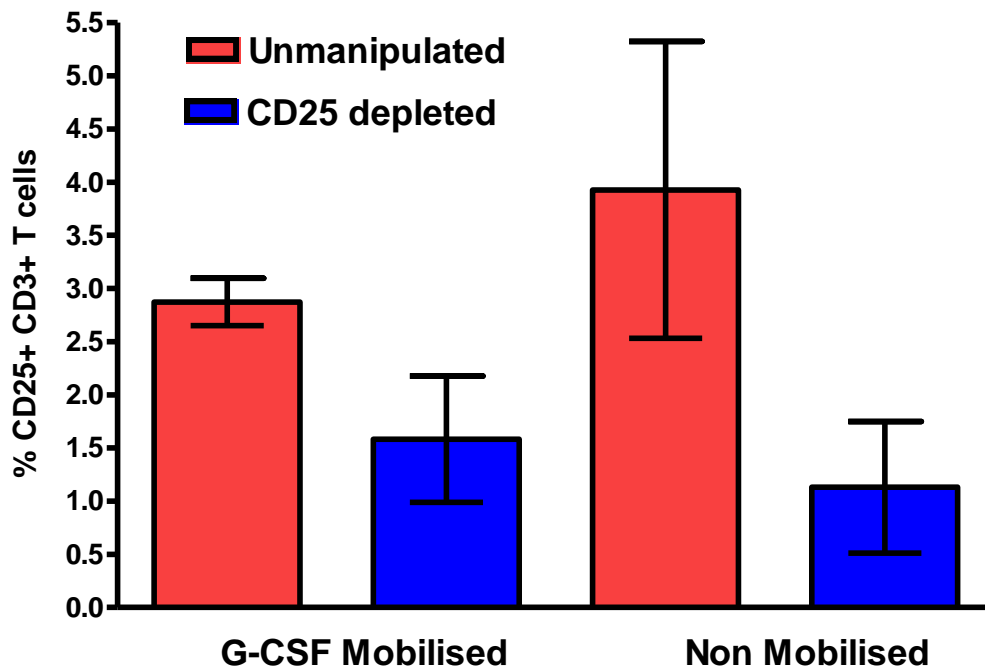
Figure 4.12 CD25+ expression after CMVpp65 stimulation in non-depleted and CD25-depleted non-mobilised and G-CSF-mobilised PBMCs:

Paired samples from both G-CSF-mobilised (n=3) and non-mobilised (n=4) PBMCs donors were stimulated for 16 hours with CMVpp65 peptides and CD25 expression analysed amongst CD3+ T cells. Bars represent the mean (± SD) percentage after subtraction of the negative control (no peptide)
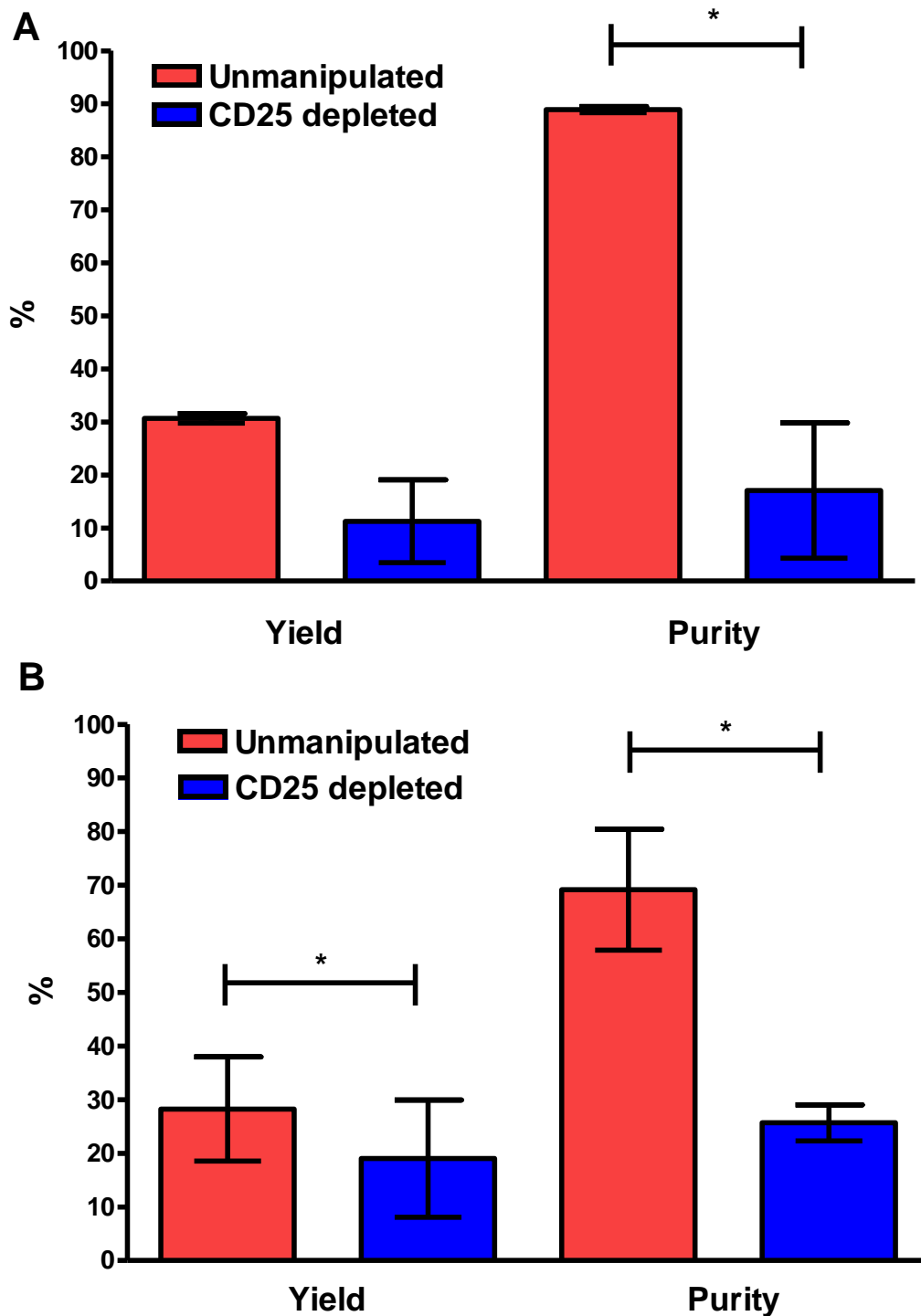
Figure 4.13 Comparison of isolation of CD25-expressing cells after CMVpp65 stimulation between non-depleted and CD25-depleted PBMCs: G-CSF-mobilised PBMCs (A) and non-mobilised PBMCs (B) were depleted of CD25-expressing cells prior to stimulation or left non-depleted before CD25 enrichment. Bars represent mean (± SD) percentage of purity and yield of CD25+ cells from within the CD3+ population. *P<0.05 in a paired *t* test

4.4.4.2 Impact on FoxP3 expression

The following experiments aimed to establish the level of FoxP3 expression in CD25+CD4+ cells after depletion of contaminating Tregs. Data presented in Section 4.4.2 showed that after CMVpp65 stimulation of G-CSF-mobilised PBMCs the mean proportion of FoxP3+ cells amongst CD25+CD4+ T cells was 43% ± 4.0. This was significantly reduced to 8.5% ± 5.4 (*p*=0.001) in G-CSF-mobilised PBMCs that had been depleted of contaminating Tregs prior to CMVpp65 stimulation. Similarly, after CD25 enrichment, FoxP3 expression amongst depleted CD25+CD4+ T cells showed a mean of 28.4% ± 12.3 compared with 73.6% ± 4.9 (*p*=0.006) in non-depleted G-CSF-mobilised PBMCs (Section 4.4.2).

In non-mobilised PBMCs post-stimulation, there was no significant difference in mean FoxP3 expression between depleted and non-depleted CD25+ CD4+ T cells (19.6% ± 9.4 vs. 20.8% ± 5.4, respectively; *p*=0.90). However, after magnetic enrichment of CD25+ CMV-T, a significant reduction in mean FoxP3 expression was observed in depleted vs. non-depleted PBMCs (14.6% ± 2.4 vs. 43.8% ± 5.6, *p*=0.009). The results from CD25 depletion experiments and the impact on FoxP3 expression is summarised in Figure 4.13, illustrating the proportion of CD25+ FoxP3 expressing cells amongst CD4+ T cells. Figure 4.14 also highlights the negative impact on the proportion of CD25+ cells after CMVpp65 stimulation and purity after CD25 enrichment.
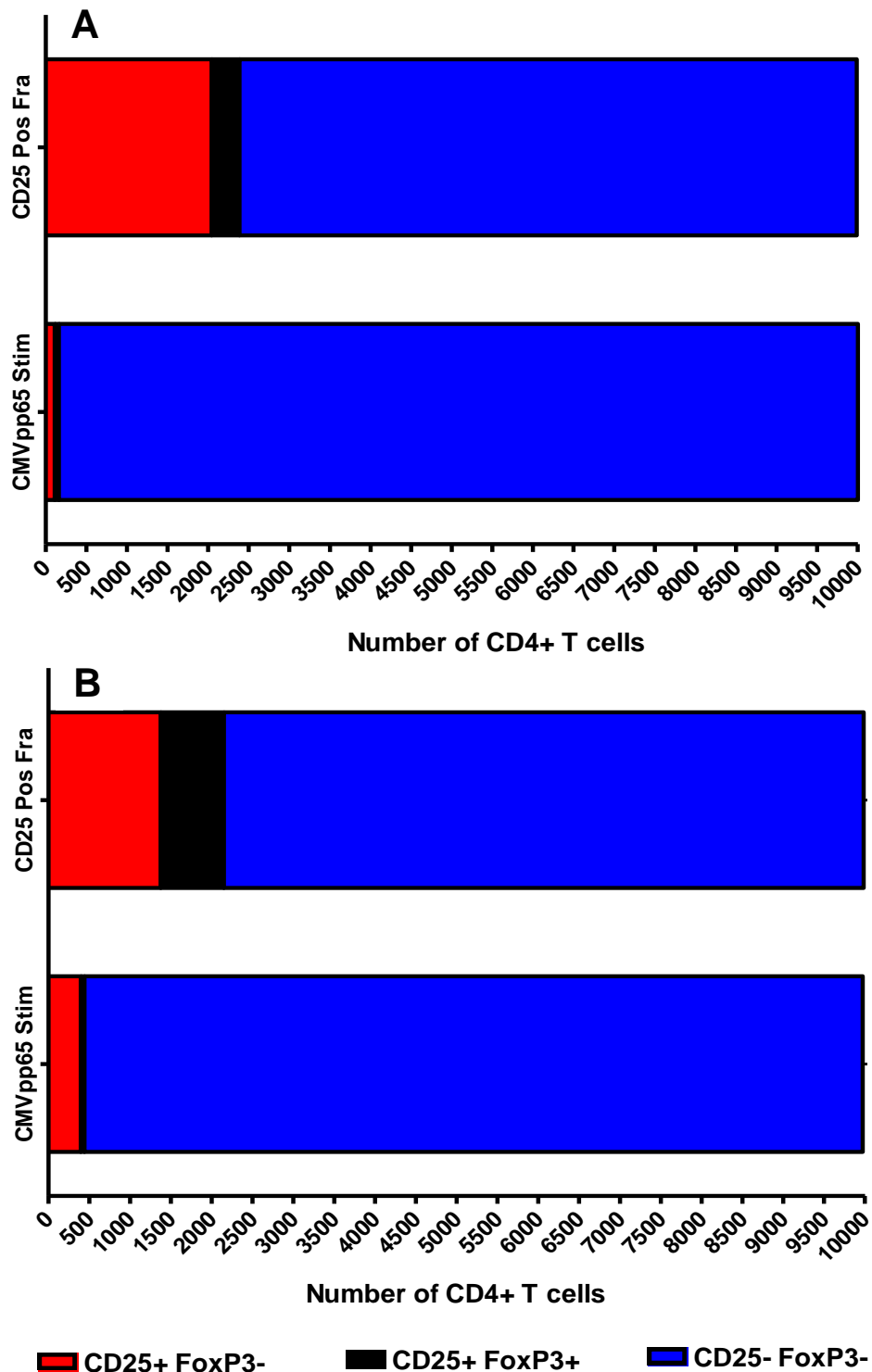
Figure 4.14 Assessment of FoxP3 expression after depletion of naturally occurring CD25-expressing cells: CD25 and FoxP3 expression was assessed per 10,000 CD4+ T cells post-CMVpp65 stimulation and post-CD25 enrichment in non-mobilised (A) and G-CSF-mobilised (B) PBMCs.

4.5   Discussion

Initial experiments targeted at the identification and isolation of CMV-T from G-CSF mobilised PBMC had highlighted two activation markers, CD25 and CD154, as candidates for further investigation and also demonstrated the sub-optimal use of IFN-γ secretion as a tool for enrichment of CMV-T. The results described in this chapter follow the isolation of CMV-T from G-CSF-mobilised through CD25 and CD154 and the subsequent functional analysis of CD25 enriched cells with regard to Tregs, due their similar CD25 expression pattern. Experiments using G-CSF-mobilised PBMCs were compared directly to non-mobilised PBMCs to allow for any significant observations to be made.

Isolation of CMV-T from G- CSF-mobilised PBMCs using both CD25 and CD154 enrichment demonstrated equivalence with non-mobilised in terms of purity. CD25- and CD154-enriched CMV-T were predominantly CD4+ cells, which is in keeping with published literature on non-mobilised PBMCs (Frentsch *et al*, 2005; Gallot *et al*, 2001). The purity of CD25-enriched CMV-T was found to be significantly higher than CMV-T isolated through CD154 expression in G-CSF-mobilised PBMCs. This difference can be explained by the increased proportion of CD25-expressing T cells prior to enrichment compared with CD154+ cells, as the efficiency of magnetic enrichment increases with the total number of target cells to be isolated. Only by performing clinical scale sorting, where the number of cells being sorted are greatly increased from ~50 x 10^6 to 1 x 10^9 cells, can the impact on purity of CD154 and CD25 be definitively evaluated.

The co-expression of CD25 and CD154 after CD154 enrichment was also investigated as CD154 cells were predominantly CD4+, suggesting the hypothesis that CD154 enrichment could reveal a population of Tregs. It has been shown relatively recently that antigen-specific CD154+ cells can be identified within a population of CD25+ Tregs after CMV stimulation, although the kinetics of CD154 expression in CD25+ Tregs differs from that in effector T cells where maximal expression is revealed at 6 hours compared with only minimal CD154+ expression in CD25+ Tregs at the same time point (Litjens *et al*, 2012). The results presented here show that in G-CSF-mobilised PBMCs, after a 6 hour stimulation with CMVpp65 peptides, CD154+ cells expressed

very little CD25 and this was continued following subsequent CD154 enrichment. In contrast, CD154 cells co-expressed CD69, demonstrating an effector T cell population, whereas CD25+ cells co-expressed minimal CD69 expression after CMVpp65 stimulation and CD25+ enrichment.

The next part of this chapter focussed on investigating the functionality of CD25 CMV-T isolated from G-CSF-mobilised PBMCs, with reference to Tregs, which also express the CD25 molecule (Sakaguchi *et al*, 1995). CD25+ cells enriched from G-CSF-mobilised PBMCs after CMV stimulation contained a high frequency (>65%) of expression of the transcription factor FoxP3, a key molecule for identification of suppressive function in Tregs (Fontenot *et al*, 2003). In comparison with G-CSF-mobilised PBMCs, CD25+ cells enriched from non-mobilised PBMCs showed a significantly reduced frequency of FoxP3 expression (<35%; $p<0.01$). There are two main conclusions that can be drawn from these findings. First, that G-CSF mobilisation increases the number of naturally circulating CD25+ Tregs; and second, that G-CSF mobilisation increases the frequency of CMVpp65-reactive Tregs. Both points raise valid arguments about the feasibility of generating a CMV-T product for clinical use that contains such a high proportion of FoxP3-expressing Tregs. To address the first point, there are very little published data investigating the impact of G-CSF mobilisation on circulating Tregs. Tayebi and colleagues (Tayebi *et al*, 2001) observed that after G-CSF mobilisation, the percentage of CD3+CD25+ cells significantly decreased in normal healthy donors, contradictory to the results seen here, and, furthermore, did not examine FoxP3 expression. Studies in rhesus macaques, have shown that the immunostimulant AMD3100, used for the effective mobilisation of CD34+ cells, also efficiently mobilised CD4+CD25^{high}FoxP3+ into the periphery significantly more than mobilisation with G-CSF (Kean *et al*, 2011). Another study that analysed Tregs after G-CSF mobilisation showed a 2-3 fold increase in CD25+ T cells that expressed high levels of FoxP3, but this investigation examined patients mobilised for autograft who were also administered with high-dose cyclophosphamide (Condomines *et al*, 2006). The cytotoxic effect of low-dose cyclophosphamide has been shown by several groups to result in the depletion of Tregs (Frentsch *et al*, 2005; Hoover *et al*, 1990; Ikezawa *et al*, 2005; Lutsiak *et al*, 2005). It has been reported in a murine study that high-dose cyclophosphamide had no effect on the absolute number of

Tregs but a reduced ratio of Tregs among CD4+ T cells (Hong *et al*, 2010). This would suggest that the increase in Treg numbers seen by Condomines and colleagues was as a result of G-CSF administration. More recently, studies investigating the impact of G-CSF-mobilisation on Treg numbers and function, showed that the number of CD4+CD25^{high}CD127- Tregs increased 1.6-fold following mobilisation and remained highly suppressive on the proliferation of effector T cells (Ukena *et al*, 2012). Although the frequency of CD25+FoxP3+ cells in non-stimulated G-CSF-mobilised PBMCs were not significantly increased when compared with non-mobilised PBMCs, the results presented here suggest a trend towards increased frequency that might attain statistical significance in a study of a larger cohort. Therefore, the hypothesis is that CD25 enrichment after CMVpp65 stimulation favours selection of CD25+ cells with a Treg phenotype due to increased numbers of Tregs in circulation after G-CSF mobilisation.

On the second point above, many studies have indicated the existence of CMV-reactive Tregs and indeed Ltjens and colleagues concluded that CD4+CD25+CD127-FoxP3+ Tregs contained antigen-specific T cells (Litjens *et al*, 2012), which is consistent with the results seen here. The effect of G-CSF mobilisation appears to elevate Treg numbers beyond that of non-mobilised PBMCs and would therefore account for the increased numbers of CD25+FoxP3+ cells observed after CMVpp65 stimulation and, more significantly, after CD25 enrichment. However, studies have also indicated that FoxP3 expression can be induced in human CD4+ effector T cells after activation (Walker *et al*, 2005) albeit in CD4+ CD25– cells. This report built on this result with the finding that FoxP3 expression is a normal consequence of CD4+ T cell activation, bringing into doubt its validity as an exclusive marker of Tregs.

In the next section of this Chapter, I investigated whether the increased numbers of CD25+FoxP3+ cells after CD25 enrichment in G-CSF mobilisation were able to function as Tregs in suppressing T cell proliferation. Initial experiments determined that naturally occurring CD25+ Tregs isolated from G-CSF-mobilised PBMCs were capable of suppressing T cell proliferation in the same manner as conventional Tregs isolated from non-mobilised PBMCs. This is an important result in view of the widespread interest in manufacturing Tregs for adoptive immunotherapy without the need for ex-vivo expansion (Ukena *et al*, 2012).

Post-CMVpp65 stimulation, CD25-enriched CMV-T from G-CSF-mobilised PBMCs were assessed for their ability to suppress T cell proliferation. Results showed that at ratios of 1:1 and 2:1 CD25+ to CFSE-labelled PBMCs, CD25+ cells were capable of suppression, which was not seen with CD25− fractions. The level of suppression observed with CD25+ CMV-T enriched from G-CSF-mobilised PBMCs was elevated compared with that of non-mobilised PBMCs. The higher level of suppression mediated with CD25+ CMV-T enriched from G-CSF-mobilised PBMCs can be explained by the significantly higher number of CD25+FoxP3+ cells isolated from G-CSF-mobilised PBMCs after CMVpp65 stimulation compared with non-mobilised PBMCs. These results would suggest that CMVpp65 stimulation of G-CSF-mobilised PBMCs reveals a population of Tregs that are not present in non-mobilised PBMCs and are preferentially enriched. CMVpp65 stimulation in G-CSF-mobilised PBMCs could induce a population of CD25+ Tregs that have antigen specificities and are able to recognize CMVpp65 peptides and possibly proliferate in response to engagement of their cognate antigen. Support for this hypothesis was obtained in a murine model of *Leishmania* infection, in which Tregs proliferated in response to recognition of parasite-derived antigen (Suffia *et al*, 2006).

Very little published data exist regarding the emergence and generation of Tregs during CMVpp65 stimulation and certainly none with regard to G-CSF mobilisation. However, Melenhorst and colleagues (Melenhorst *et al*, 2008a) have demonstrated the emergence of a significant population of FoxP3+CD4+ and FoxP3+CD8+ T cells after CMVpp65 stimulation in non-mobilised PBMCs but failed to demonstrate any subsequent suppression. However, CD4+CD27-CD28- cells sorted from CMVpp65-stimulated PBMCs was shown to include a population of cells with the capacity to inhibit *de novo* CMV-specific proliferation of autologous PBMCs (Tovar-Salazar *et al*, 2010) which would support the hypothesis that adaptive Tregs may arise as a consequence of antigenic stimulation.

The hypothesis that CMVpp65 stimulation of G-CSF-mobilised PBMCs results in the emergence of a population of CD25+FoxP3+ cells with a suppressive capacity clearly requires further experimental investigation. However, in the context of manufacturing CMV-T for adoptive immunotherapy, it highlights potential problems with targeting

CD25 as an activation marker. With this in mind, the experiments in the final Results section sought to investigate the impact of depleting CD25-expressing cells prior to CMVpp65 stimulation in order to eliminate the high level of CD25+FoxP3+ suppressive cells in the enriched fractions from G-CSF-mobilised PBMCs. Results showed a reduction in CD25 expression post-CMVpp65 stimulation in both G-CSF-mobilised PBMCs and non-mobilised PBMCs, which had a negative impact on both purity and yield after CD25 enrichment. The reduction in CD25 expression is partly explained by the elimination of contaminating Tregs prior to CMVpp65 stimulation, as indeed was the aim, but could also be attributed to a population of CMV-T that are in an activated state and express CD25 prior to CMVpp65 stimulation *in vitro*. The significant decrease in purity post-CD25 enrichment observed in both G-CSF-mobilised and non-mobilised PBMCs can be explained firstly by the reduced proportion of CD25-expressing cells and, secondly, by a possible reduction in the intensity of CD25 expression which would negatively effect magnetic enrichment.

The impact on FoxP3 expression in cells depleted of contaminating CD25+ Tregs was also investigated. Although a significant reduction in FoxP3 expression was observed in CD25-depleted PBMCs post-CMVpp65 stimulation, FoxP3 expression post-CD25 enrichment was still detectable. These results suggest either the emergence of newly expressing FoxP3 cells or persistence of FoxP3+CD25− cells following the depletion step. These results observed with PBMCs isolated from G-CSF-mobilised donors is in keeping with the findings of Melenhorst and colleagues (Melenhorst *et al*, 2008a) , and suggests that removal of Tregs using CD25 depletion prior to CMVpp65 is not an effective strategy for boosting the purity of CMV-specific cells isolated through CD25 expression.

The results from this Chapter illustrate the feasibility of isolating CMV-T from G-CSF-mobilised PBMCs through both CD154 and CD25, although the overwhelming Treg phenotype observed when using CD25 appears to rule out its use. However, Lugthart and colleagues (Lugthart *et al*, 2012), using non-mobilised PBMCs, demonstrated that multivirus-specific T cells isolated through CD25 expression contain a population of Tregs and yet secrete IFN-γ and lyse virally infected target cells. Furthermore, they argued that it is unlikely that infused Tregs will prevent proliferation of virus-specific T

cells; a hypothesis that was supported by studies in mice showing that co-infusion of Tregs and conventional T cells enhances virus-specific immune reconstitution in the HSCT setting (Nguyen *et al*, 2008). These findings encouraged the further investigation of the functional capacity of CD154+ and CD25+ CMV-T isolated from G-CSF-mobilised PBMCs, which is described in the following Chapter.

Chapter 5

Expansion of CD154+ and CD25+ CMV-T isolated from G-CSF-mobilised PBMCs and assessment of cytotoxic effector function

5.1   Introduction

In order to manufacture CMV-T isolated from G-CSF-mobilised PBMCs as a therapeutic alternative to CMV-T isolated from non-mobilised PBMCs, the functional assessment of CD25+ and CD154+ CMV-T after short term culture is essential. The *in vitro* expansion of CMV-T isolated from G-CSF-mobilised PBMCs together with the retention of potent anti-viral properties, determined by cytokine secretion and cytotoxic capabilities, would demonstrate the feasibility of manufacturing CD25- and CD154-selected CMV-T at a clinical scale for therapeutic use.

It has yet to be successfully demonstrated that anti-viral T cells isolated from G-CSF-mobilised PBMCs are capable of either expansion in short term culture or cytotoxicity when re-challenged with CMV loaded target cells. However, clinical-scale isolation of γδ T cells from G-CSF-mobilised donors showed a level of cytotoxicity towards the target erythroleukaemic cell line K562 and the neuroblastoma line NB-1691(Otto *et al*, 2005) that was comparable to γδ T cells isolated from non-mobilised PBMCs (Schilbach *et al*, 2001). Although there is limited published data comparing the efficacy and toxicity of G-CSF-mobilised DLI with non-mobilised DLI, G-CSF-mobilised DLI are routinely used in several transplant centres for the successful resolution of mixed chimerism. A recent study comparing G-CSF-mobilised and non-mobilised DLI therapy following allogeneic HSCT reported similar therapeutic activity and also a similar incidence of GvHD (Abbi *et al*, 2013), suggesting G-CSF-mobilised PBMCs retain a comparable functional capacity when compared with non-mobilised PBMCs.

Several groups have shown that anti-viral T cells isolated from non-mobilised PBMCs through CD154 isolation are capable of expansion and reveal a high level of specificity

when re-challenged (Frentsch *et al*, 2005; Cohen *et al*, 2005; Kirchhoff *et al*, 2007; Khanna *et al*, 2011). Expansion of CD154 anti-viral T cells from non-mobilised PBMCs was shown to have a predominance of CD4+ T cells after enrichment. However, Khanna and colleagues (Khanna *et al*, 2011) showed that the low frequency of CD8+ anti-viral T cells in CD154 T cell lines did not hamper the effectiveness of target cell killing compared with isolated CD8+ T cells alone in adenovirus (AdV)- and EBV latent membrane protein 2 (LMP2)-specific lines. The study of Frentsch and colleagues (Frentsch *et al*, 2005) was the first publication to demonstrate in non-mobilised PBMCs that CD154-isolated CMV-T expanded in short term culture contained a population of cells capable of secreting high levels of IFN-γ upon antigenic re-challenge, thereby illustrating their potential cytotoxic nature. A comprehensive study of the cytokine profile secreted by CD154-expanded CMV-T has yet to be reported; this will be addressed in the current Chapter.

The expansion of anti-viral T cells with cytolytic activity from non-mobilised PBMCs selected on the basis of CD25 expression has also been successfully demonstrated after isolation from non-mobilised PBMCs (Gallot *et al*, 2001; Lugthart *et al*, 2012). However, no studies have determined the survival of CD25+ FoxP3-expressing Tregs that are also enriched after antigenic stimulation. This consideration becomes more important when attempting to expand CD25+ CMV-T from G-CSF-mobilised PBMCs, as the results reported in Chapter 4 (section 4.4.2) revealed a significant increase in the proportion of FoxP3- expressing cells after CD25+ enrichment compared with non-mobilised PBMCs. Co-infusion of Tregs and conventional T cells has been shown to enhance recovery of virus-specific immune reconstitution in murine models (Nguyen *et al*, 2008). However, the hypothesis that the adoptive transfer of CD25+ CMV-T that contained a proportion of Tregs would benefit the donor, in terms of GvHD prevention, without risk to restoration of cellular immunity has not been tested. A determination of the proportion of FoxP3+ Tregs retained in CD25 CMV-T cultures following isolation and expansion from G-CSF-mobilised PBMCs is vital in assessing the feasibility of manufacturing these cells for clinical use.

Data presented in Chapter 4 showed that after CD25+ and CD154+ enrichment following CMVpp65 stimulation, CD4+ T cells are the predominant T cell subset.

CD4+ T cells are considered for the most part to be dedicated towards the orchestration of cytotoxicity, rather than being directly responsible for cytotoxic activity. The *in vivo* existence of CD4+ T cells capable of mediating killing has been the subject of much debate in the past and because observations of such cytotoxic activity had been restricted to CD4+T cell lines and clones, it had been considered by some to be an artefact (Fleischer, 1984). More recently, the identification and characterisation of CD4+ cytotoxic T lymphocytes (CTLs) has confirmed the existence of such a population of T cells through the study of perforin expression, a molecular mechanism of cytotoxicity (Appay *et al*, 2002b). The identification of a subset of CMV-specific CD4+ CTLs in the peripheral blood in response to CMV antigenaemia (Suni *et al*, 2001; van Leeuwen *et al*, 2004) has aided the emerging hypothesis of a role for CD4+ T cells as mediators of protection against viral infections. Indeed, this hypothesis is strengthened by the mechanism of immune evasion of viruses such as CMV, which impair the CD8+ T cell response of MHC class I antigen processing and presentation (Petersen *et al*, 2003), thus presenting a cytotoxic role for CD4+ T cells. Clinical trials using both CD4+ T cell clones (Perruccio *et al*, 2005) and CD4+ T cell lines (Einsele *et al*, 2002) manufactured from non-mobilised PBMCs have demonstrated effective control of CMV disease. Therefore, an assessment of the cytotoxic capacity of CD25+ and CD154+ CMV-T isolated from G-CSF-mobilised PBMCs, in which CD4+ cells are the predominant T cell subset, was a key objective of this Chapter.

5.2   Aims of Experiments Described in this Chapter

The initial aims of this Chapter were to expand both CD25+ and CD154+ CMV-T in short term culture following magnetic enrichment, and subsequently to test the CMV specificity of expanded cells through antigenic re-challenge. Expansion was investigated in both G-CSF-mobilised and non-mobilised PBMCs to ascertain the impact G-CSF may have on the potential for CMV-T expansion. Following short term culture, expanded cells were re-challenged with autologous PBMCs loaded with CMVpp65 antigens to ascertain the specificity of expanded cells. The cytokine profile of expanded cells from G-CSF-mobilised PBMCs was investigated in response to

CMVpp65 re-challenge and compared directly against non-mobilised PBMCs, using both CBA and ICS.

Expanded CD25+ CMV-T were tested to identify whether FoxP3 expressing Tregs were maintained in short term culture and if any suppressive capacity existed.

I also aimed to identify the cytolytic activity of expanded CMV-T from both CD25+ and CD154+ enrichments using a calcein-based cytotoxicity assay. Comparisons between both CD154 and CD25 CMV-T isolated from non-mobilised and G-CSF-mobilised PBMCs were made to ascertain any differences in functionality. In addition, cultures were monitored for CD57 expression, a marker associated with replicative senescence and activation-induced apoptotic death (Brenchley *et al*, 2003). It has been proposed in some studies that chronic stimulation of T cells, such as that which occurs with CMV, can result in a reduced capacity for cell division in both CD4+ (Fletcher *et al*, 2005) and CD8+ (Voehringer *et al*, 2001) T cell subsets. Determining the level of CD57 expression in expanded cells will indicate the potential capacity for replication *in vivo*, were they to be used in a clinical setting.

A final aim of this Chapter was to identify which activation marker would be the best candidate for clinical scale manufacture of CMV-T after isolation from G-CSF-mobilised PBMCs.

The experimental aims of the Chapter were as follows:

1. To expand CMV-T isolated from G-CSF-mobilised PBMCs through CD154 or CD25 and directly compare against expanded CMV-T isolated from non-mobilised PBMCs.

2. To establish the specificity of expanded CMV-T through antigenic re-challenge.

3. To compare the functional profile of expanded CMV-T from G-CSF-mobilised PBMCs though cytokine profiling after re-challenge against that of non-mobilised PBMCs.

4. To compare the cytotoxic capability of expanded CMV-T from G-CSF-mobilised PBMCs in response to co-culture with CMV-pulsed target cells against that of non-mobilised PBMCs.

5.3   **Materials and Methods**

CD25 and CD154 expressing cells magnetically enriched following CMVpp65 peptide stimulation were counted and resuspended at a concentration of 0.25 x 10^6/ml, and incubated in 24-well plates in the presence of irradiated autologous PBMCs at a ratio of 50:1 (feeders:CMV-T) for up to 23 days. Cultures were supplemented with 10ng/ml of IL-7 and IL-15, and the medium changed every 3-4 days. Cultures were counted and the T cell phenotype assessed by flow cytometry every 3 days and cultures split when the concentration exceeded 3 x 10^6/ml, usually around day 7–10. In cultures where the total cell number exceeded 30 x 10^6, cells were transferred from 24-well plates into T-25 culture flasks (Nunc).

Expanded cells were harvested no later than day 23 for final phenotypic analysis, antigenic re-challenge and cytotoxicity assays. Autologous PBMCs, previously cryopreserved, were thawed prior to re-challenge and loaded with CMVpp65 peptides, or left untouched as a negative control. In some experiments, autologous PBMCs were loaded with CMV-IE peptides to allow the assessment of specificity. Re-challenge experiments consisted of co-culture of autologous PBMCs with expanded cells at a ratio of 2.5:1 to 5:1 (PBMCs:CMV-T), dependent upon availability of cell number, in 12-well plates for 6 hours in the presence Brefeldin A (1µg/ml) to allow for ICS. Secretion of IL-2, TNF-α, IFN-γ, Granzyme B and, in the case of CD25-expanded CMV-T, IL-10 were assessed by flow cytometry after antigenic re-challenge. Aliquots of supernatants from re-challenge cultures were frozen for subsequent use in a CBA assay for further determination of cytokine secretion. Surface expression of CD25 and CD154 was also assessed after re-challenge in the absence of Brefeldin A, after 6 hours. CD25-expanded cells were assessed for FoxP3 expression and their suppressive capacity determined using the CFSE suppression assay targeted towards SEB-stimulated autologous PBMCs, as previously described in Chapter 4 (section 4.4.3).

Autologous PBMCs were stimulated with PHA (3µg/ml) for 24 hours, followed by 20IU/ml of IL-2 for 72 hours to use as targets in a cytotoxicity assay. PHA blasts were then loaded with CMVpp65 peptides or left untouched before labelling with Calcein-

AM (Neri *et al*, 2001) and incubating with CMV-T-expanded cells in 24-well plates for 4 hours at E:T ratios ranging from 0.5:1 to 20:1. Lysis of target cells was determined by fluorescence using a microplate fluorescence spectrophotometer. In some experiments, target cells were blocked from engaging CD4+ or CD8+ T cells or both using purified HLA class I and class II antibodies after calcein labelling, to assess the T cell subset predominantly responsible for cytotoxicity.

In both CD25 and CD154 expansions, experiments using G-CSF-mobilised PBMCs were compared directly with conventional non-mobilised PBMCs and any statistical difference determined using an unpaired *t*-test.

5.4   Results

5.4.1   Expansion of CD154+ and CD25+ CMV-T

Figure 5.1 illustrates the expansion of CMV-T after CD154 selection from G-CSF-mobilised donors (Figure 5a) and non-mobilised donors (Figure 5b). CD154+ CMV-T from G-CSF-mobilised PBMCs were cultured for up to 23 days and showed no significant difference (p=0.75) in their mean proliferative capacity (90.4-fold expansion ± 25.6) compared with cells from non-mobilised PBMCs (104.4-fold expansion ± 37.4). Both G-CSF-mobilised and non-mobilised expanded cells were >90% CD3+ and were predominantly CD4+ (mean, 98%) in all cultures, although a small fraction of expanded cells expressed CD8 (mean, 2%). Representative staining for CD3, CD4 and CD8 over 21 days expansion of CD154+ CMV-T from a G-CSF-mobilised donor is illustrated in Figure 5.2. This highlights the elevated lymphocyte purity of expanded cells and also the high CD3+ purity coupled with the predominance of CD4+ T cells.

The mean proliferative capacity of CD25+ CMV-T expanded from G-CSF-mobilised PBMCs (90.3-fold expansion ± 30.0) was similar to that of CD154+ CMV-T. However, a trend (p=0.06) towards an increased mean proliferative capacity of CD25+ CMV-T was observed in non-mobilised PBMCs (175.2-fold expansion ± 21.0) vs. G-CSF-mobilised PBMCs. In contrast to CD154+ CMV-T, CD25+ expanded cells were a mixture of both CD4+ and CD8+ T cells. G-CSF-mobilised CD25+ CMV-T revealed a higher mean proportion of CD4+ T cells compared with non-mobilised PBMCs (44.13% ± 17.04 vs. 13.7% ± 7.07; p=0.18); and a trend towards a significantly reduced proportion of CD8+ T cells compared with non-mobilised PBMCs (53.0% ± 17.31 vs. 80.2% ± 11.18; p=0.06). Interestingly, in two of the five CD25 expansion experiments performed from G-CSF-mobilised donors, the CD8+ subset at day 22 accounted for >90% of CD3+ T cells compared with <5.5% CD4+, which was contradictory to the results seen in the remaining three CD25 expansions from G-CSF-mobilised donors. However, in both the predominantly CD8+ expanded cell lines, the CD25+ fractions isolated after magnetic enrichment were comparable to the CD25+ fractions from the other three expansions, with respect to having a predominantly CD4+ fraction. The

change of phenotype over short term culture, from predominantly CD4+ to predominantly CD8+, is illustrated in Figure 5.4 and will be discussed later in this Chapter.
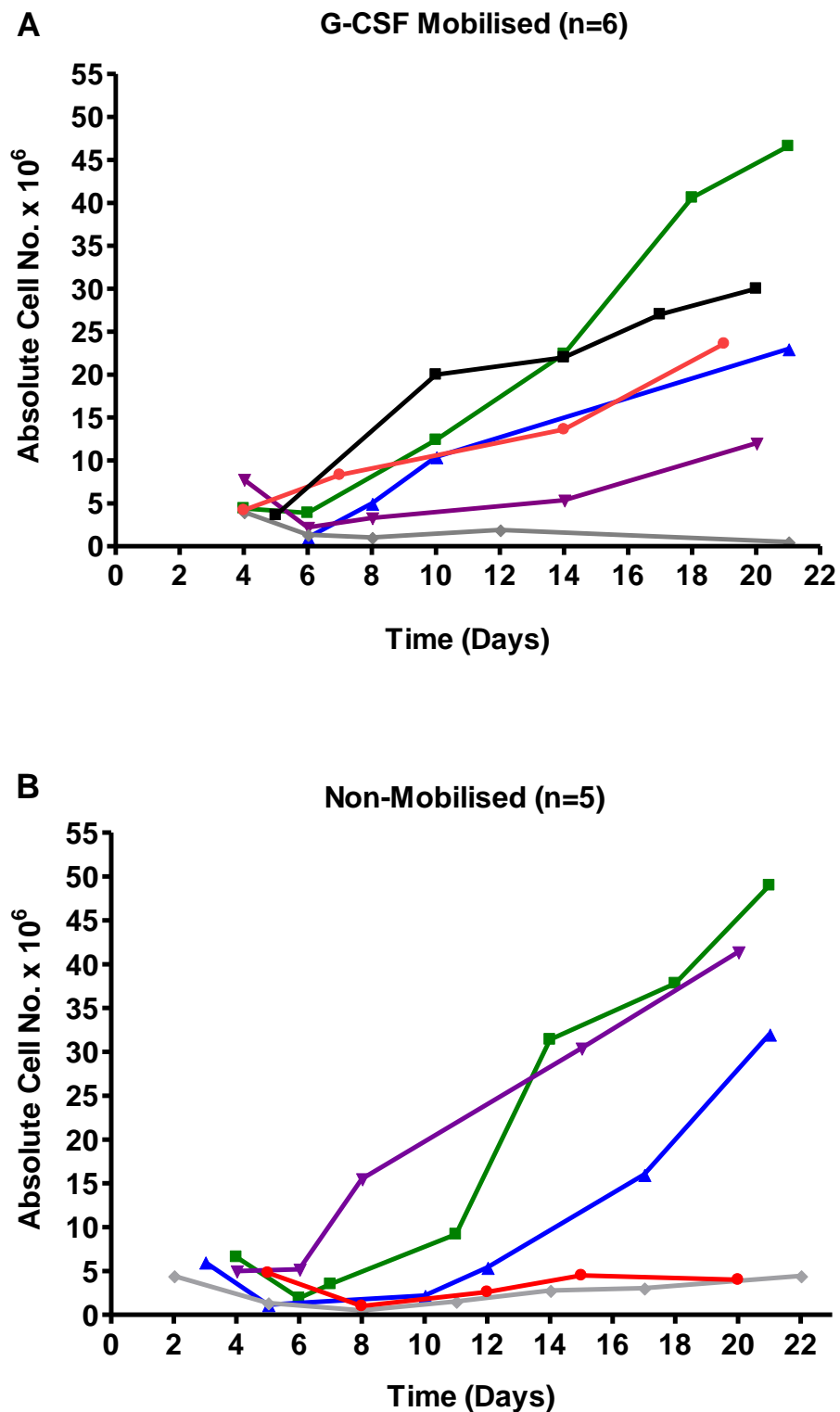
Figure 5.1 Expansion of CD154+ CMV-T in short term culture

CD154+ CMV-T were expanded in short term culture for up to 23 days in the presence of IL-7, IL-15 and 12.5 x 10^6 irradiated autologous feeder cells from both G-CSF-mobilised (A) and non-mobilised (B) donors.
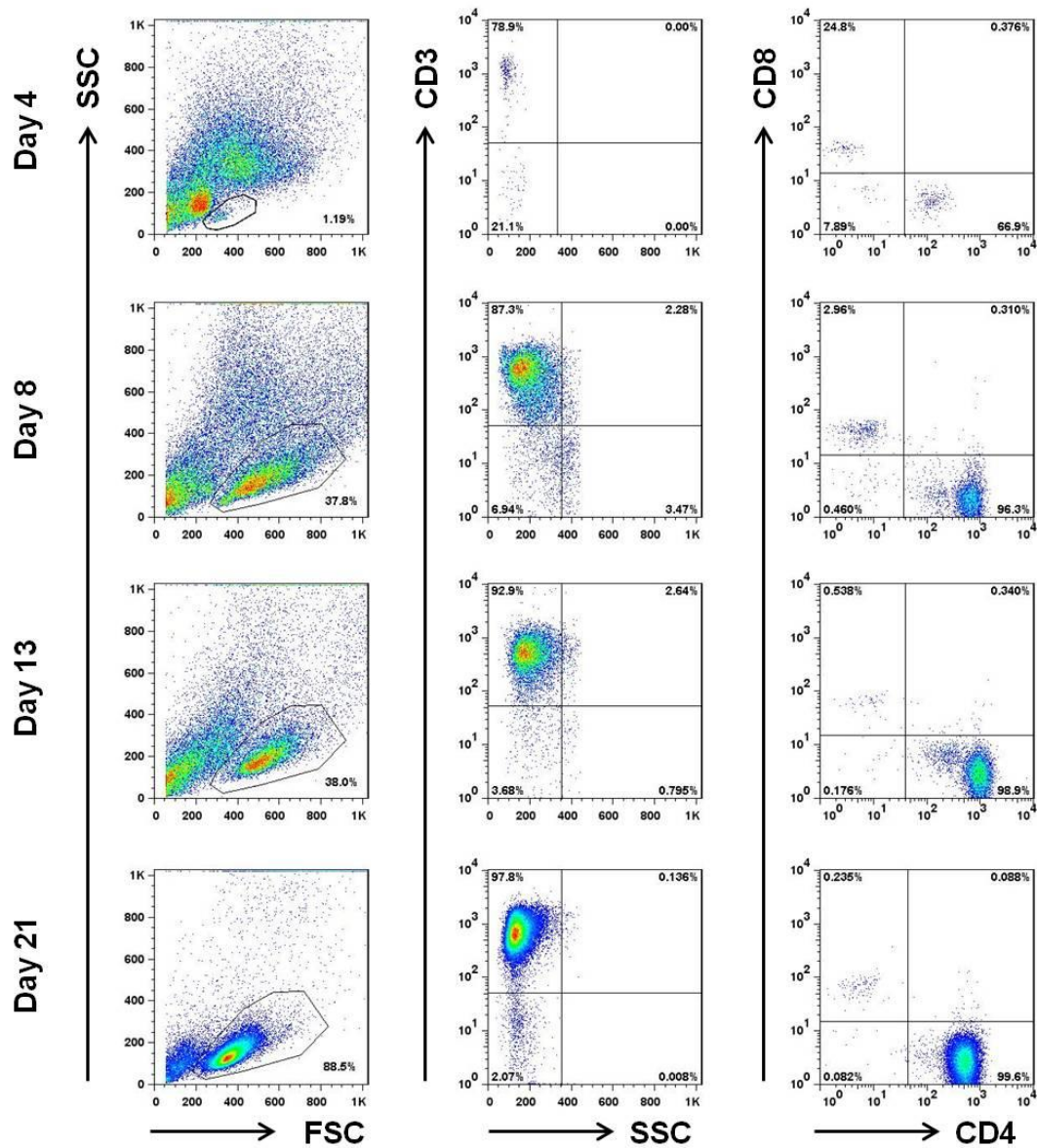
Figure 5.2 T cell phenotype of CD154+ CMV-T isolated from G-CSF-mobilised PBMCs cultured over 21 days

Freshly isolated CMVpp65-stimulated CD154+ cells were expanded in short term culture over 21 days in the presence of IL-7, IL-15 and irradiated feeder cells. Lymphocyte purity was assessed together with CD4+ and CD8+ T cell phenotype at 4 different time points.
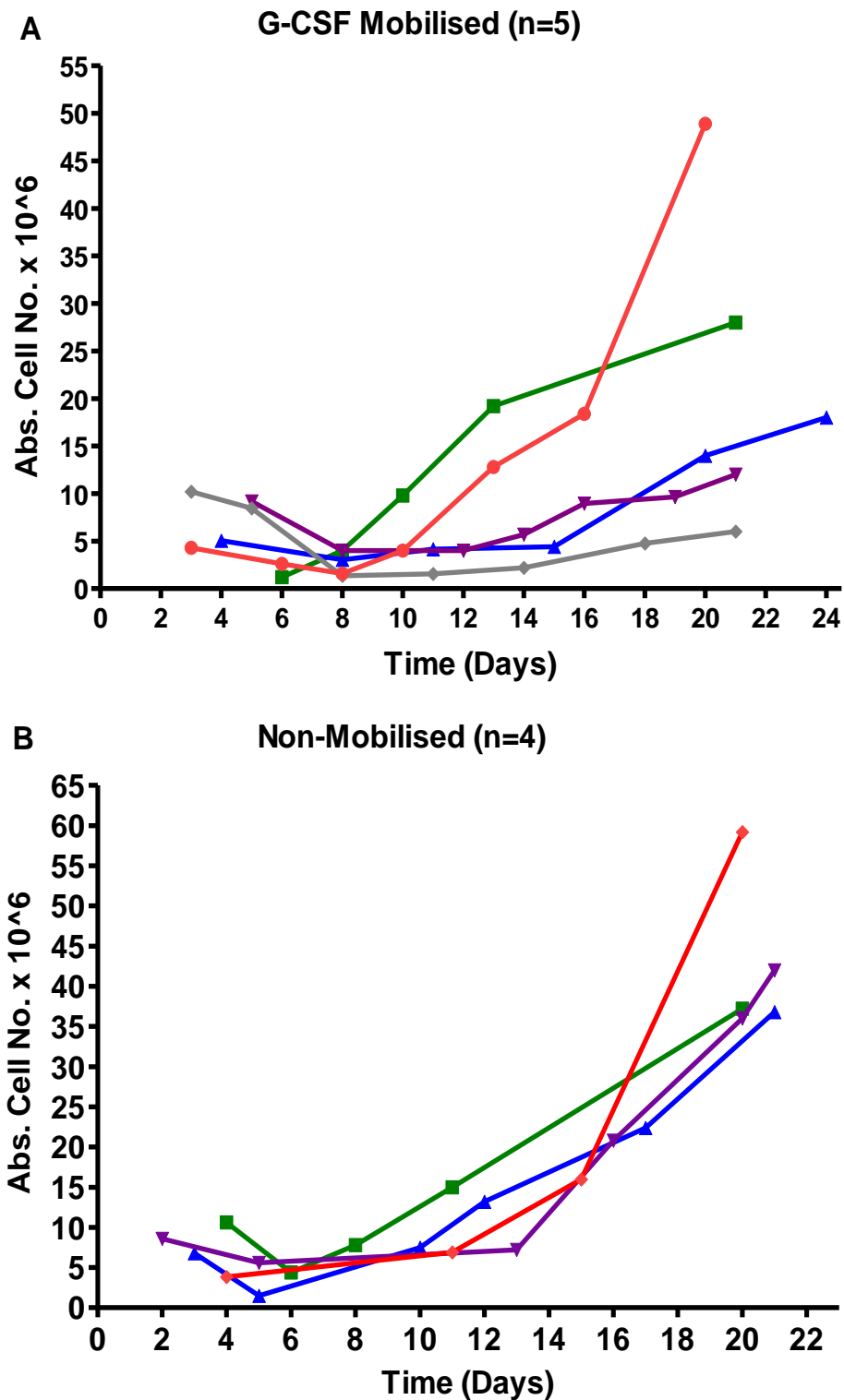
Figure 5.3 Expansion of CD25+ CMV-T in short term culture

CD154+ CMV-T were expanded in short term culture for up to 24 days in the presence of IL-7, IL-15 and 12.5 x 10^6 irradiated autologous feeder cells from both G-CSF-mobilised (A) and non-mobilised (B) donors.
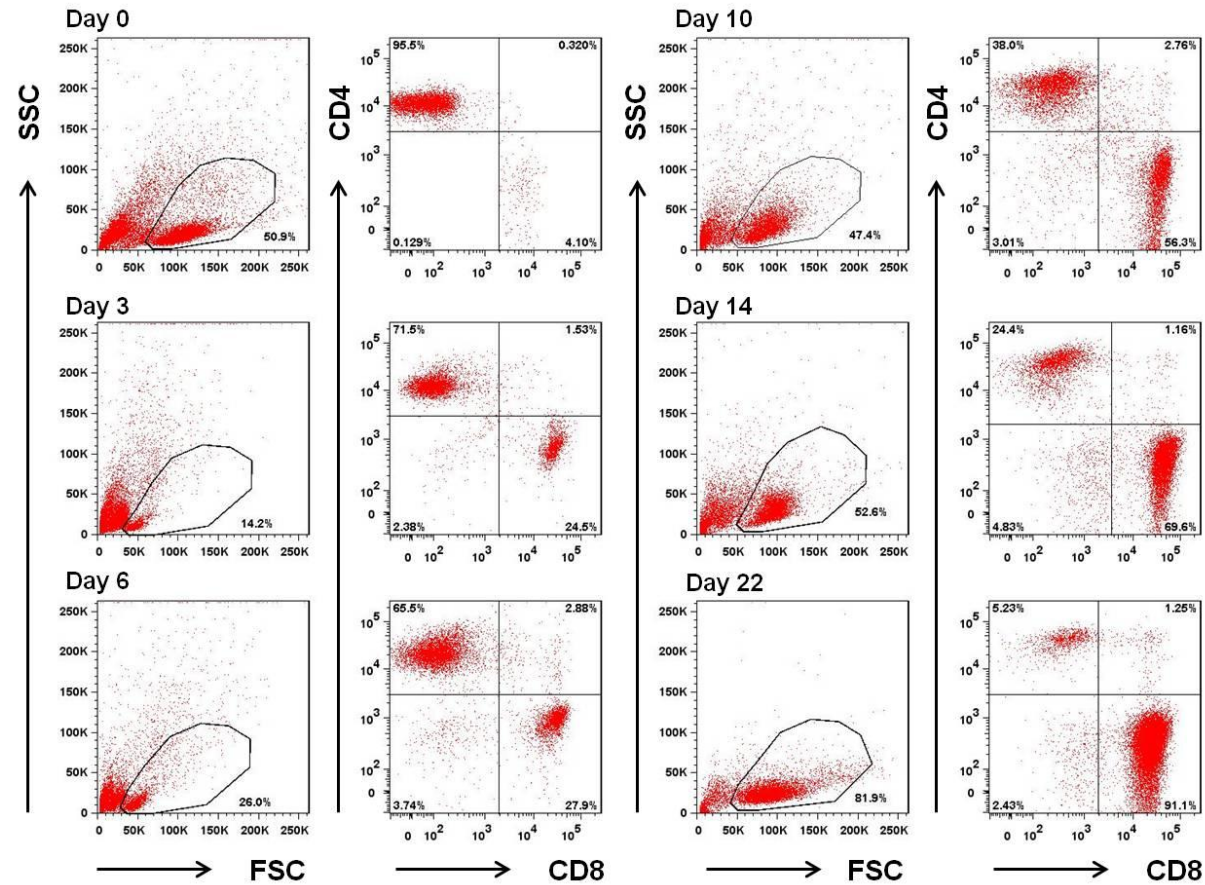
Figure 5.4 T cell phenotype of CD25+ CMV-T isolated from a G-CSF-mobilised donor cultured over 22 days

Freshly isolated CMVpp65-stimulated CD25+ cells were expanded in short term culture over 22 days in the presence of IL-7, IL-15 and irradiated feeder cells. Lymphocyte purity was assessed together with CD4+ and CD8+ T cell phenotype at 6 different time points.

5.4.2   Antigenic re-challenge of expanded cells

To determine the specificity of CD154 and CD25 expanded cells, the cytokine profile was examined in response to CMVpp65 peptide re-challenge together with Granzyme B secretion and co-expression of CD69. Where sufficient cells were available, expanded cells were re-challenged with an irrelevant CMV peptide to determine specificity to CMVpp65.

5.4.2.1 CD154

Figure 5.5 illustrates representative data showing the specificity of CD154+ expanded CMV-T after CMVpp65 re-challenge in one G-CSF-mobilised donor and one non-mobilised donor. Experiments to determine the specificity of CD154+ T cells after CMVpp65 re-challenge of expanded cells are summarised in Figure 5.6. Mean levels of CD154+ CD69+ co-expression in both G-CSF-mobilised (83.3% ± 5.03) and non-mobilised (60.96% ± 15.5) PBMCs, showed specificity for CMVpp65 upon re-challenge with autologous PBMCs loaded with CMVpp65 peptides. Re-challenge of expanded cells with autologous PBMCs alone did not induce CD154 expression in either G-CSF-mobilised or non-mobilised PBMCs. Stimulation of expanded cells with autologous PBMCs loaded with CMV IE-1 peptides also revealed low mean levels of CD154 expression in both G-CSF-mobilised (4.24% ± 2.9) and non-mobilised (5.83% ± 2.7) PBMCs.

Figure 5.5 Re-challenge of CD154+ CMV-T expanded from one G-CSF-mobilised and one non-mobilised donor
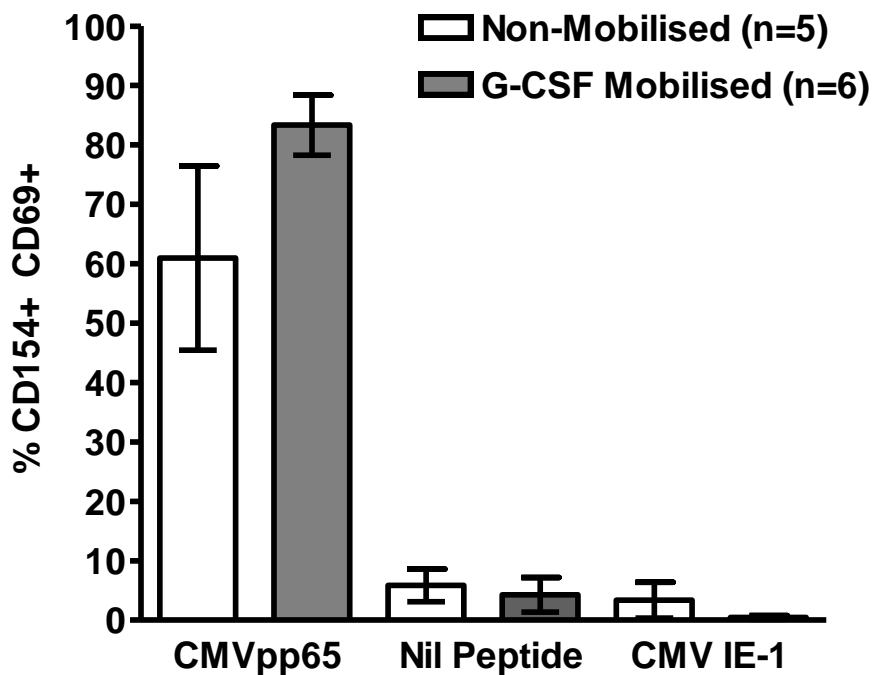
Figure 5.6 Summary of CD154 and CD69 expression after antigenic re-challenge

Expanded cells were also re-challenged to assess the secretion of effector cytokines by ICS determined by flow cytometry. Figure 5.8 shows representative data comparing cytokine secretion in one G-CSF-mobilised and one non-mobilised donor after re-challenge with CMVpp65 peptides. The combined assessment of IL-2, TNF-α, IFN-γ and Granzyme B after re-challenge is illustrated in Figure 5.7 using data generated from expanded cells in six G-CSF-mobilised and five non-mobilised donors. Expanded cells from both G-CSF-mobilised and non-mobilised PBMCs synthesised and secreted similar mean levels of IL-2 (25.3% of CD3+ ± 11.8 vs. 38.3% ± 12.1; p=0.47), TNF-α (24.4% ± 7.3 vs. 25.5% ± 8.1; p=0.93) and IFN-γ (69.5% ± 9.3 vs. 54.1% ± 14.3; p=0.37) with no significant differences observed. Expanded cells were also highly positive for Granzyme B both after re-challenge with CMVpp65 peptides, indicating that cells possessed the effector molecules necessary for cytotoxic activity. Comparison between mean Granzyme B expression in G-CSF-mobilised (79.4% ± 2.7) and non-mobilised (83.5% ± 4.1) PBMCs also showed equivalence with no significant difference detected (p=0.53). In experiments where expanded cells were unstimulated or incubated with CMV-IE peptides, minimal cytokine secretion was observed.
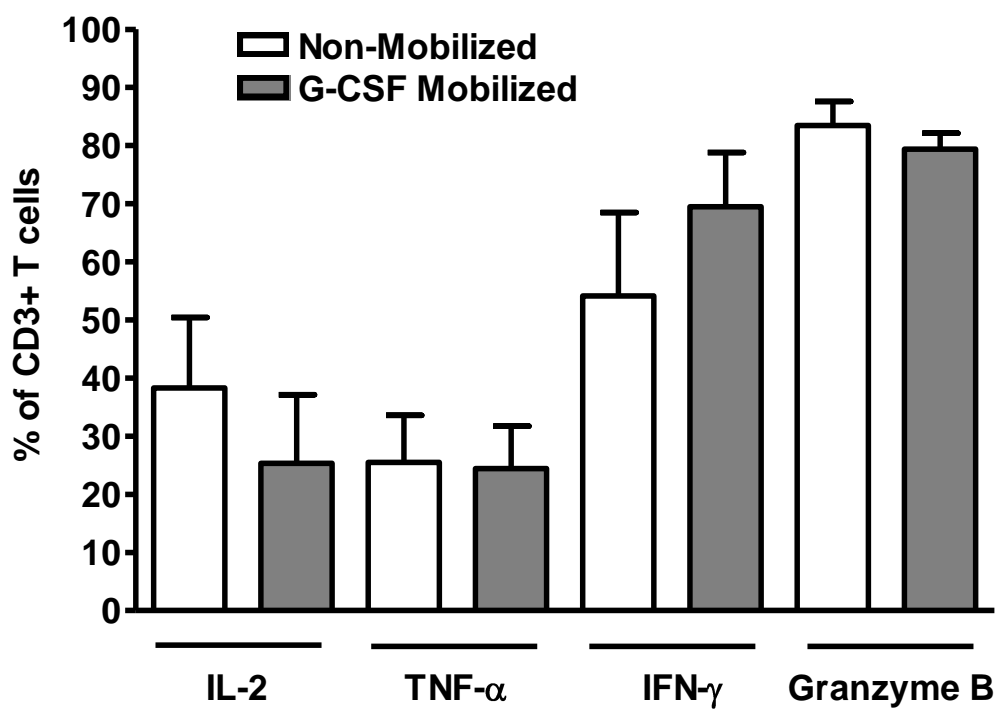
Figure 5.7 Summary of cytokine secretion after antigenic re-challenge from CD154+ CMV-T
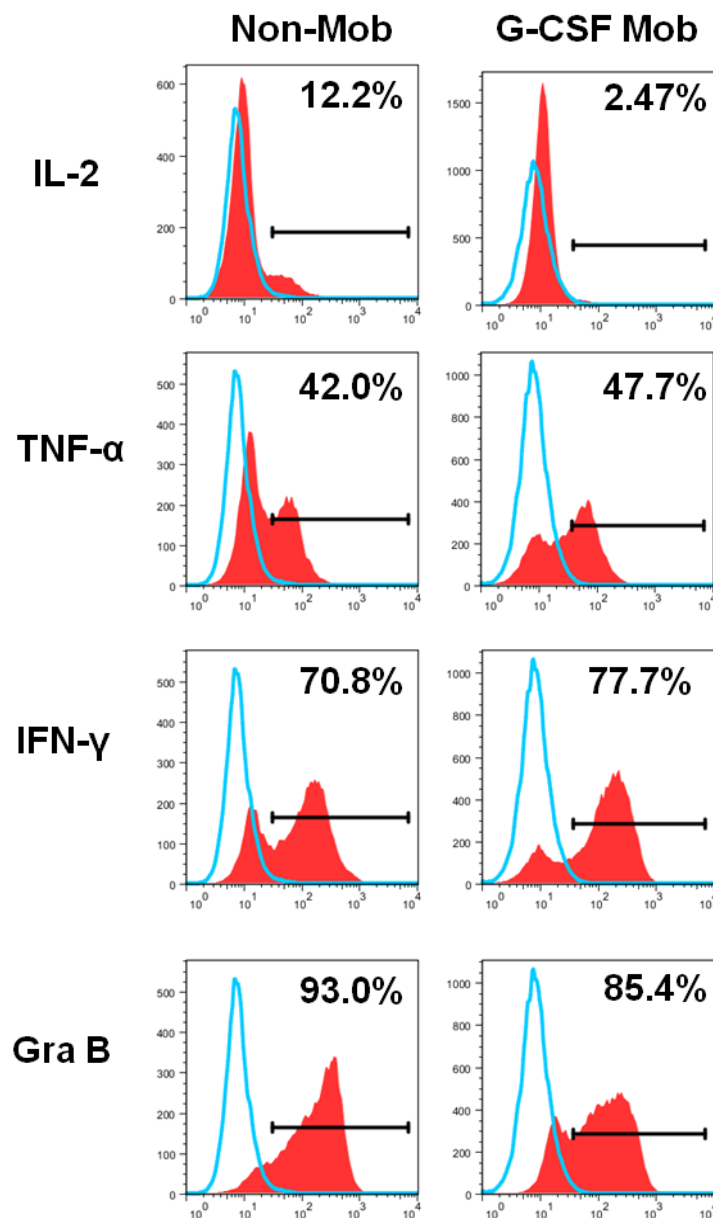
Figure 5.8 Cytokine secretion in re-challenged CD154+ CMV-T from one G-CSF-mobilised and one non-mobilised donor

Histograms are generated after live cell gating on lymphocytes using FSC vs. SSC plots and after subsequent gating on CD3+SSC^{low} events. Open peaks represent matched isotype controls and filled peaks represent test samples.

Having demonstrated synthesis and secretion of effector molecules by CD154+ expanded cells using ICS, cytokine secretion was further assessed through the analysis of culture supernatants after re-challenge with CMVpp65 peptides. Cytokine concentration was measured for IL-2, TNF-α, IFN-γ, IL-4, IL-5 and IL-10 using the CBA assay and results are summarised in Figure 5.9. Cytokine levels were again comparable in expanded cells from G-CSF-mobilised and non-mobilised PBMCs, with no significant differences observed. Analysis of mean cytokine levels in expanded cells from G-CSF-mobilised PBMCs revealed a predominantly cytotoxic phenotype, with significant levels of TNF-α (26,960 ± 8667 pg/ml), IL-2 (7791 ± 5400 pg/ml), and IFN-γ (8091 ± 1457 pg/ml;) secretion. Low levels of IL-5 (237 ± 140 pg/ml) and IL-10 (35 ± 16 pg/ml) secretion were observed and interestingly a high concentration of IL-4 secretion (14,759 ± 7065 pg/ml).

The CMVpp65 specificity of CD154+ expanded cells is evident when comparing cytokine levels in these cells against those in PBMCs following CMVpp65 stimulation, as reported in Chapter 3 (section 3.4.1). In CD154+ expanded CMV-T vs. G-CSF-mobilised PBMCs (Table 5.1), mean IL-2 secretion increased 22-fold, mean IFN-γ secretion increased 15-fold, whilst mean TNF-α secretion increased by 88-fold. In CD154+ expanded CMV-T vs. non-mobilised PBMCs, mean IL-2 secretion also increased 22-fold, mean IFN-γ secretion increased 4.5-fold, and mean TNF-α secretion increased 23-fold. Most striking were the 5904-fold increase of IL-4 secretion in CD154+ expanded CMV-T vs. G-CSF-mobilised PBMCs, and the 659-fold increase of IL-4 secretion in CD154+ expanded CMV-T vs. non-mobilised PBMCs.
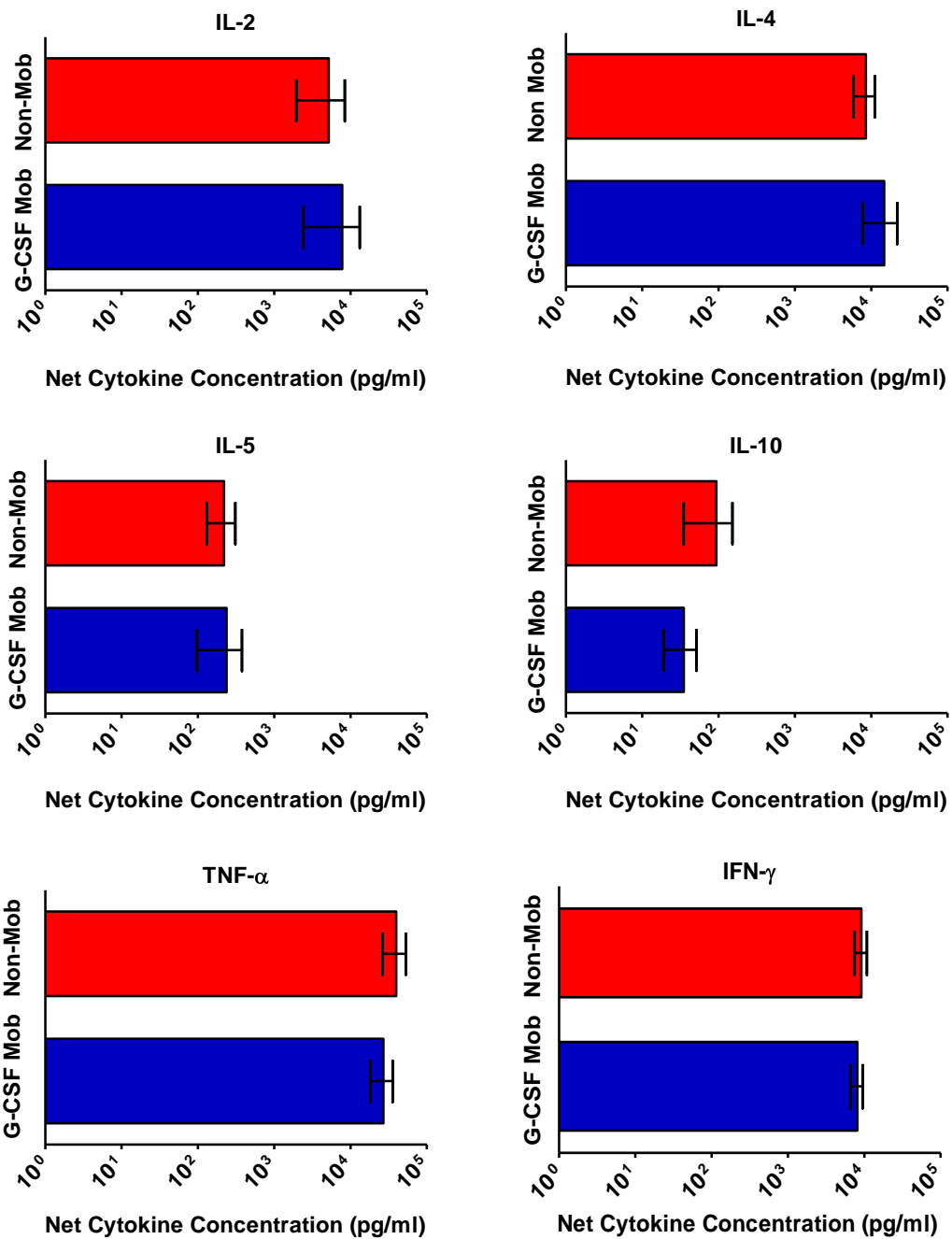
Figure 5.9 Cytokine secretion detected in culture supernatant of CD154+ expanded cells after CMVpp65 re-challenge

Quantitative assessment of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ detected in the supernatant of cultures after a 6-hour re-challenge with autologous PBMCs loaded with CMVpp65 peptides. Performed by CBA in G-CSF-mobilised (n=5) and non-mobilised (n=4) donors

A

G-CSF-Mobilised

	IL-2		TNF-α		IFN-γ		IL-4	
	PBMC	**Exp**	**PBMC**	**Exp**	**PBMC**	**Exp**	**PBMC**	**Exp**
Mean Conc. (pg/ml)	354	7791	306	26960	548	8091	3	14760
Range (pg/ml)	30 – 1328	607 – 29165	52 – 1247	4053 – 48906	140 – 1195	5572 – 13540	0 – 6	80 – 40839
Fold Increase		22		88		15		5904

B

Non-Mobilised

	IL-2		TNF-α		IFN-γ		IL-4	
	PBMC	**Exp**	**PBMC**	**Exp**	**PBMC**	**Exp**	**PBMC**	**Exp**
Mean Conc. (pg/ml)	235	5178	1756	39604	2028	9137	13	8497
Range (pg/ml)	27 – 450	584 – 14659	37 – 8129	589 – 61353	497 – 5753	4143 – 10721	0 – 48	683 – 12090
Fold Increase		22		23		4.5		659

Table 5.1 Comparison of mean cytokine concentration between PBMCs and CD154+ expanded CMV-T (Exp) following CMVpp65 stimulation in both G-CSF-mobilised PBMCs (A; n=5) and non-mobilised PBMCs (B; n=5)

5.4.2.2 CD25

Expanded CD25+ CMV-T were re-challenged with autologous PBMCs loaded with CMVpp65 peptides to determine their CMV specificity and cytokine repertoire, as described for expanded CD154+ CMV-T. The results obtained were also compared directly with those of expanded CD154+ CMV-T to ascertain the suitability of CD25 as a target molecule for the effective manufacture of CMV-T. Figure 5.10 illustrates representative CD25+ CD69+ co-expression after antigenic re-challenge in expanded CD25+ CMV-T from one G-CSF-mobilised donor and one non-mobilised donor. Figure 5.11 shows a summary of CD25+ CD69+ co-expression after re-challenge in all CD25 CMV-T expansion experiments. Mean levels of CD25+ CD69+ co-expression in CD25+ expanded CMV-T from both G-CSF-mobilised (64.9% ± 15.7) and non-mobilised (38.0% ± 8.0) PBMCs showed specificity for CMVpp65. The reduction in CD25+ CD69+ expression observed in CD25+ expanded CMV-T from non-mobilised vs. mobilised PBMCs was not statistically significant ($p=0.23$). Re-challenge with autologous PBMCs alone produced a low mean level of CD25+ CD69+ co-expression in CD25+ expanded CMV-T from G-CSF-mobilised (10.0% ± 4.9) and non-mobilised (3.7% ± 1.4) PBMCs.

Determining the cytokine profile of CD25+ expanded CMV-T was achieved through ICS with the addition of IL-10 staining. The rationale for determining the level of IL-10 secretion was based on results presented in Chapter 4 indicating that after magnetic enrichment following CMVpp65 stimulation, CD25-expressing cells contained a significant population of FoxP3-expressing Tregs. Tregs are associated with a suppressive function, which is achieved partly through IL-10 secretion (Groux *et al*, 1997).

Figure 5.12 illustrates representative data from one G-CSF-mobilised and one non-mobilised donor after CMVpp65 re-challenge. The cumulative data from re-challenge of CD25+ expanded cells in five G-CSF-mobilised and four non-mobilised donors is summarised in Figure 5.13. CD25+ expanded CMV-T revealed low to undetectable mean levels of IL-10 secretion after CMVpp65 re-challenge from both G-CSF-mobilised (0.11% ± 0.05) and non-mobilised PBMCs (0.41% ± 0.10). A trend towards

increased mean cytokine secretion in expanded CD25+ CMV-T from G-CSF-mobilised PBMCs vs. non-mobilised PBMCs was revealed for IL-2 (11.5% ± 4.5 vs. 4.7% ± 2.4, respectively; $p=0.25$) and TNF-α (36.0% ± 12.9 vs. 25.0% ± 7.6, respectively; $p=0.51$). This difference became significant ($p=0.02$) when comparing mean IFN-γ secretion between CD25+ expanded cells from G-CSF-mobilised (78.4% ± 5.2) and non-mobilised (50.0% ± 9.1) PBMCs after CMVpp65 re-challenge. A comparison of mean Granzyme B expression in G-CSF-mobilised (66.8% ± 16.2) and non-mobilised (73.8% ± 17.5) PBMCs revealed no significant difference ($p=0.79$). Similar to the results observed after CMVpp65 re-challenge of CD154+ expanded cells, CD25+ expanded cells exhibited minimal cytokine secretion when cells were unstimulated or incubated with CMV-IE peptides.
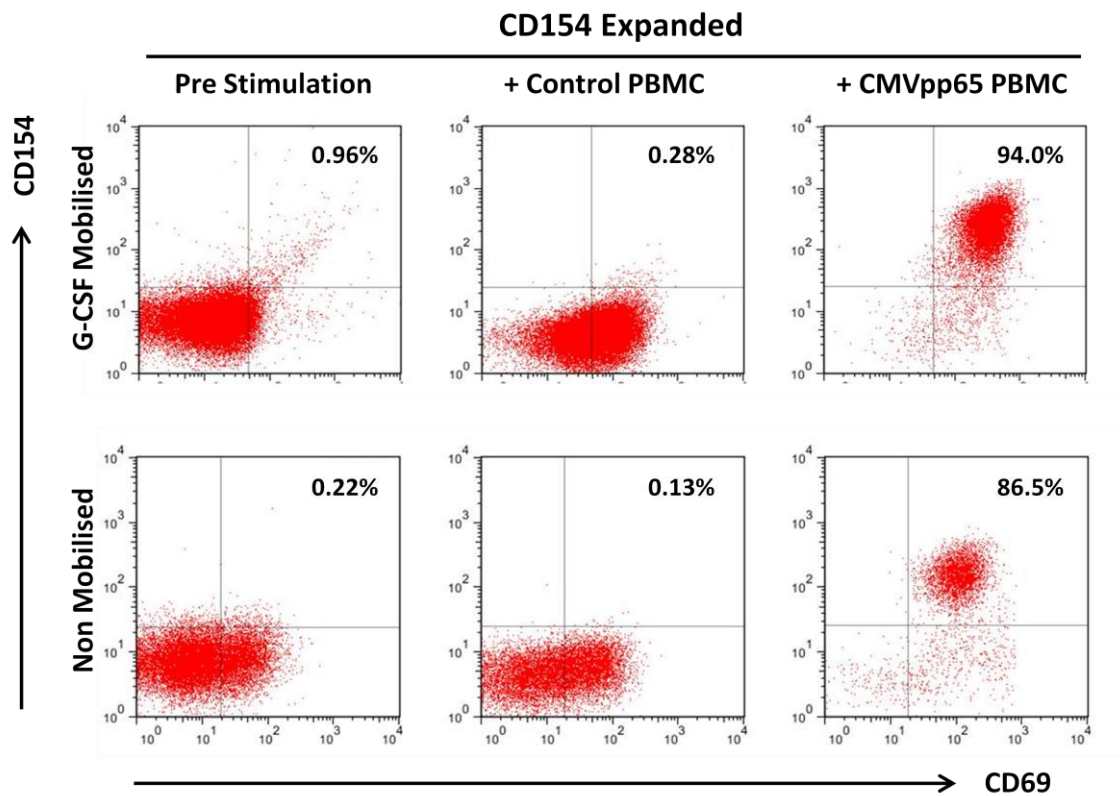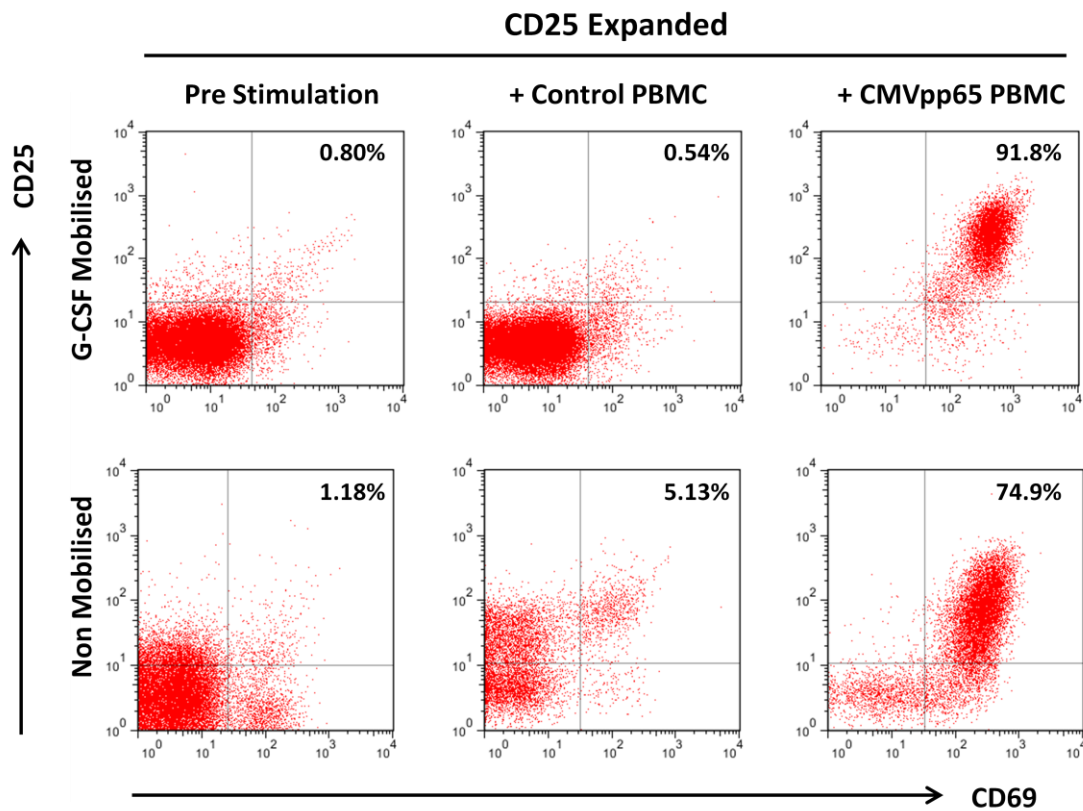
Figure 5.10 Re-challenge of CD25+ CMV-T expanded from one G-CSF-mobilised and one non-mobilised donor
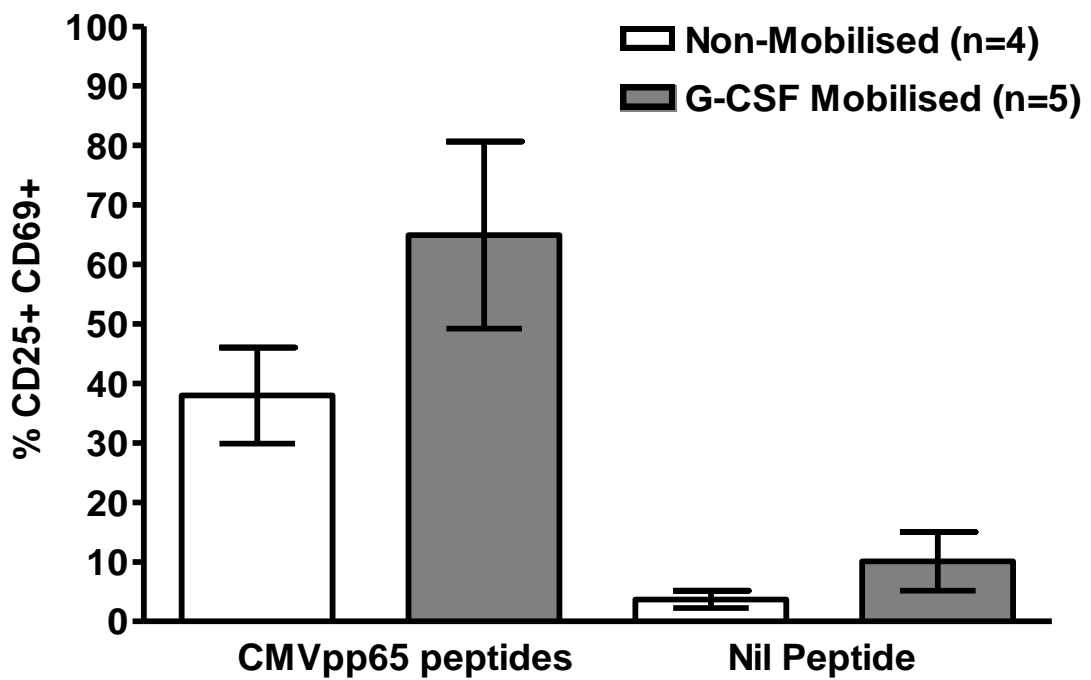
Figure 5.11 Summary of CD25 and CD69 expression after antigenic re-challenge
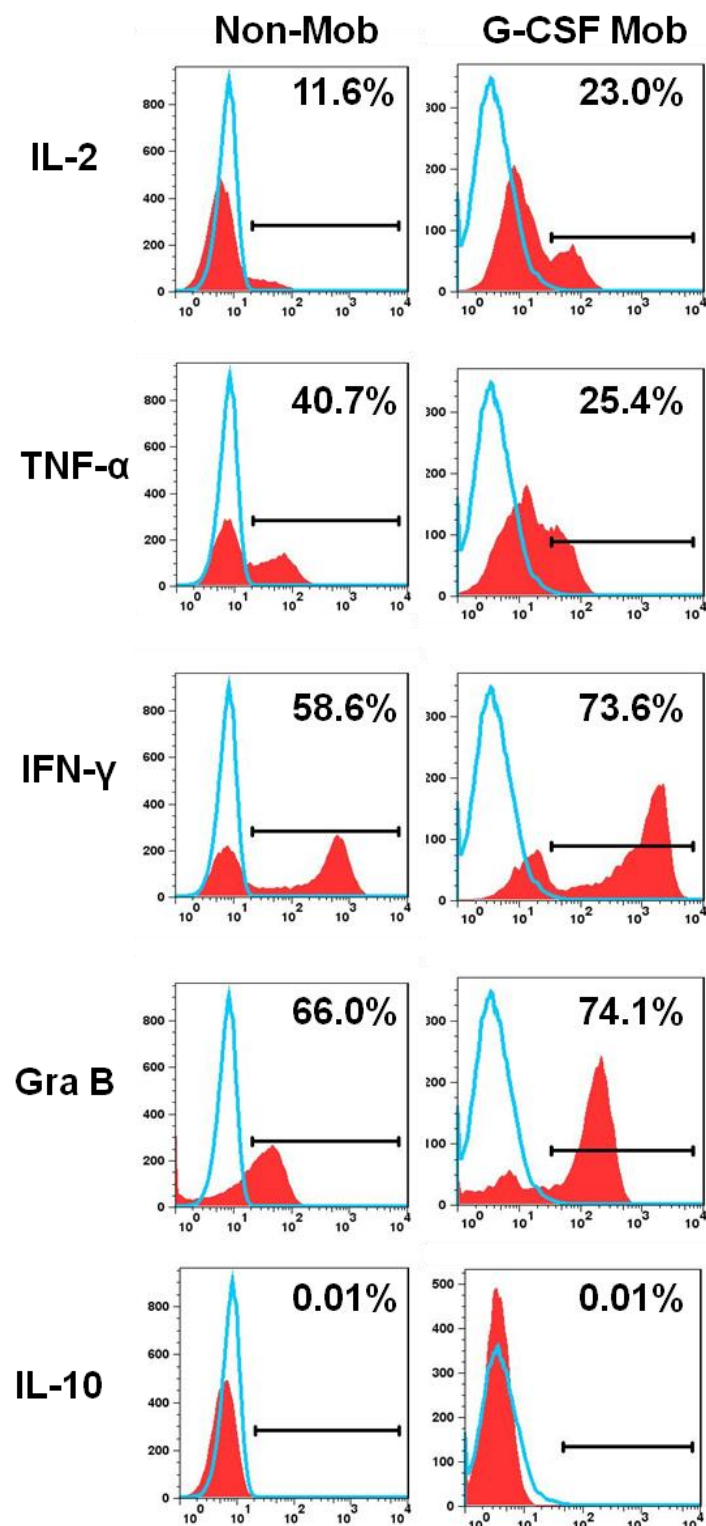
Figure 5.12 Cytokine secretion after CMVpp65 re-challenge in CD25+ CMV-T from both a G-CSF-mobilised donor and a non-mobilised donor

Histograms are generated after live cell gating on lymphocytes using FSC vs. SSC plots and after subsequent gating on CD3+SSC^low events. Open peaks represent matched isotype controls and filled peaks represent test samples.

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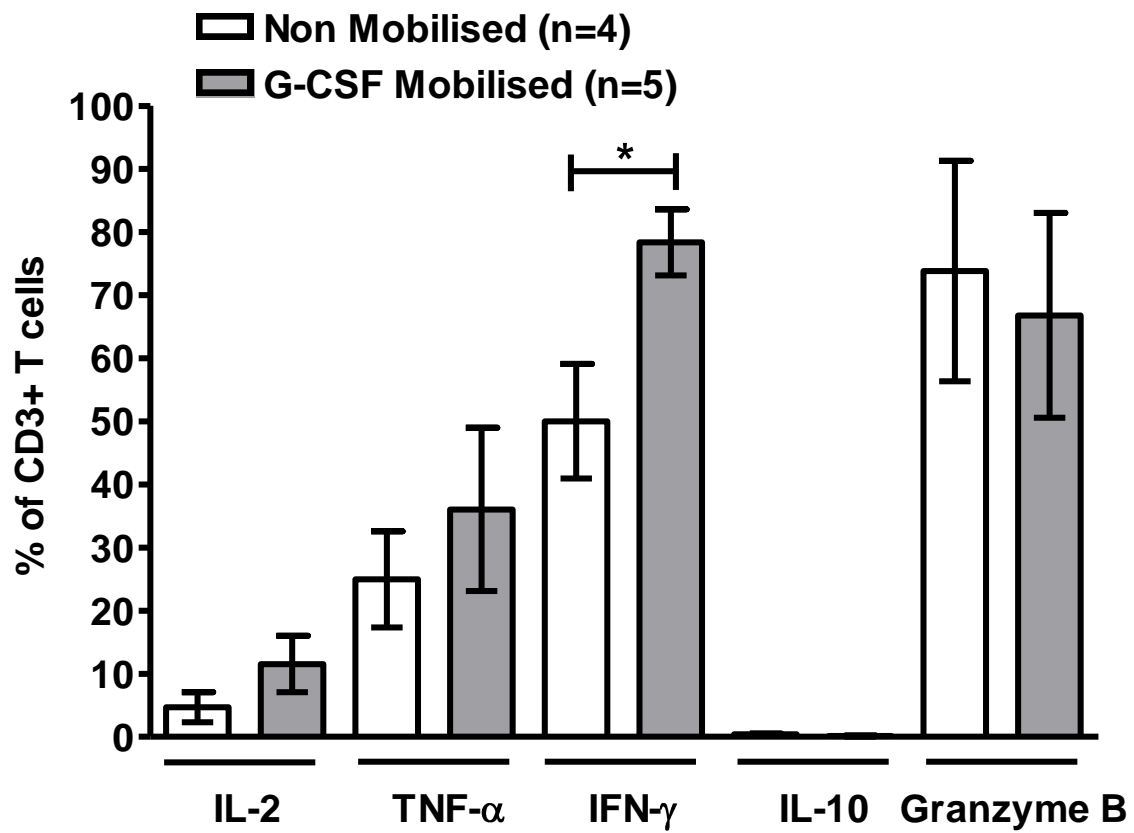
Figure 5.13 Summary of cytokine secretion after antigenic re-challenge in CD25+ CMV-T

The CBA assay was used to further determine the concentration of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ secreted after re-challenge (Figure 5.14). In line with the results observed with CD154+ expanded cells, CD25+ expanded cells from G-CSF-mobilised PBMCs showed no statistical difference compared with non-mobilised PBMCs in the levels of any of the cytokines. Furthermore, analysis of CD25+ expanded CMV-T from G-CSF-mobilised PBMCs revealed a similar profile to that of CD154+ expanded CMV-T, in mean concentration of TNF-α (27,430 ± 12540 pg/ml), IL-2 (5849 ± 4866 pg/ml) and IFN-γ (12,620 ± 859 pg/ml) secretion. The mean concentration of IL-4 levels (10,960 ± 5974 pg/ml) from G-CSF-mobilised PBMCs was comparable to that of IL-2 and IFN-γ, as was observed with CD154+ expanded CMV-T. IL-5 (48 ± 30 pg/ml) and IL-10 (29 ± 13 pg/ml) were both secreted at low levels compared with the other cytokines. Taken together, these findings support a polarisation towards a population of cytotoxic CMV-T and argue against the existence of a significant population of IL-10-secreting Tregs.

Table 5.2 compares cytokine levels in CMVpp65-stimulated PBMCs and CD25+ expanded cells from G-CSF-mobilised and non-mobilised PBMCs. Analysis showed a greater increase relative to PBMCs in CD25+ expanded cells from G-CSF-mobilised vs. non-mobilised PBMCs: IL-2 (16.2-fold vs. 6-fold, respectively), TNF-α (90-fold vs. 19-fold, respectively), IFN-γ (22-fold vs. 5-fold, respectively) and IL-4 (3935-fold vs. 65-fold, respectively).
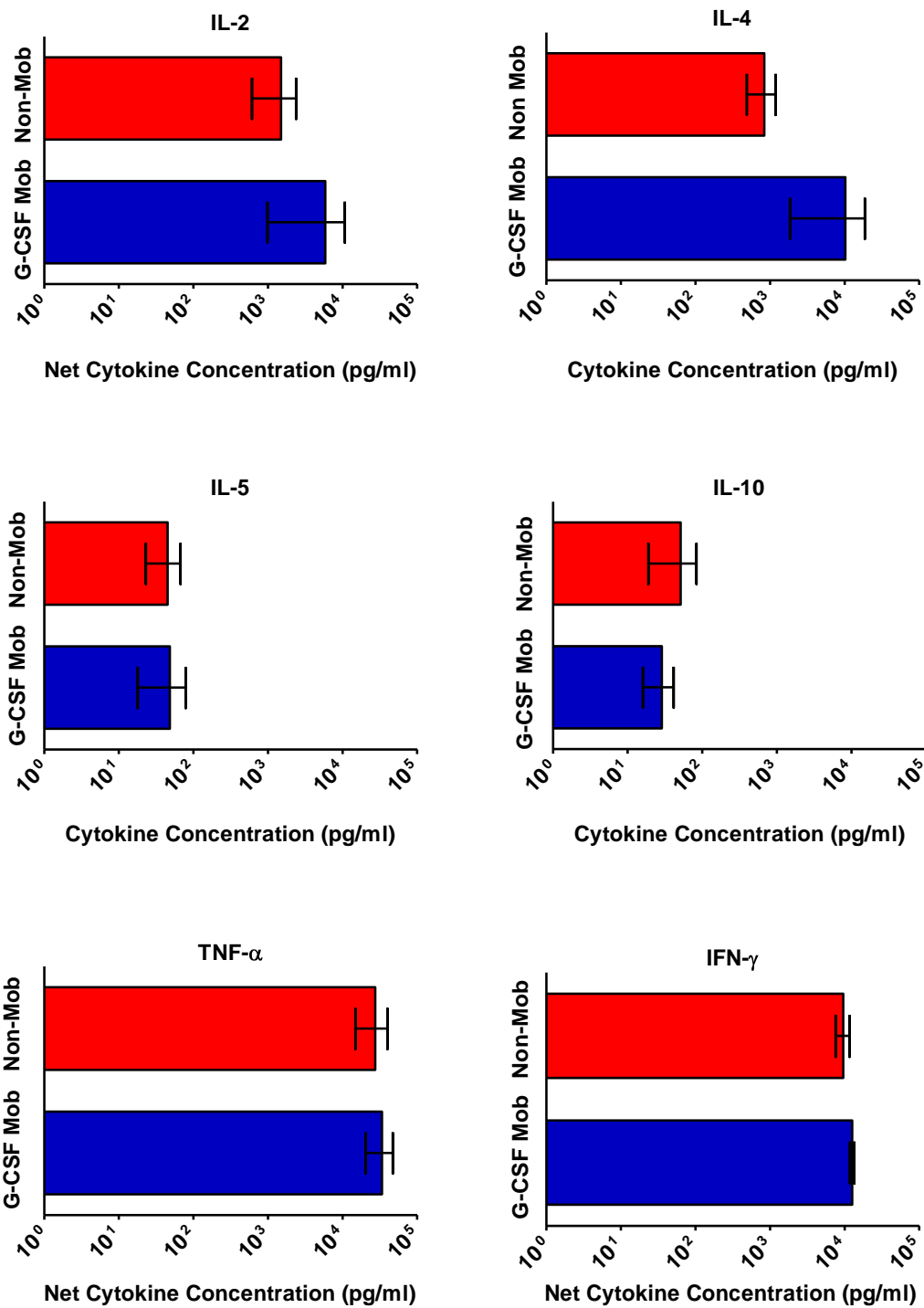
Figure 5.14 Cytokine secretion detected in culture supernatant of CD25+ expanded cells after CMVpp65 re-challenge

Quantitative assessment by CBA of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ detected in the supernatant of cultures after a 6-hour re-challenge with autologous PBMCs loaded with CMVpp65 peptide in G-CSF-mobilised (n=3) and non-mobilised (n=3) donors

A

G-CSF-Mobilised

	IL-2		TNF-α		IFN-γ		IL-4	
	PBMC	**Exp**	**PBMC**	**Exp**	**PBMC**	**Exp**	**PBMC**	**Exp**
Mean Conc. (pg/ml)	354	5849	306	27430	548	12260	3	10960
Range (pg/ml)	30 – 1328	499 – 15565	52 – 1247	13134 – 58625	140 – 1195	11204 – 14169	0 – 6	255 – 26896
Fold Increase		16.5		90		22		3935

B

Non-Mobilised

	IL-2		TNF-α		IFN-γ		IL-4	
	PBMC	**Exp**	**PBMC**	**Exp**	**PBMC**	**Exp**	**PBMC**	**Exp**
Mean Conc. (pg/ml)	235	1496	1756	33760	2028	9569	13	837
Range (pg/ml)	27 – 450	58 – 3110	37 – 8129	735 – 24488	497 – 5753	5619 – 12211	0 – 48	146 – 1284
Fold Increase		6		19		5		65

Table 5.2 Comparison of mean cytokine concentration between PBMCs and CD25+ expanded CMV-T (Exp) following CMVpp65 stimulation in both G-CSF-mobilised PBMCs (A; n=3) and non-mobilised PBMCs (B; n=3)

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5.4.3   FoxP3 expression in CD25+ expanded CMV-T

Data presented in Chapter 4 indicated that CD25+ cells enriched from G-CSF-mobilised PBMCs, following a 16-hour incubation with CMVpp65 peptides, contain a significant population of FoxP3 expressing cells. Therefore, FoxP3 expression was analysed amongst the CD4+ population of CD25+ expanded cells from four G-CSF-mobilised donors, after 14-21 days in culture. The mean CD25+ FoxP3+ expression was 4.28% ± 1.92, a significant reduction compared with CD25+ FoxP3+ expression prior to expansion (64.24% ± 4.88; $p<0.0001$). Figure 5.15 illustrates the reduced frequency observed in FoxP3 expression in two representative experiments.
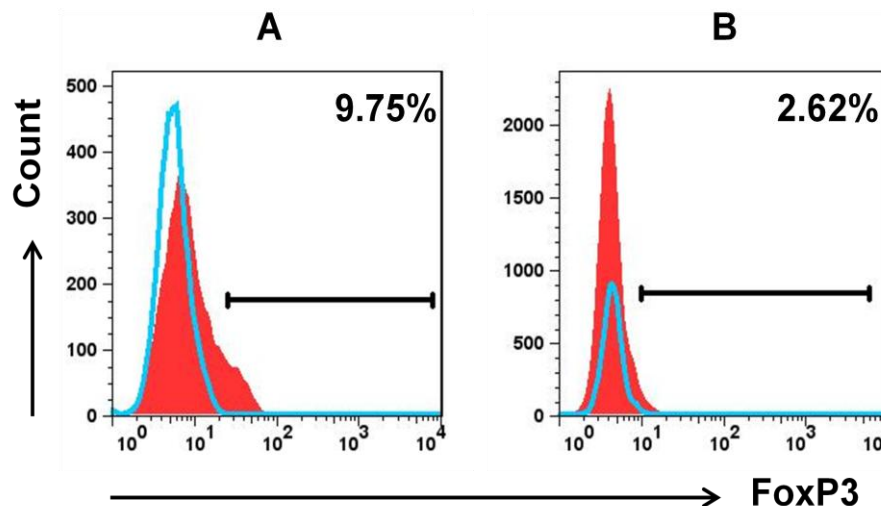
Figure 5.15 FoxP3 expression in CD25+ expanded cells in two G-CSF-mobilised donors (A and B)

Open peaks represent matched isotype controls and filled peaks represent test samples.

5.4.4   Cytotoxicity of expanded CMV-T

Cytotoxicity was evaluated in CD154+ and CD25+ expanded CMV-T from both G-CSF-mobilised and non-mobilised PBMCs, using autologous PHA blasts loaded with CMVpp65 peptides and labelled with Calcein-AM dye as targets. Both CD154+ and CD25+ expanded cells from G-CSF-mobilised PBMCs effectively lysed CMVpp65 loaded targets at all E:T ratios in a dose-dependent manner that was comparable with lysis by CD154 and CD25 CMV-T expanded from non-mobilised PBMCs (Figures 5.16 and 5.17).
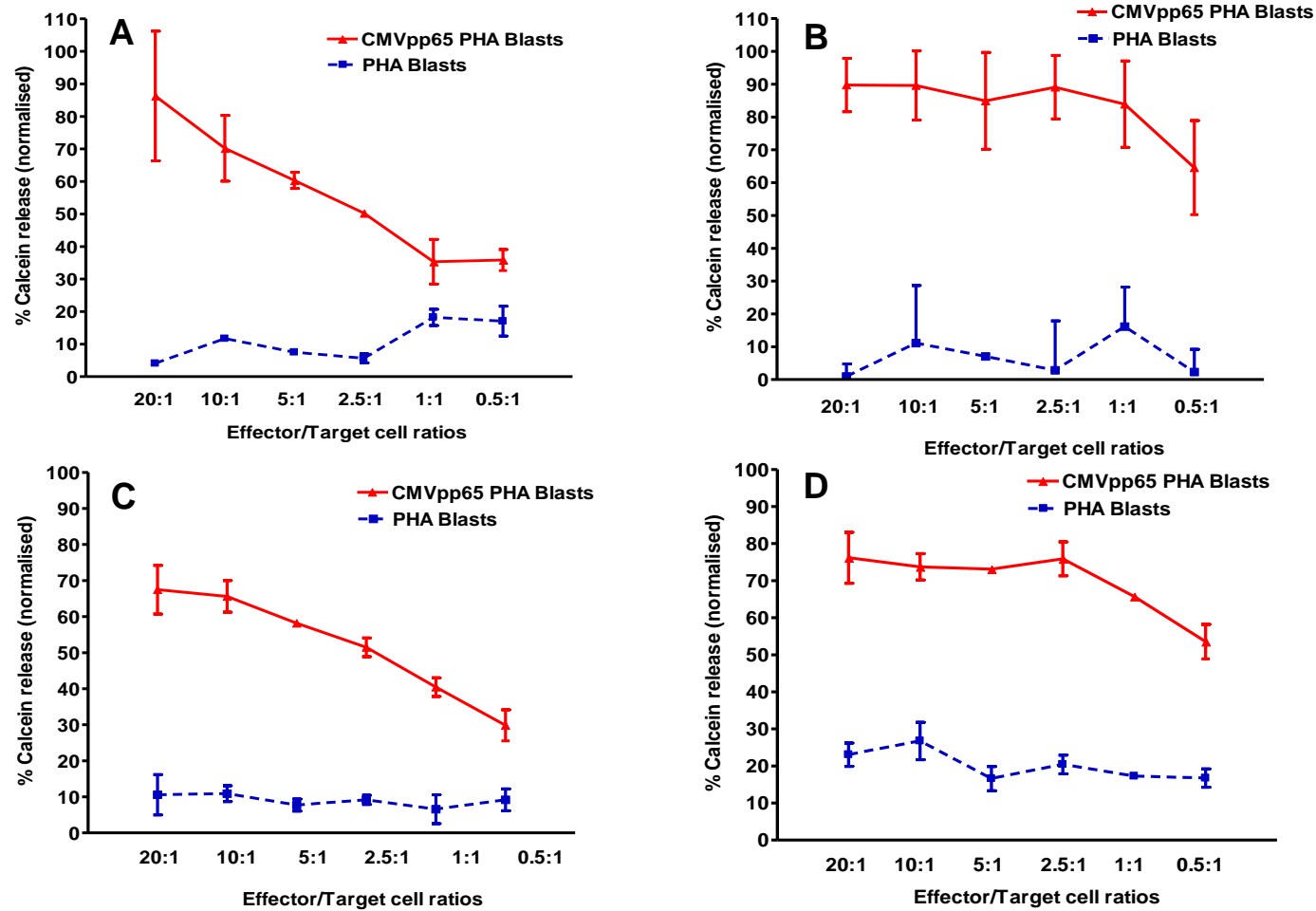
Figure 5.16 Lysis of CMVpp65 targets by CD154+ CMV-T expanded from G-CSF-mobilised PBMCs (A, B) and non-mobilised PBMCs (C, D)

Graphs show the percentage of calcein released from target cells after normalisation in the supernatants of cultures measured by fluorescence
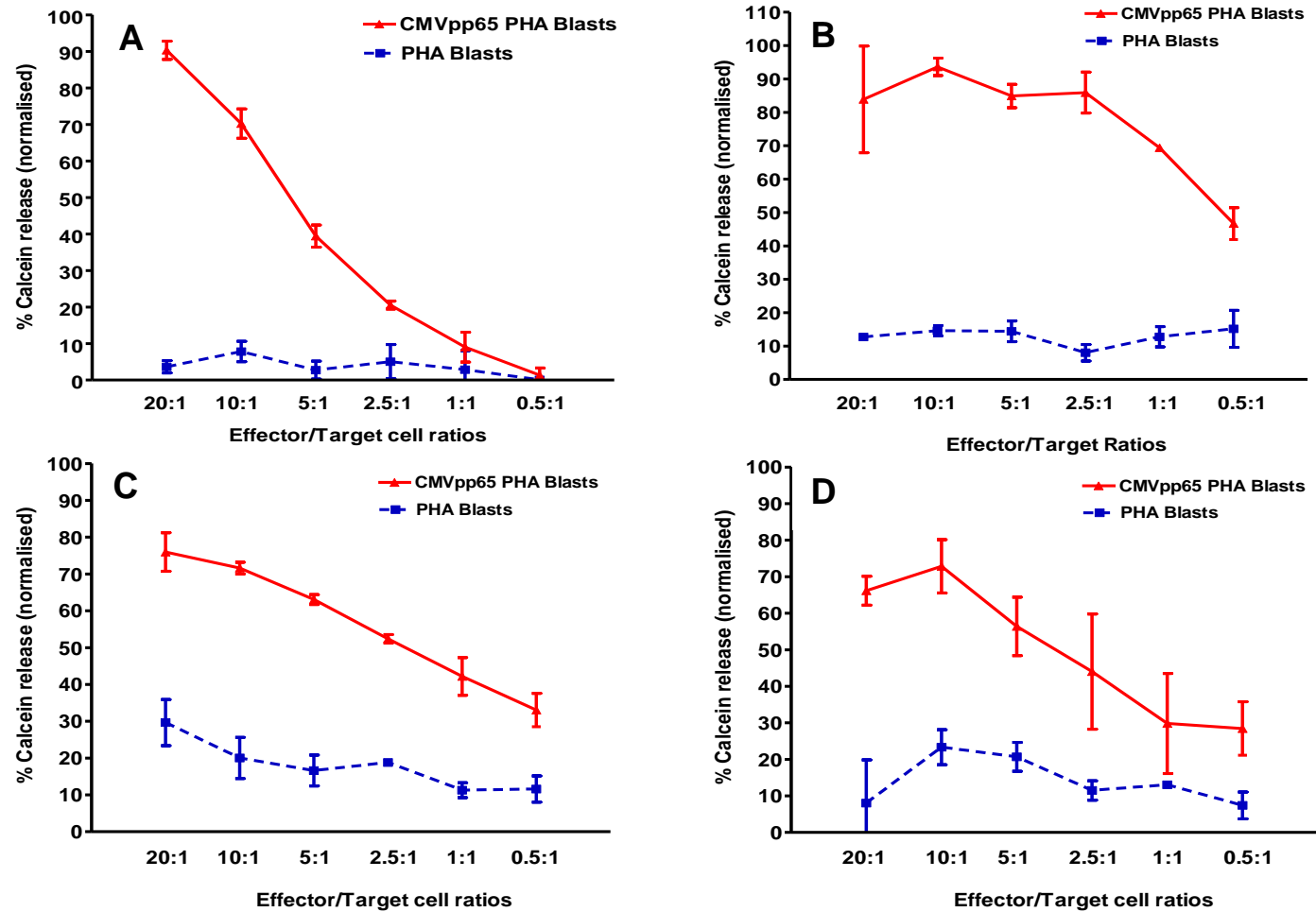
Figure 5.17 Lysis of CMVpp65 targets by CD25+ CMV-T Expanded from G-CSF-mobilised PBMCs (A, B) and non-mobilised PBMCs (C, D)

Graphs show the percentage of calcein released from target cells after normalisation in the supernatants of cultures measured by fluorescence

5.4.5 Suppression by CD25+ expanded CMV-T from G-CSF-mobilised PBMCs

FoxP3 expression and IL-10 secretion were shown to be detected at very low levels in CD25+ expanded CMV-T from G-CSF-mobilised PBMCs, suggesting that Tregs present prior to expansion do not survive in short-term culture. To further elucidate whether this is indeed the case, CD25+ expanded CMV-T were co-cultured with autologous PBMCs in the presence of anti-CD3 antibody, to assess whether expanded CD25+ CMV-T exerted any suppressive capacity. Expanded CD25+ CMV-T from two experiments showed greater suppressive capacity than the negative control at all ratios. Following anti-CD3 antibody stimulation, undivided CD3+ T cells in the two experiments were 10.3% and 17.9%, respectively. In the presence of CD25+ CMV-T expanded from G-CSF-mobilised PBMCs at a ratio of 1:1 (PBMCs:CD25+ CMV-T), undivided CD3+ T cells were 44.4% and 54.5% respectively. At a ratio of 1:2, the proportion of undivided CD3+ T cells was 46.9% and 59.2% respectively. Figure 5.19A illustrates suppression of CD3+ T cells at a ratio of 1:4 (43.2% undivided), which was also evident at a ratio of 1:5 (63.5% undivided), as shown in Figure 5.19B. It can be considered a limitation of the suppression assays that comparator CD25-autologous PBMCs were not used due to the availability of cell numbers. This would have allowed the assessment of proliferation in co-culture with autologous PBMCs, in comparison with expanded CD25+ CMV-T.

A comparison of mean suppression exerted by CD25+ enriched CMV-T (at a ratio of 1:1) prior to expansion (59.8% undivided; Chapter 4.4.3) and CD25+ CMV-T post-expansion (49.5% undivided) shows a trend towards a decrease in mean suppression exerted by expanded CD25+ CMV-T. The mean fold-increase, relative to autologous PBMCs, in the proportion of undivided CD3+ T cells alone (at ratios of 1:1 and 1:2) was 5.09 and 5.66, reectively, for CD25+ enriched CMV-T; compared with 3.65 and 3.90 , respectively, for expanded CD25+ CMV-T.
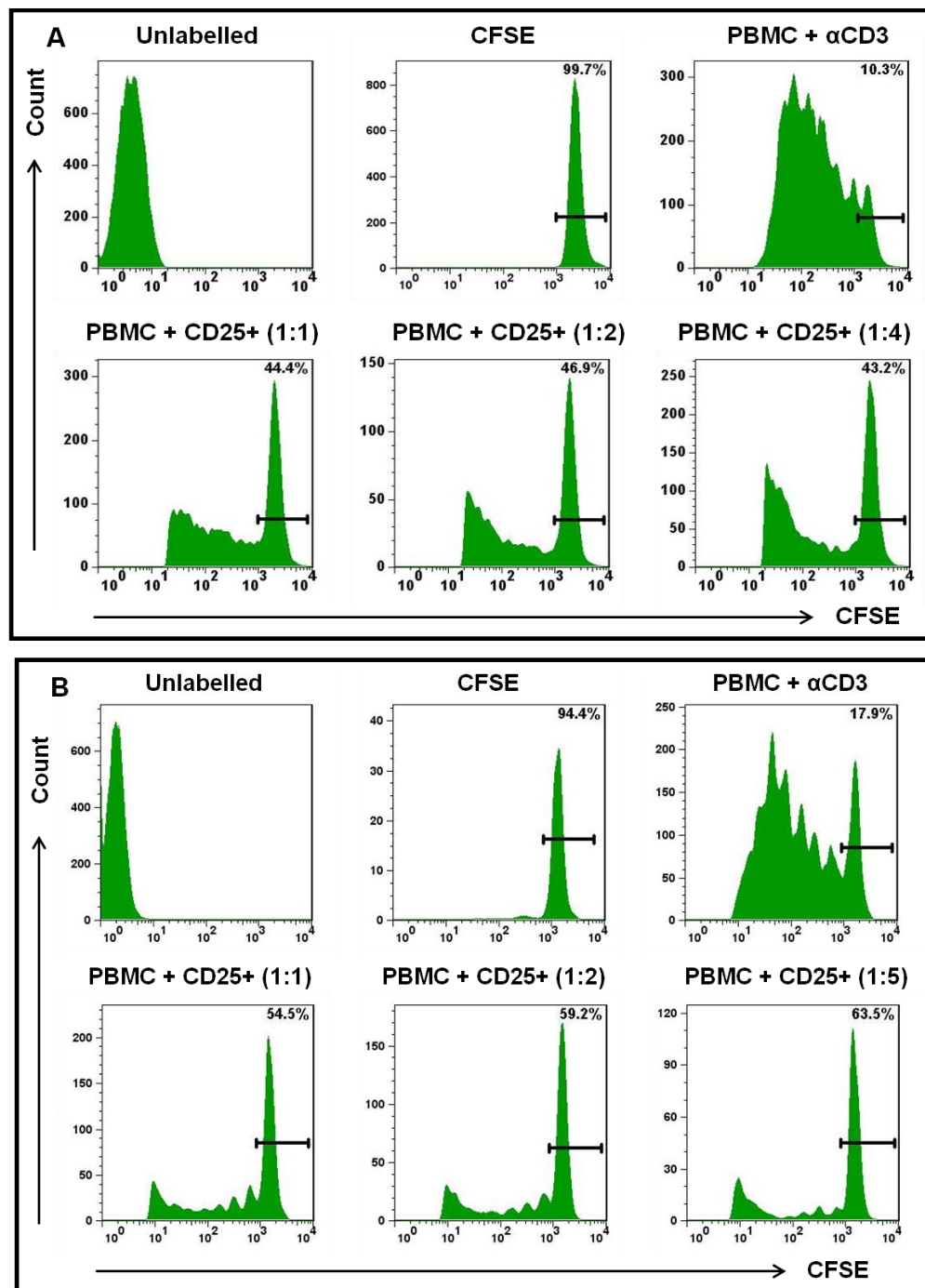
Figure 5.18 Figure 4.9 Suppression of αCD3 stimulated autologous G-CSF-mobilised PBMCs by CD25+ CMV-T in two donors (A and B):

Dose-dependent suppression of T cell proliferation in CFSE-labelled PBMCs by expanded CD25+ CMV-T was assessed at 3 ratios in the presence of αCD3 antibody for 5 days. Unlabelled PBMCs, PBMCs alone (CFSE) and PBMCs with αCD3 were cultured as experimental controls.

5.4.6   CD57 expression in expanded CMV-T

CD57 expression after expansion of CD154 and CD25 CMV-T from both G-CSF-mobilised and non-mobilised PBMCs was assessed in order to determine the level of replicative senescence of expanded cells. No significant difference was observed in baseline CD57 expression in resting G-CSF-mobilised (17.96% ± 7.05) vs. non-mobilised (12.21% ± 3.19) PBMCs prior to CMVpp65 stimulation and subsequent expansion (Figure 5.20). Further analysis showed CD57 expression to be comparable between G-CSF-mobilised and non-mobilised PBMCs in both CD4+ (3.94% ± 1.80 vs. 2.53% ± 0.85; *p*=0.47) and CD8+ (14.06% ± 5.79 vs. 9.68% ± 2.90; *p*=0.49) T cell subsets. In both G-CSF-mobilised and non-mobilised PBMCs, a trend was identified towards increased CD57 expression in the CD8+ T cell subset compared with CD4+ (*p*=0.10 and *p*=0.05 respectively).

As summarised in Figure 5.21, analysis of mean CD57 expression in CD154 expanded CMV-T from G-CSF-mobilised PBMCs (56.03% ± 4.04) showed a significant increase compared with non-mobilised CD154 CMV-T (18.67% ± 5.31; *p*=0.005). Analysis also revealed a significant increase of CD57 expression in CD154 expanded CMV-T from G-CSF-mobilised PBMCs compared with resting PBMCs (56.03% vs. 17.96%; *p*=0.008). Representative CD57 staining in expanded CMV-T is illustrated in Figure 5.20. Comparison of CD57 expression between CD25 expanded CMV-T from G-CSF-mobilised PBMCs (16.83% ± 8.17) showed no significant difference compared to non-mobilised CD25 CMV-T (27.09% ± 10.71; *p*=0.49)
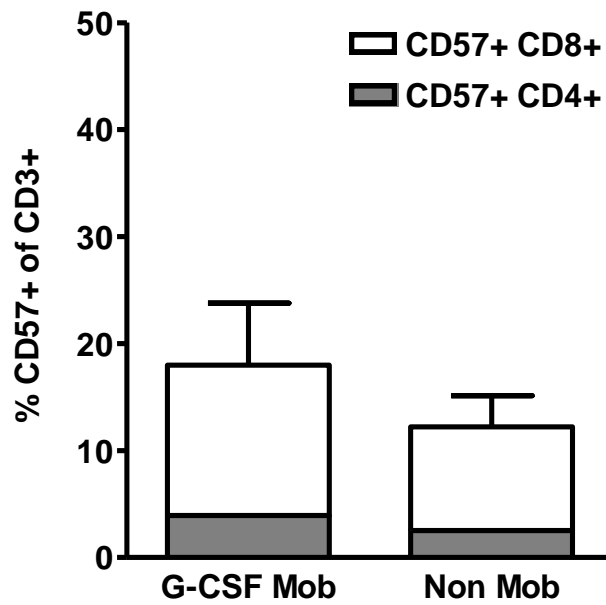
Figure 5.19 Mean CD57 expression in resting PBMCs from G-CSF-mobilised (n=5) and non-mobilised (n=6) donors
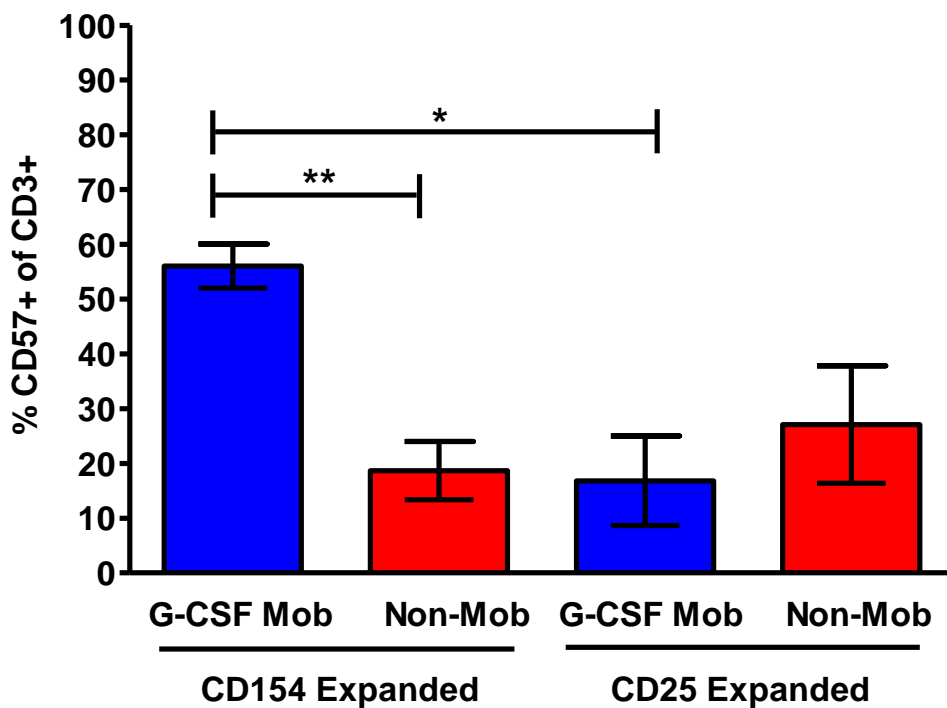
Figure 5.20 Mean CD57 expression post CMV-T expansion in CD25+ and CD154+ G-CSF-mobilised (n=3) and non-mobilised donors (n=3).

*P<0.05; **P<0.01
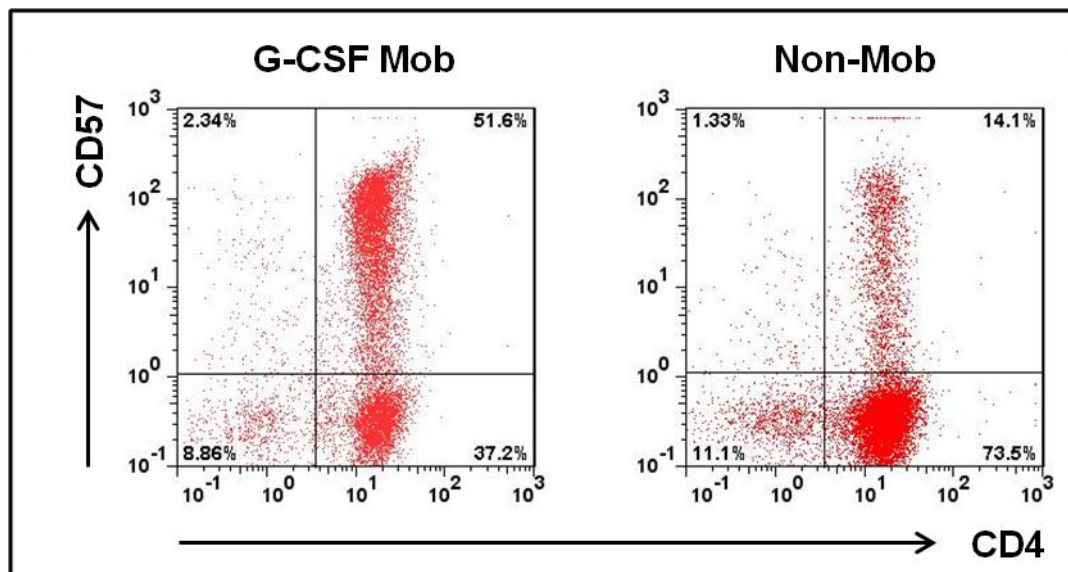
Figure 5.21 Phenotypic analysis of CD57 expression after CD154 CMV-T expansion in a G-CSF-mobilised and non-mobilised donor.

Plots are derived after gating on lymphocytes using a FSC vs. SSC plot and on CD3+ events. CD154 CMV-T are presented as CD57 vs. CD4 cell subset

5.4.7   Memory phenotype of G-CSF-mobilised expanded CMV-T

CD25+ and CD154+ CMV-T expanded from G-CSF-mobilised PBMCs were assessed for memory phenotype using anti-CD45RA and anti-CCR7 antibodies to distinguish between effector memory (CCR7- CD45RA-), central memory (CCR7+CD45RA-) and naïve (CCR7+CD45RA+) subsets. The memory phenotype of both CD154+ and CD25+ CMV-T were predominantly effector memory (72.4% ± 7.98 and 73.63% ± 11.09 respectively) and to a lesser extent, central memory (26.67% ± 7.73 and 24.20% ± 10.64 respectively). Figure 5.23 shows the accumulative data analysing memory phenotype from four CD25+ CMV-T and three CD154+ expansions. Figure 5.24 shows representative staining from one CD25+ and one CD154+ expansion from G-CSF-mobilised PBMCs.
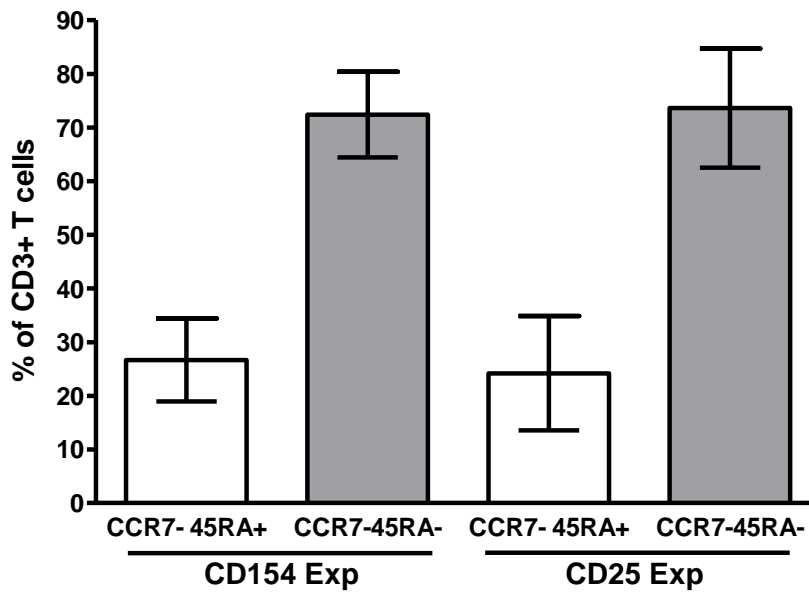
Figure 5.22 Classification of memory phenotype of CD25+ (n=4) and CD154+ (n=3) CMV-T expanded from G-CSF-mobilised PBMCs
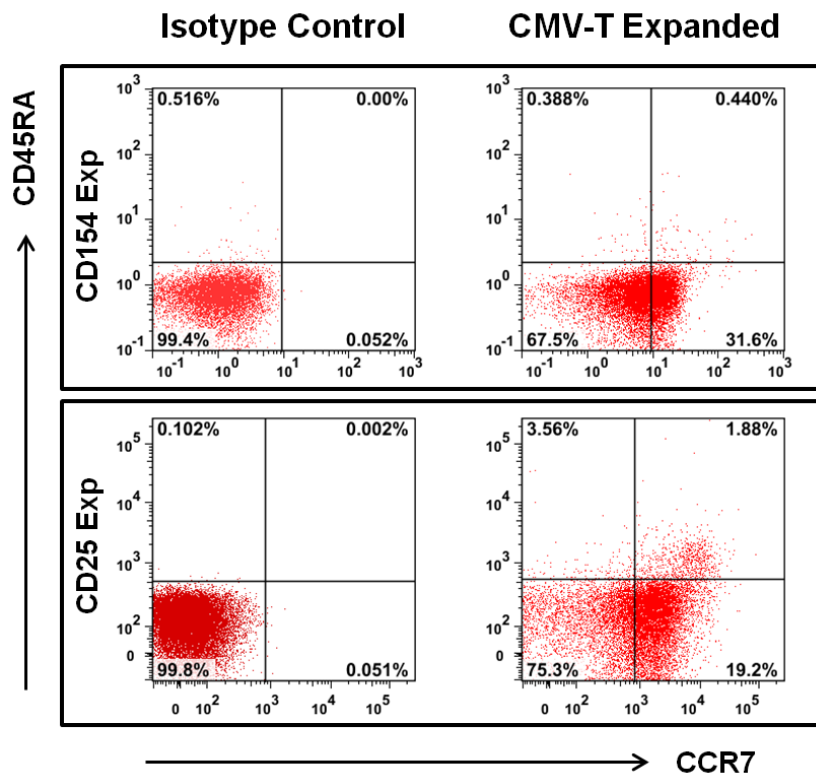
Figure 5.23 Phenotypic analysis of memory phenotype in one CD154+ and one CD25+ CMV-T expansion from G-CSF-mobilised donor

Plots were derived after gating on lymphocytes using a FSC vs. SSC plot and on CD3+ events.

Matched isotype controls were used to enable gating and are shown on the left-hand side.

5.5   Discussion

The initial aims of this chapter were to expand CD25+ and CD154+ CMV-T following magnetic enrichment in short term culture and then to re-challenge the expanded cells to demonstrate CMVpp65 specificity. Expansions were performed for CD25+ and CD154+ CMV-T isolated from G-CSF-mobilised PBMCs and compared directly against CMV-T from non-mobilised PBMCs. Cytotoxicity was examined in expanded cells to demonstrate and compare functionality in G-CSF-mobilised and non-mobilised PBMCs. Analysis of CD25+ enriched CMV-T from G-CSF-mobilised PBMCs in Chapter 4 had shown a significantly high level of FoxP3-expressing cells that were capable of suppressing T cell proliferation. Therefore, a further aim of this chapter was to assess FoxP3 expression and suppressive capacity of CD25+ CMV-T following expansion in G-CSF-mobilised PBMCs.

The expansion of CD154+ CMV-T from G-CSF-mobilised PBMCs was shown to be comparable with that of non-mobilised PBMCs, demonstrating that G-CSF mobilisation had no detrimental effect on the proliferative capacity of CMV-T after enrichment through CD154 expression. The mean expansion of 54-fold from G-CSF-mobilised PBMCs and 60-fold from non-mobilised PBMCs after 14 days was comparable to the expansion of CD154+ CMV-T of 41-fold and 58-fold after 14 days reported by Khanna and colleagues (Khanna *et al*, 2011). However, the level of expansion reported here and by Khanna *et al.* are markedly lower than that of Frentcsh *et al.* (Frentsch *et al*, 2005) who reported expansion of at least 1,000-fold within 14 days. A possible explanation for this discrepancy is that the analysis of expansion by Fentsch *et al.* used CD154+ CD4+ T cells together with the absolute number of CD154+ cells that were isolated, whereas the analysis described here utilised CD154+ CD3+ T cells and the absolute number of cells isolated. The absolute number of cells would be significantly higher than that of CD154+ cells; and, given that the mean purity of CD154+ cells from G-CSF-mobilised PBMCs was 48.9%, would therefore be consistent with a reduced level of expansion relative to Frentcsh *et al.* (Frentsch *et al*, 2005).

Analysis of mean CD25+ CMV-T expansion, following a 3 week culture period, showed a trend (p=0.06) towards increased expansion in non-mobilised PBMCs compared with G-CSF-mobilised PBMCs (175-fold vs. 90-fold expansion respectively). Similar to the findings for CD154+ CMV-T, these levels of expansion are markedly lower than that in the published literature. Gallot and colleagues (Gallot *et al*, 2001) reported data from one non-mobilised donor, showing an amplification factor of 583-fold in CD25+ CMV-T after 13 days in culture. It must be pointed out, however, that culture conditions were very different from that in the present study, as was the method of CMVpp65 stimulation, which makes comparisons between the two studies difficult to interpret.

Following CD25+ enrichment, G-CSF-mobilised PBMCs contained significantly more FoxP3-expressing Tregs that demonstrated superior suppressive capacity than CD25+ enriched from non-mobilised PBMCs. The increased number of Tregs in CD25+ enriched T cells from G-CSF-mobilised PBMCs could account for the difference in expansion between G-CSF-mobilised and non-mobilised PBMCs. There are two possible hypotheses to consider here. Firstly, that the higher proportion and the superior suppressive capacity of Tregs in CD25+ enriched from G-CSF-mobilised PBMCs reduces the total number of non-Tregs (CD25+ CMV-T) and inhibits their expansion compared with non-mobilised PBMCs. Secondly, Tregs do not survive the culture process and therefore the number of CD25+ T cells capable of proliferating in the G-CSF-mobilised setting is significantly lower than that of the non-mobilised setting. This second hypothesis is supported by the decrease in the mean proportion of CD4+ T cells from G-CSF-mobilised PBMCs following expansion (44.1%) compared with pre-expansion (97.5%). FoxP3 expression and IL-10 secretion levels were both low to undetectable in CD25+ expanded CMV-T from G-CSF-mobilised PBMCs, also suggesting that Tregs present after CD25+ enrichment do not survive in long-term culture. However, expanded CD25+ CMV-T still demonstrated suppression of T cell proliferation after polyclonal activation, which is suggestive of a population of FoxP3– IL10– Tregs in the expanded cells.

The finding, reported here, of an increased number of Tregs in G-CSF-mobilised PBMCs following CMVpp65 stimulation, is supported by the results of a recent study

by Ukena and colleagues (Ukena *et al*, 2012). In contrast, however, this study also reported that CD25+ enriched T cells from G-CSF-mobilised PBMCs were capable of a higher level of expansion than non-mobilised PBMCs after 14 days. In this regard, it should be noted that culture conditions were different from those reported here, and CD25+ enrichment was performed in the absence of antigenic stimulation prior to enrichment.

Both CD25+ and CD154+ expanded cells from G-CSF-mobilised PBMCs showed functional specificity against CMVpp65, demonstrated by the high levels of IFN-γ, TNF-α and IL-2 secretion, which were statistically equivalent to those of non-mobilised PBMCs. Cytokine profiling by CBA supported these results, demonstrating a cytotoxic effector function in expanded CMV-T. The most striking result observed through cytokine profiling of expanded CD25+ and CD154+ CMV-T, was the highly significant increase in IL-4 secretion compared with both G-CSF-mobilised and non-mobilised PBMCs. Mean IL-4 secretion levels were 3935-fold higher in CD25+ CMV-T and 5904-fold higher in CD154+ CMV-T from G-CSF-mobilised PBMCs compared with G-CSF-mobilised PBMCs prior to magnetic enrichment. IL-4 is recognized as a key pleiotropic cytokine promoting the development of the TH_2 cell subset (Hsieh *et al*, 1992; Swain *et al*, 1990) which, in contrast to TH_1 cells, do not secret IFN-γ (Abbas *et al*, 1996). Both CD25+ and CD154+ expanded CMV-T secreted similar levels of IFN-γ, suggesting that expanded cells give rise to polarized differentiation, producing a mixture of TH_1 and TH_2 cells amongst the CD4+ T cell population. One study has described the down-regulation of CD8 expression induced by IL-4 secretion during *in vitro* activation, (Kienzle *et al*, 2005), which could explain the predominantly CD4+ phenotype of CD154+ expanded CMV-T, but not the CD25+ expanded CMV-T phenotype, which contained a proportionally more balanced population of CD4+ and CD8+ T cells. Future experiments investigating the expression of GATA3, a transcription factor associated with TH_2 cells and necessary for IL-4 secretion (Zheng & Flavell, 1997), could determine the subset of CD4+ cells expanded in this culture system.

CD25+ and CD154+ expanded CMV-T both contained high levels of Granzyme B, indicating a possible mechanism for cytotoxicity through the process of directed

exocytosis, resulting in apoptosis of the target cells (Adrain *et al*, 2005; Darmon *et al*, 1995). The cytokine profile of expanded cells together with the identification of the lytic granule Granzyme B are in keeping with a population of anti-viral CD4+ cytotoxic T cells (CTLs) characterised in previous studies (Appay *et al*, 2002b) and reminiscent of anti-viral CD8+ T cells. Furthermore, cytotoxicity was directly demonstrated by using a Calcein-AM cytotoxicity assay (Neri *et al*, 2001) to show that both CD25+ and CD154+ CMV-T expanded from G-CSF-mobilised PBMCs can successfully lyse CMVpp65 peptide pulsed target cells. The memory phenotype of CD154+ CMV-T expanded from G-CSF-mobilised PBMCs was predominantly effector memory and, to a lesser extent, central memory determined by staining for CD45RA and CCR7 by flow cytometry. The effector memory phenotype is consistent with previous findings that CCR7 is down regulated during prolonged culture (Zandvliet *et al*, 2009). The functional expansion of a population of anti-viral CD4+ CTLs could have been strengthened through the demonstration of enhanced cell lysis after effective blocking of HLA class I on target cells. Although experiments demonstrated ligation of HLA class I and HLA class II with anti-HLA DR,DP,DQ and anti-HLA A,B,C antibodies, lysis of target cells was not affected.

In addition to direct cytotoxicity, the secretion by expanded CD154+ CMV-T of IFN-γ and TNF-α, cytokines that inhibit CMV replication (Hengel *et al*, 1994), further supports the existence of a predominant CD4+ CTL cell subset. It is important to note that in various anti-viral immune responses, maintenance of CD8+ CTL depends greatly on CD4+ T cell function (Gallot *et al*, 2001; Komanduri *et al*, 2001), demonstrated by the increase of CD8+ CMV-T after adoptive transfer of CD4+ CMV-T *in vivo* (Einsele *et al*, 2002). In contrast, the expansion of CD25+ CMV-T in both G-CSF-mobilised and non-mobilised PBMCs contained a mixture of both CD4+ and CD8+ T cells. The use of CMV-T comprising a mixture of CD4+ and CD8+ T cells is in line with several studies which demonstrate the effective treatment of CMV disease after adoptive transfer of CMV-T (Peggs *et al*, 2003; Peggs *et al*, 2011; Rauser *et al*, 2004; Feuchtinger *et al*, 2010).

The expression of CD57 as a marker of replicative senescence in HIV-specific CD8+ T cells was demonstrated through an inability to proliferate after antigenic stimulation

(Brenchley *et al*, 2003). CD154+ CMV-T expanded from G-CSF-mobilised PBMCs expressed CD57 at significantly higher levels than CD154+ CMV-T from non-mobilised PBMCs and CD25+ CMV-T from G-CSF-mobilised and non-mobilised PBMCs. Over half of CD154+ CMV-T expanded from G-CSF-mobilised PBMCs expressed the CD57 antigen (mean, 56%). However, expansion in CD154+ CMV-T from G-CSF-mobilised PBMCs was equivalent to that of CD154+ CMV-T from non-mobilised PBMCs, indicating that their proliferative capacity is not effected. This could be attributed to the predominant CD4+ phenotype in CD154+ CMV-T, in contrast to the CD8+ subset studied by Brenchley and colleagues. Future experiments to sort CD57+ cells at the end of the culture period and assess their proliferation in response to antigenic challenge could allow further understanding of CD57 as a marker of replicative senescence in CD4+ CMV-T cells.

An additional experimental idea is the investigation of telomere length, which is associated with the proportion of expanded cells that are non-functional and will become senescent or apoptotic (Verdun & Karlseder, 2007). Telomere length has been demonstrated to be reduced in CD8+ CD28− T cells, a population of cells associated with activation, compared with that of the naïve CD8+ CD28+ T cell subset (Batliwalla *et al*, 2000) and also after CMV infection (van de Berg *et al*, 2010). Investigating telomere length offers further understanding of the replicative capacity of expanded cells after adoptive transfer.

The results presented in this Chapter illustrate that CMV-T isolated from G-CSF-mobilised PBMCs through both CD154+ and CD25+ activation-dependent expression are capable of expanding in short term culture at a comparable level to that of conventional non-mobilised PBMCs. Expanded cells secreted and synthesised cytokines associated with a cytotoxic function and were effective in lysing CMVpp65 loaded target cells in a dose-dependent manner. CMV-T expanded from G-CSF-mobilised PBMCs following CD25+ enrichment did not retain a Treg phenotype, as indicated by the loss of FoxP3 expression and the lack of IL-10 secretion, but were, nevertheless, capable of exerting a suppressive capacity on autologous PBMCs, as determined by a CFSE proliferation assay. In conclusion, these results suggest that manufacturing a CMV-T immunotherapy product from G-CSF-mobilised PBMCs to

clinical scale could be effective in the treatment of CMV disease following allogeneic HSCT. Clinical scale-up would allow for the rigorous assessment of the purity, yield and absolute numbers of CMV-T necessary for the successful translation of this approach as a routine therapy for patients with CMV disease.

Chapter 6

Development of CD154-based Selection of CMV-T to Clinical Scale

6.1 Introduction

The development of a strategy for CD154-based selection of CMV-T from G-CSF-mobilised PBMCs requires process validation at a clinical scale in order for this methodology to be approved as a clinically applicable protocol. The direct selection of CMV-T for clinical use has relied primarily on the use of HLA-peptide multimers (Cobbold *et al*, 2005) or IFN-γ secretion (Feuchtinger *et al*, 2010; Peggs *et al*, 2011) in order to manufacture clinically relevant doses for adoptive transfer. Direct selection of CMV-T using the IFN-γ secretion protocol has also been used as a platform for the subsequent expansion of CMV-T, thereby demonstrating a second approach to the production of CMV-T that can be translated into clinical use (Rauser *et al*, 2004b). IFN-γ secretion has also been utilised for the isolation and expansion of adenovirus-specific CD4+ and CD8+ T cells, which were safely administered to nine children with lymphopenia following HSCT (Feuchtinger *et al*, 2004). However none of these protocols resolves the complex issues surrounding successive donation following donation of the original HSC graft.

Results reported in Chapter 3 showed that the IFN-γ secretion method for CMV-T isolation is sub-optimal when using G-CSF-mobilised PBMCs as a starting material. However, the use of G-CSF-mobilised PBMCs as a starting material for the generation of CMV-T to clinical scale has recently been reported in 11 donors (Clancy *et al*, 2013). In this study, seven patients received CMV-T lines expanded from an aliquot of the original HSCT graft. This study adopted the approach of using monocyte-derived APCs transfected with a clinical grade adenovirus vector (Ad5F35pp65) encoding the entire CMVpp65 protein in order to initiate CMV-T expansion from unmanipulated aliquots of G-CSF-mobilised PBMCs. This approach is labour-intensive, requires several weeks for the generation of a clinically relevant product, and would potentially require laboratories to hold both gene therapy and cell therapy licences, which from a

regulatory standpoint is extremely difficult to achieve outside of an industrial setting. Therefore, the feasibility of this approach to CMV-T manufacture is not one that is easily applicable to the widespread cell therapy community and patient-directed therapies. An alternative method of direct selection, the isolation of CMV-T through cell surface activation markers, has been successfully demonstrated at a clinical scale using CD25-based selection (Lugthart *et al*, 2012).

GMP-compliant magnetic isolation of cell subsets has been enhanced through the availability of instruments such as the CliniMACS (Miltenyi Biotec) and Isolex 300 (Baxter Healthcare) devices. The CliniMACS device has now become a routine instrument in cellular therapy laboratories since its inception for the isolation of CD34+ cells from apheresis products (Schumm *et al*, 1999). Over the past decade its use in process development for the manufacture of novel cell therapies, including the generation of anti-viral T cells for adoptive transfer, has resulted in the CliniMACS becoming the primary device used in direct selection (Cobbold *et al*, 2005; Feuchtinger *et al*, 2004; Feuchtinger *et al*, 2010; Meij *et al*, 2012; Peggs *et al*, 2011; Rauser *et al*, 2004a; Lugthart *et al*, 2012). Therefore, the CliniMACS lends itself to the process development of CD154-based enrichment of CMV-T and the potential translation of this methodology into routine clinical therapy.

The manufacture of cell therapies, including CMV-T, has changed substantially over the past decade and as a consequence, EU legislation was laid down to govern such products. CMV-T manufactured by any process other than direct selection using multimers, are classified in Europe as advanced therapeutic medicinal products (ATMPs) as an amendment to the EU Directive 2001/83/EC, under the classification of a somatic cell therapy product (Annex 1, Part IV), defined in Regulation 1394/2007/EC. In the UK, the Medicines and Healthcare products Regulatory Agency (MHRA) is the competent authority responsible for issuing manufacturing licences for ATMPs and subsequently accrediting laboratories to undertake such manufacturing. The manufacture of ATMPs must be in compliance with the principles of good manufacturing practice (GMP), as set out in Directive 2009/94/EC, which demands the use of validated procedures and processes with product definition, sterility testing, pharmaceutical-grade raw materials and records of reagents used (Dwenger *et al*, 2010;

Botta & Migliaccio, 2011). Currently there is no commercially available GMP-grade anti-CD154 antibody nor associated CD154 magnetic bead conjugate for use in clinical grade immunoselection. Neither is there a GMP-grade purified anti-CD40 antibody available. Using an anti-biotin bead conjugate is a potential solution to performing clinical scale enrichment of CD154+CMV-T, and is commercially available to GMP-grade (Miltenyi Biotec). Although the remaining reagents needed for a GMP-compliant process validation are not available, the use of non-GMP-qualified reagents is not precluded once they have been subjected to suitable risk assessments (Lowdell *et al*, 2012b). Process validation of CD154+ CMV-T manufacture, together with appropriate risk assessments of non-GMP-qualified reagents, is an important step in assessing the feasibility of both this approach to CMV-T manufacture and its successful transfer into routine clinically practice as an ATMP.

6.2   Aims of Experiments Described in this Chapter

The initial aim of this Chapter was the assessment of anti-biotin microbeads for the enrichment of CD154+ CMV-T following CMVpp65 peptide stimulation. This aim was assessed in three G-CSF-mobilised PBMC donors to investigate whether this approach was comparable with previous experiments using anti-PE microbeads and could, therefore, be used in a clinical scale experiment. Next, a G-CSF-mobilised PBMC donor was pre-screened for CD154 activation-induced expression after CMVpp65 peptide stimulation to assess whether previously cryopreserved PBMCs would be a feasible approach for use in the clinical scale-up.

The secondary aim of this Chapter was the process development of a CD154-based selection strategy for isolation of CMV-T from a G-CSF-mobilised donor at a clinical scale. This process validation aimed to test current standard operating procedures (SOPs) for the stimulation, labelling and selection of anti-viral T cells that would allow the process to be translated into a "clean-room" laboratory for manufacture. The process validation allowed yield and purity at clinical scale to be tested and compared to current practice. In addition, total cell numbers were measured to ascertain whether clinically relevant doses of CMV-T can be manufactured in this system. CD154+ CMV-T were

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also rested after selection and re-challenged with CMVpp65 peptides to assess the specificity of the product. Process validation is a critical step in testing novel therapies to ascertain the feasibility of translation to full GMP-compliance. The final aim of this Chapter was to ascertain what aspects of the protocol could be changed and improved in future clinical scale experiments to facilitate the development of the process and expedite translation to the clinic.

The experimental aims of the Chapter were as follows:

1. To optimise CD154+ CMV-T isolation from G-CSF-mobilised PBMCs using anti-biotin microbeads and compare against isolation using PE-microbeads

2. To identify a suitable G-CSF-mobilised donor for clinical scale CD154+ CMV-T manufacture.

3. To assess yield, purity and cell numbers in clinical scale CD154+ CMV-T manufacture.

4. To assess the functionality of CD154+ CMV-T following clinical scale manufacture.

6.3   Materials and Methods

6.3.1   Anti-Biotin Microbeads for Small Scale Enrichment

Optimisation of the biotin-based selection strategy was performed in three G-CSF mobilised donors. CMVpp65 peptide stimulation was performed as previously described for CD154 activation-induced expression at an optimal time point of 6 hours. Following a 6-hour stimulation, PBMCs were centrifuged at 300g for 10 minutes at room temperature and the cell pellet was resuspended at a concentration of 10^7 cells/100µl of CliniMACS PBS/EDTA buffer. PBMCs were labelled in 15ml conical tubes with 10µl of anti-CD154 Biotin (Miltenyi Biotec)/10^7 total cells and incubated for 10 minutes at 4–8°C in the dark. The supernatant was removed and the cell pellet resuspended at 10^7 cells/80µl in CliniMACS buffer, labelled with 20µl of anti-Biotin microbeads (Miltenyi Biotec)/10^7 cells and incubated for 15 minutes at 4–8°C in the dark. Cells were diluted to 15ml with CliniMACS buffer, centrifuged at 300g for 10 minutes at room temperature, and enrichment performed using MS columns as previously described in Chapter 2.10.1. Following enrichment, samples from the positive, negative, and pre-sort fractions were labelled with anti-Biotin PE (Miltenyi Biotec) for assessment of CD154 purity and yield by flow cytometry, as described in Chapter 2.7.

6.3.2   Preparation of PBMCs for Clinical Scale Enrichment

A previously cryopreserved G-CSF-mobilised apheresis collection of 100ml, signed off for discard, was identified as starting material for the clinical scale-up experiment. Pre-screening was performed on a cryopreserved aliquot from the identified donor, to determine CD154 expression in response to CMVpp65 stimulation. The cryopreserved sample was thawed at 37°C by gentle massage in a waterbath, before transferring into 20 x 50ml falcon tubes with 5ml per tube. Thawed PBMCs were diluted to 50ml in pre-warmed CM and centrifuged for 10 minutes at 200g. Supernatant was removed using a 25ml pipette and cell pellets resuspended in 2-3ml of CM and passed through a cell

strainer (Becton Dickenson). Cells were diluted to a volume of 10ml in CM, before re-combining all cells and transferring into a 600ml transfer bag (Baxter).

6.3.3 Isolation of PBMCs for Clinical Scale Enrichment

Isolation of PBMCs was performed on the COBE 2991 cell processor using a COBE processing set (both BCT Caridian). The cell suspension was layered onto an equal volume of Lymphoprep, using a peristaltic pump (Watson Marlow) and pump tube (Quest Biomedical) at a speed of 25rpm and subsequently centrifuged at 2000rpm for 20 minutes. The mononuclear cell (MNC) fraction was collected and washed in 1 litre of buffer, consisting of 500ml HSA 4.5% and 500ml saline (Baxter), on the COBE 2991. Washed cells were resuspended in wash buffer and transferred to a 50ml falcon tube and centrifuged for 10 minutes at 250g. Supernatant was removed and the cell pellet resuspended in CM at a concentration of 10^7/ml, before transferring into a 100ml cell expansion bag (Miltenyi Biotec) and incubating overnight at 37°C/5%CO_2. Following the overnight incubation, cells were transferred into four falcon tubes and washed in CM to remove any cell clumps. Supernatants were removed and cell pellets resuspended in CM, then re-combined and transferred into a cell expansion bag at a concentration of 10^7/ml.

6.3.4 *In vitro* Stimulation of PBMCs for Clinical Scale Enrichment

G-CSF mobilised PBMCs were stimulated for 6 hours with CMVpp65 Peptivator at a final concentration of 0.6nmol (approximately 1µg) of each peptide per ml of cells, in the presence of 1µg/ml of anti-CD40 antibody. As a negative control, 1×10^6 PBMCs were incubated in one well of a 96-well plate in the presence of anti-CD40 antibody only and incubated at 37°C/5%CO_2.

6.3.5 Clinical Scale CD154 Enrichment

Following CMVpp65 stimulation PBMCs were transferred into 2 x 50ml Falcon tubes, washed in cold CliniMACS PBS/EDTA buffer (Miltenyi Biotec) supplemented with 0.5% HSA at 300g for 10 minutes, the supernatants removed and the cell pellets

resuspended in 2ml of buffer and recombined. The cell suspension was sampled for counting and 1ml of anti CD154-Biotin antibody added before incubation at 4-8°C for 10 minutes. PBMCs were diluted to 50ml in buffer, centrifuged at 300g for 10 minutes, the supernatant removed and the cell pellet resuspended in 2.5ml of buffer. PBMCs were incubated at 4-8°C for 15 minutes with 2ml of anti-biotin microbeads, then washed in 50ml of buffer and resuspended in a final volume of 100ml in buffer for selection. A sample was taken for pre-sort counting and immunophenotyping, before loading onto the CliniMACS^{PLUS} with 1 litre of CliniMACS PBS/EDTA buffer supplemented with 0.5% HSA using a CliniMACS tubing set (all Miltenyi Biotec). The Enrichment 2.1 programme was used for the selection of labelled cells with low antigen expression, and the positive fraction eluted into a 250ml collection bag (Miltenyi Biotec). CD154-positive and CD154-negative fractions were detached from the CliniMACS tubing set and their volumes recorded. Samples were taken from both fractions for counting and immunophenotyping in order to establish yield and purity. Both fractions were transferred to 50ml falcon tubes and centrifuged at 300g for 10 minutes, the supernatant removed and cells resuspended in CM at 2×10^6 cells/ml for culture.

Table 6.1 shows a list of reagents and consumables used in the clinical scale CD154 enrichment and Figure 6.1 (A–D) illustrate the instrumentation involved in clinical scale manufacture. Analysis of pre-stimulation, post-stimulation, CD154-positive and CD154-negative samples were analysed for viability using the carbocyanine monomer nucleic stain TO-PRO-3 Iodide (Life Technologies) as a dead cell indicator that was resolved in FL4 by flow cytometry.

Reagent/Consumable	No. of units	Lot Number	Expiry Date	Supplier
Anti CD154 Biotin	1	5121128075	29/11/13	Miltenyi Biotec
Anti Biotin Beads	1	130-090-485	17/08/13	Miltenyi Biotec
CMVpp65 Peptivator (60nmol)	1	130-093-435	29/11/14	Miltenyi Biotec
5L RPMI + Glutamax	1	1156093	05/2013	Invitrogen
100ml AB Serum	3	10169	06/2017	BioSera
250ml Lymphoprep	1	12CK515	10/2013	Axis Shield Diagnostics
Purified anti CD40	1	B147482	-	BioLegend
COBE processing set	3	06S15004	10/2014	Caridian BCT
100ml cell expansion bag	3	6061009007	11/2013	Miltenyi Biotec
1L CliniMACS PBS/EDTA buffer	3	D0467	02/2014	Miltenyi Biotec
CliniMACS Tubing Set	1	B1915	06/2015	Miltenyi Biotec
Pre System Filter	1	219802	07/2017	Miltenyi Biotec
150ml transfer bag	1	110721F2	06/2014	Miltenyi Biotec
Leur/Spike interconnector	1	12A03V233	12/2016	Miltenyi Biotec
Injection site coupler	5	FA11I10049	08/2016	Baxter
500ml 4.5% HSA	1	ADAN9424	01/2015	BPL
100ml 20% HSA	1	ABCN8943	09/2013	BPL
500ml sodium chloride	1	12E03E42	04/2014	Baxter
1L transfer bags	2	FA11H01115	08/2014	Fenwal Inc.
600ml transfer set	4	1412020115	01/2015	Baxter
50ml syringe	5	1211001	10/2017	Terumo
pump tube	1	10G0379	07/2015	Quest Biomedical
2 port to spike	2	H17014	10/2014	OriGen Biomedical
10ml Sterile water ampoule	1	12266012	05/2015	Braun
Air Inlet	2	12I04V725	08/2017	Baxter
50ml Falcon tube	10	17812011AA	-	BD

Table 6.1 List of reagents and consumables used in clinical scale-up
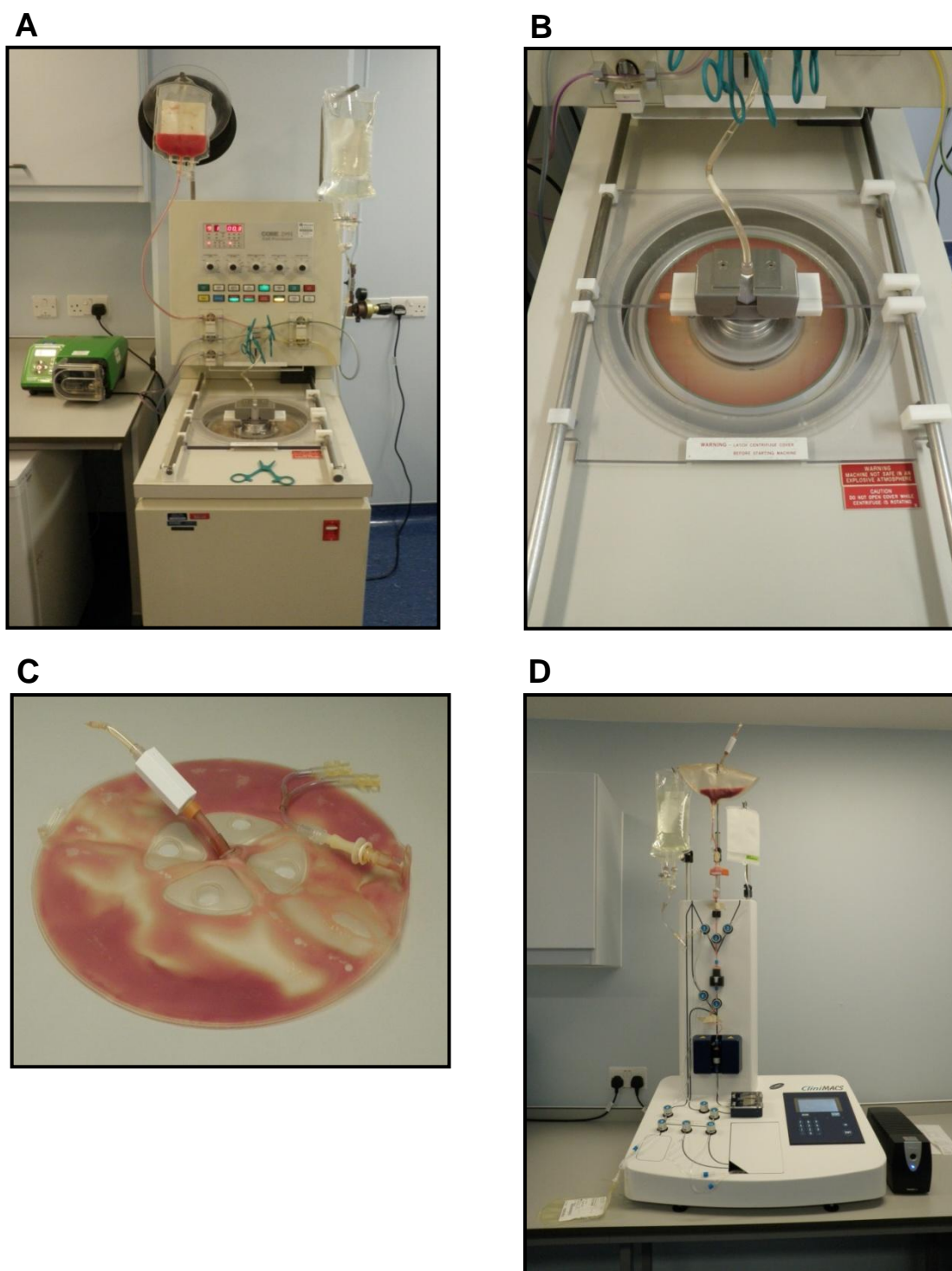
Figure 6.1 Processing of CD154+ CMV-T from G-CSF-mobilised PBMCs

G-CSF-mobilised PBMCs were loaded onto the COBE 2991 cell processor for washing following thawing and MNC preparation (A-B). The resulting MNC fraction was recovered (C) and, following labelling with anti-CD154 Biotin and anti-Biotin microbeads, was loaded onto the CliniMACS for CD154 selection (D).

6.3.6 Culture of CD154+ Fraction

CD154-positive and negative fractions were immediately washed in CliniMACS PBS/EDTA buffer + 0.5% HSA following clinical scale enrichment then resuspended at 2×10^6/ml and sampled for flow cytometry. The remaining cells were rested for 6 days in 24-well plates in CM supplemented with 10ng/ml of IL-7 and IL-15. CD154+ cells were cultured in the presence of irradiated autologous feeder cells at a ratio of 1:1, before assessment of anti-CMV responses using intracellular cytokine staining and proliferation assays, as previously described in Chapter 2.

6.4 Results

6.4.1 Small scale enrichment of CD154+ CMV-T using anti-biotin microbeads

Three small scale enrichments of CD154+ CMV-T were performed in G-CSF-mobilised PBMCs, for assessment of the efficiency of the selection methodology and for comparison with CD154+ CMV-T enrichment using PE-microbeads reported in Chapter 4. Figure 6.2 illustrates CD154+ enrichment in two donors using anti-biotin microbeads and Figure 6.3 summarises the comparative CD154+ enrichment data using PE- and anti-biotin microbeads. Anti-biotin enrichment technology showed an increased mean CD154+ purity vs. PE-microbeads (71.13% ± 12.64 vs. 48.94% ±12.80, respectively; *p*=0.29), but a decreased mean CD154+ yield was observed (20.87% ± 9.59 vs. 30.80% ± 7.95, respectively; *p*=0.46). These changes were not statistically significant. Analysis of T cell subsets after CD154+ enrichment using anti-biotin microbeads showed no significant difference with PE-microbead enrichment when comparing CD4+ T cells (67.77% ± 13.42 vs. 45.38% ± 11.70, respectively; *p*=0.27) and CD8+ T cells (3.36% ± 0.96 vs. 3.57% ± 1.59, respectively; *p*=0.93).
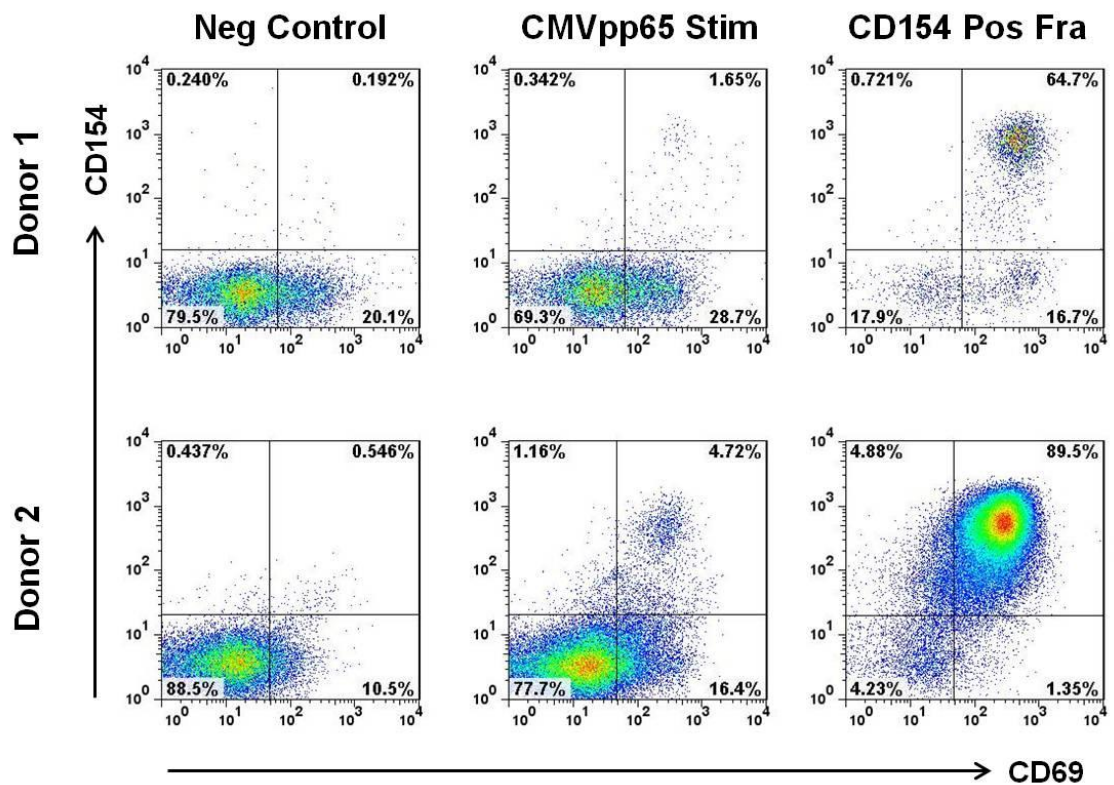
Figure 6.2 Isolation of CD154+ CMV-T using anti-biotin microbeads in G-CSF-mobilised PBMCs

PBMCs isolated from G-CSF-mobilised donors were stimulated for 6 hours in the presence of anti-CD40 antibody, labelled with CD154-biotin and anti-biotin microbeads prior to magnetic enrichment. Cells were assessed for phenotype using anti-biotin-PE for the detection of CD154+ cells.

Fra, fraction; Neg, negative; Pos, Positive.
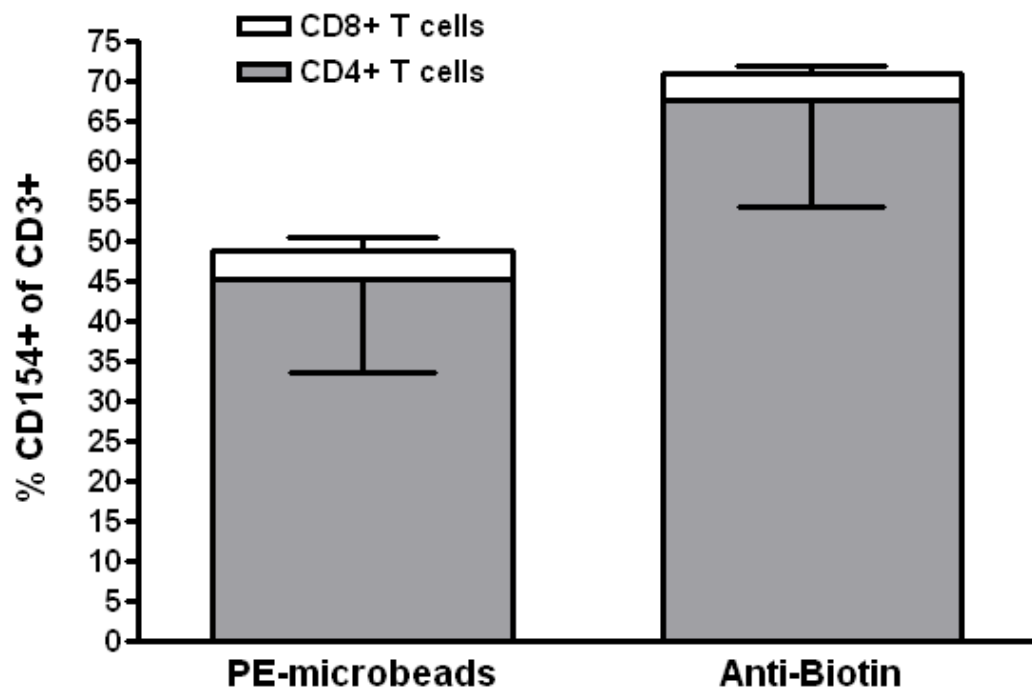
Figure 6.3 Comparison of CD154+ isolation using PE-microbead (n=5) and anti-biotin (n=3) technology for the enrichment of CMV-T in G-CSF-mobilised PBMCs CD154+ purity was measured following magnetic enrichment and CD4+ and CD8+ T cell populations assessed.

6.4.2   CD154+ pre-screening of G-CSF-mobilised donor for clinical scale-up

A CMV+ G-CSF-mobilised donor was selected from a bank of previously cryopreserved PBMCs to demonstrate an effective CD154+ T cell response to CMVpp65 peptide stimulation and establish suitability for clinical scale-up. CD154 expression was analysed pre-CMVpp65 stimulation, post-stimulation and in response to the superantigen SEB. A negative control (non-stimulated) was also included to ascertain the specificity of the CMV response using CD154 detection. Figure 6.4 shows the results from the pre-screening experiment analysing the CD154 response to CMVpp65 peptide stimulation. Minimal background CD154 expression was observed pre-stimulation (0.29%) and in the non-stimulated negative control (0.27%). Following CMVpp65 peptide stimulation, CD154 expression was 2.47% (1.83% CD4+, 0.64% CD8+), demonstrating donor suitability suitable for use in a clinical scale-up experiment. CD154 expression after SEB stimulation in the same donor was 14.41% (CD4+, 13.0%, CD8+, 1.41%).
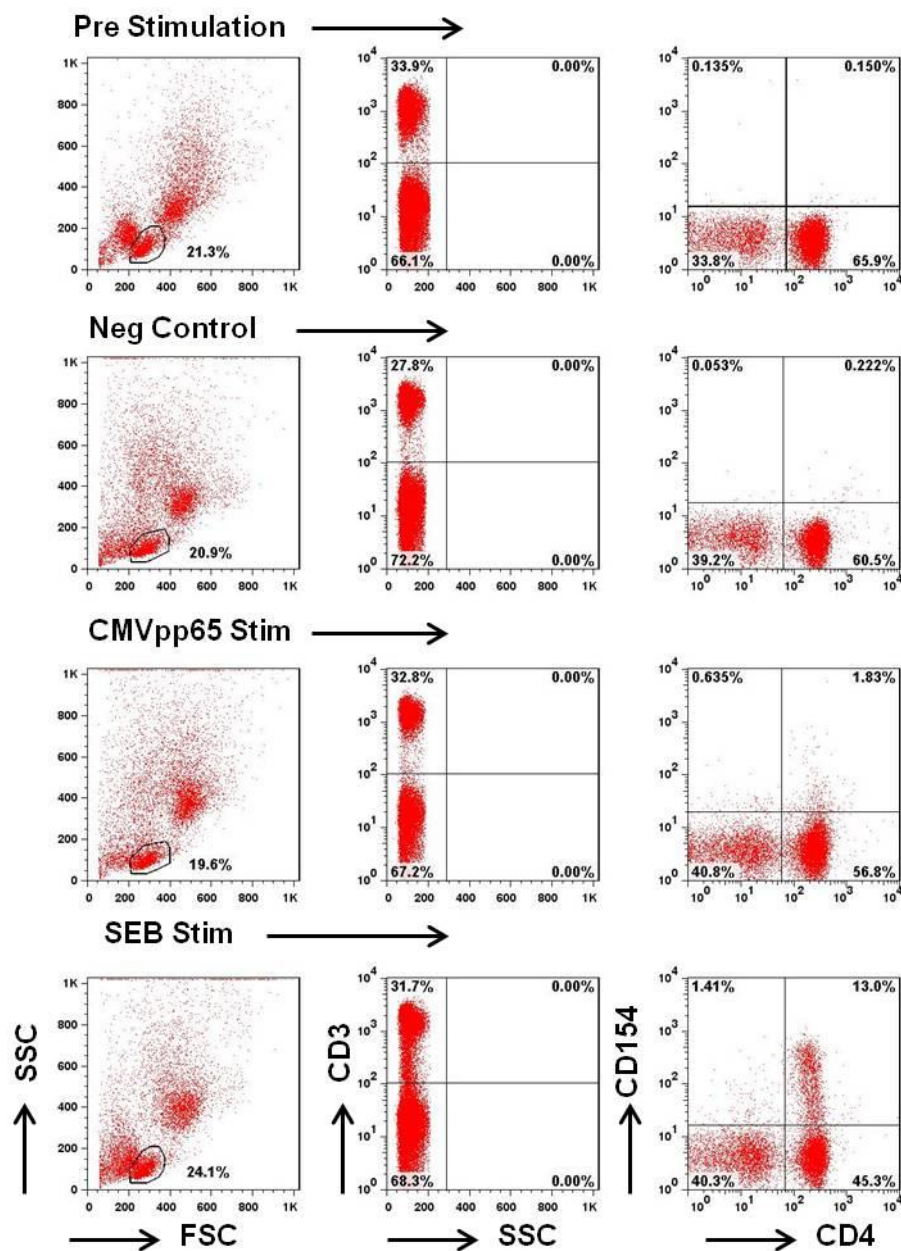
Figure 6.4 Pre-screening CD154 expression in a G-CSF-mobilised donor to ascertain suitability for clinical scale-up

G-CSF-mobilised PBMCs were stimulated in a 96-well plate with SEB, CMVpp65 peptides or left untouched (non-stimulated) as a negative control before analysis of CD154 expression amongst the CD3+ T cell population. Background CD154 expression was analysed pre-stimulation in order to ascertain CD154 CMV specificity.

6.4.3   Clinical scale enrichment of CD154+ CMV-T

6.4.3.1 CD154+ Selection

PBMCs from a previously cryopreserved G-CSF-mobilised CMV+ donor were thawed out for subsequent use in a clinical scale process for the isolation of CD154+ CMV-T. The target total cell number for stimulation in the clinical scale process run had been set at 1000 x 10^6 as this was the figure reported in a previously published clinical scale manufacture of CMV-T using the IFN-γ catch method (Peggs *et al*, 2011). Table 6.2 shows that our clinical scale process run did not achieve that threshold. The total number of cryopreserved cells was 6000 x 10^6, but this decreased almost 10-fold to 678 x 10^6 prior to CMV peptide stimulation. Viability was also found to diminish during the process, stabilising at around 50% at pre-enrichment.

	Cryo'd	Post-Thaw	Post-MNC prep	Pre-Stim (Post O/N rest)	Post-Stim	Pre-Sort	CD154 Pos Fra	CD154 Neg Fra
Viability (%)	90	70	60.0	57.0	50.0	49.7	21.9	51.5
Total Cell Number (x 10^6)	6000	3000	1340	678	310	275	29.5	132

Table 6.2 Cell recovery and viability during clinical scale enrichment of CD154+ CMV-T

Viability and total cell number were recorded during each step of the process prior to enrichment. Viability was determined using TO-PRO cell dye for staining as a dead cell indicator and cell counts were performed on a haematology analyser.

Cryo'd, cryopreserved; Fra, fraction; MNC, mononuclear cells; Neg, negative; O/N, overnight; prep, preparation; Pos, positive; Stim, stimulated;

Following enrichment, the total cell number in the CD154+ fraction was calculated to be 0.21×10^6, compared to 0.45×10^6 in the pre-enrichment sample (Table 6.3). This represented a CD154+ cell yield of 46.6%. The total cell number of the CD154-negative fraction was calculated at 0.15×10^6. CD154+ purity after enrichment was 54.55%, of which 45.3% (83% of total CD154+) were CD4+ and 9.25% (17% of total CD154+) were CD8+, as determined by flow cytometry. This was in keeping with the proportion of CD4+ (87.5% of total CD154+) and CD8+ (12.5% of total CD154+) T cells in the pre-enrichment sample. Viability diminished following enrichment from 49.7% to 21.9% in the CD154 positive fraction, whereas viability in the negative fraction remained stable (51.5%). Both the lymphocyte population and CD3+ T cell content were reduced following enrichment when compared with pre-enrichment (36.6% vs. 18.7% and 53.2% vs. 32.3% respectively). Figure 6.5 illustrates the results from the clinical scale process validation after flow cytometric analysis.

	Pre-CD154 Enrichment	CD154+ Fraction	CD154– Fraction
Cell concentration Volume	5.5×10^6/ml 50ml	0.95×10^6/ml 31ml	0.44×10^6/ml 300ml
Total Cell Number	275×10^6	29.5×10^6	132×10^6
Viability (%)	49.7	21.9	51.5
Lymphocytes (%)	36.6	18.7	53.0
CD3+ (%)	53.2	32.3	55.7
Total CD3+ Cell Number	26.6×10^6	0.39×10^6	20.1×10^6
CD4+ CD154 (%)	1.48	45.3	0.59
CD8+ CD154 (%)	0.21	9.25	0.17
Total CD154 (%)	1.69	54.55	0.76
Total CD154+ Cell Number	0.45×10^6	0.21×10^6	0.15×10^6

Table 6.3 Enumeration of post-magnetic enrichment of CD154+ CMV-T isolated from a single cryopreserved G-CSF-mobilised CMV+ donor

Values were determined using a dual-platform method by flow cytometry and manual cell counts using a haematology analyser. Viability was determined using TO-PRO cell dye for staining as a dead cell indicator and gating on TO-PRO negative events prior to phenotypic analysis
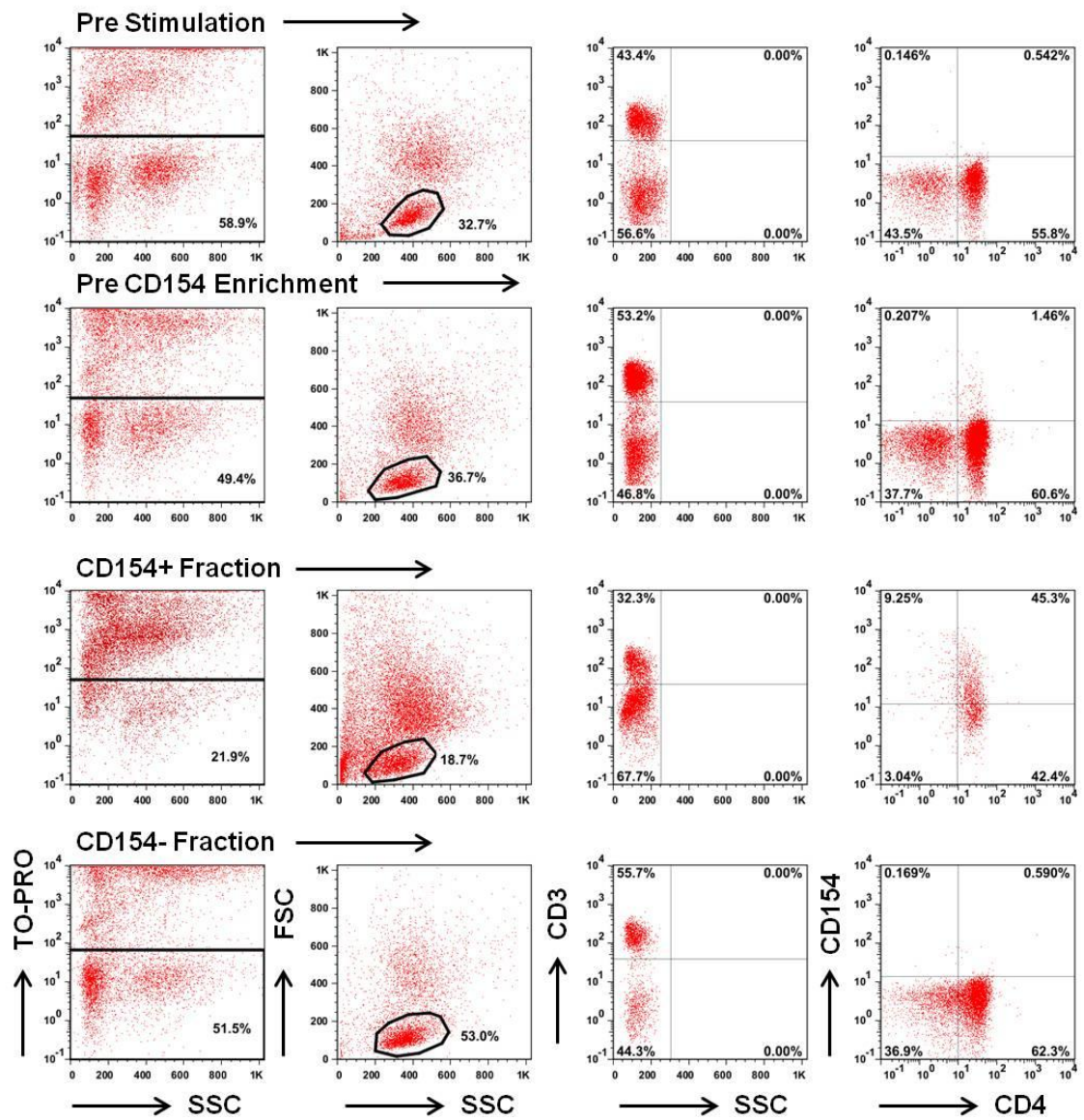
Figure 6.5 Phenotypic analysis of clinical scale CD154 enrichment following CMVpp65 peptide stimulation in a G-CSF-mobilised donor

CD154 analysis was performed by gating on TO-PRO negative events to exclude dead cells, prior to lymphocyte gating using FSC vs. SSC.

6.4.3.2 Functional analysis of CD154+ following clinical scale enrichment

Following enrichment of CD154+ CMV-T, the CD154+ positive fraction was cultured in the presence of irradiated negative feeders (1:1), 10ng/ml of IL-7 and IL-15, and rested for 6 days before carrying out the functional analysis. At day 6, CD154+ CMV-T cultures were 97.6% CD3+ T cells of which 98.1% were CD4+ and 0.57% CD8+, determined by flow cytometry. CD154+ CMV-T were re-challenged with CFSE-labelled autologous PBMCs loaded with CMVpp65 peptides at a ratio of 1:2 (CD154+ CMV-T:autologous PBMCs) before analysis of cytokine secretion using both ICS and CBA. Re-challenge with CFSE-labelled autologous PBMCs without peptide was included as a negative control. ICS revealed a high level of Granzyme B secretion compared to the negative control (44.8% vs. 6.1%, respectively) and a low level of IFN-γ secretion (8.3% vs. 0.7%, respectively). TNF-α secretion (1.8% vs. 0.7%) and IL-2 secretion (0.6% vs. 0.5%) were both at low to undetectable levels after CMVpp65 peptide re-challenge (Figure 6.6). CD69 analysis after re-challenge revealed an increase in surface expression from 39.2% in the negative control (PBMCs alone) to 75.5% after CMVpp65 peptide re-challenge.

Supernatants were collected and analysed by CBA for quantification of cytokine secretion in response to CMVpp65 peptide re-challenge. Analysis revealed an increased secretion of IFN-γ and TNF-α compared with the negative control (1055 vs. 412 pg/ml; and 970 vs. 609 pg/ml respectively). Secretion levels of IL-2, IL-4, IL-5 and IL-10 were minimal and is summarised in Figure 6.7.

Memory phenotype was performed following CD154+ enrichment on the CD3+ T cell population (Figure 6.8) and revealed a mixed population of naive CD45RA+CCR7+ (45.5%) and central memory CD45RA-CCR7+ (42.8%), with a small population of effector memory CD45RA-CCR7- (10.4%). Following 6 day rest in culture, CD154+ CMV-T were predominantly central memory (67.7%) and to a lesser extent effector memory (27.3%).
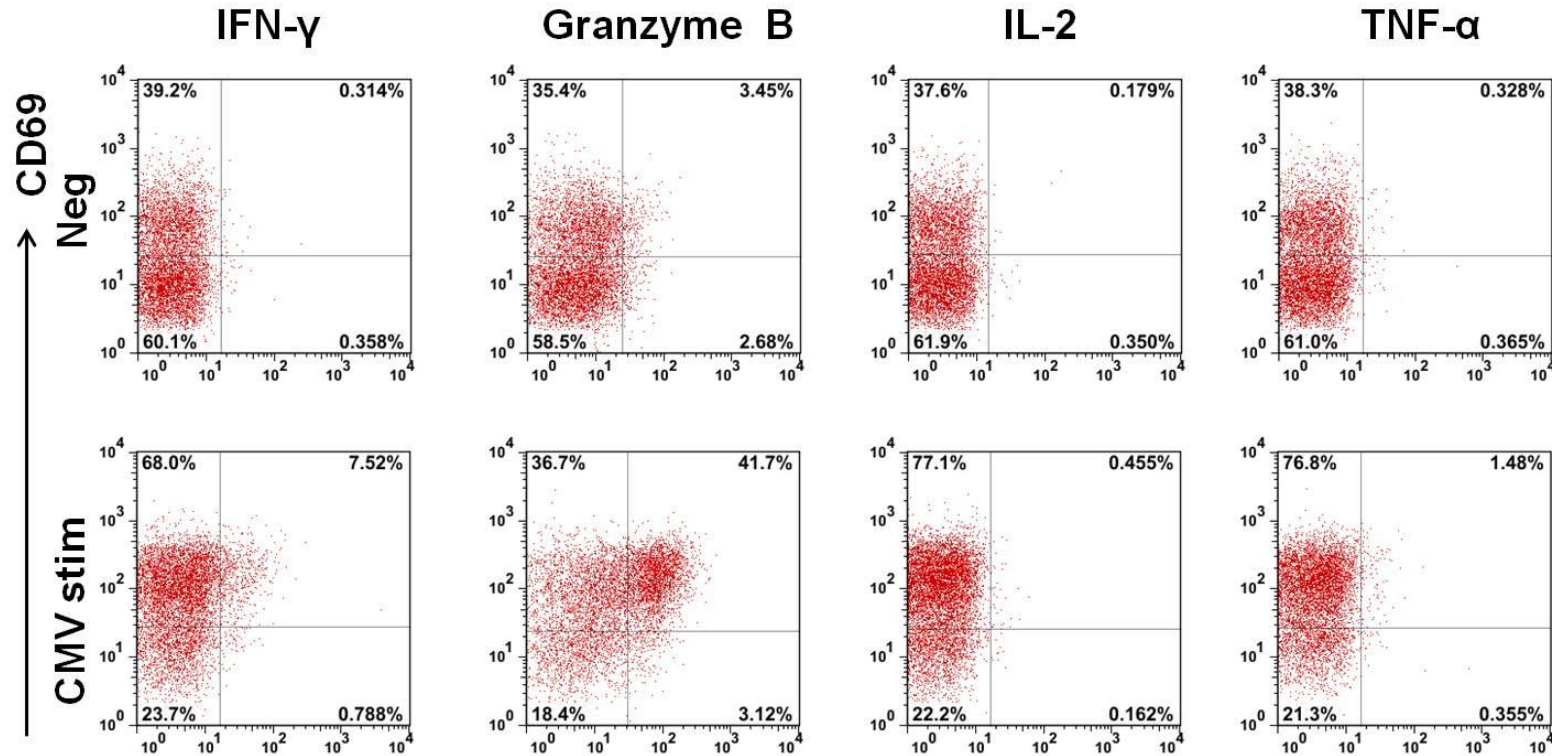
Figure 6.6 Cytokine profile of CMV-T after clinical scale CD154 enrichment

CD154+ cells were co-incubated with autologous PBMCs with or without loaded CMVpp65 peptides for 6 hours and cytokine secretion measured using ICS by flow cytometry.
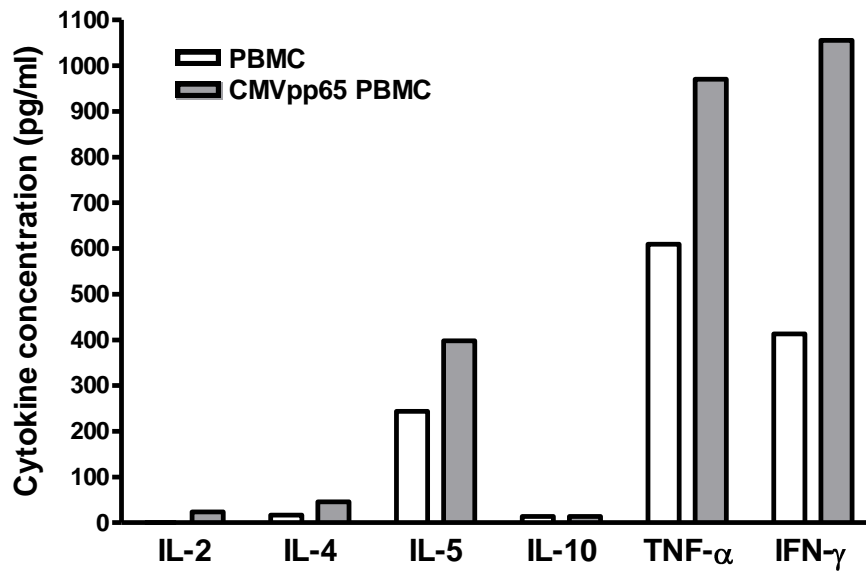
Figure 6.7 Cytokine profile of CMV-T by CBA following re-challenge

CMV-T were co-incubated with autologous PBMC loaded with CMVpp65 peptides or PBMC alone. Supernatant was collected after 6 hour re-challenge for analysis of cytokine secretion by CBA.
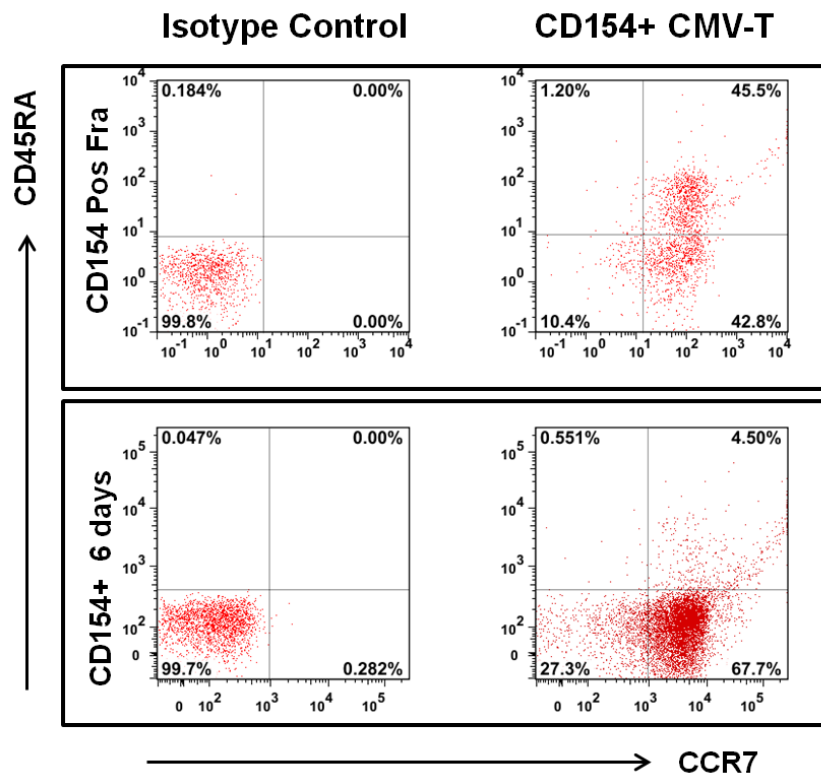
Figure 6.8 Classification of memory phenotype after CD154+ enrichment and following 6 days rest in culture

Plots were derived after gating on lymphocytes using a FSC vs. SSC plot and on CD3+ events. Matched isotype controls were used to enable gating and are shown on the left-hand side.

6.5   Discussion

The main aims of this Chapter were to develop CD154-based magnetic selection of CMV-T from G-CSF-mobilised PBMCs at a clinical scale, and to assess whether this process yielded an immunotherapeutic product that was clinically relevant in terms of cell numbers and demonstrated CMV-specificity upon re-challenge. In common with the work described in Chapters 3, 4 and 5 of this thesis, all cell selections utilised the Miltenyi/MACS immunomagnetic enrichment system. Therefore, in order to test the feasibility of CD154-based enrichment of CMV-T at a clinical scale, the CliniMACS^{PLUS} device (Miltenyi Biotec) was used for the scale-up. Previous results reported in Chapter 4 showed that it was possible to enrich for CMV-T through CD154 activation-induced expression from G-CSF-mobilised PBMCs. Mean CD154+ purity was 48.9%, mean yield 30.8% and the mean proportion of CD4+ and CD8+ T cells 92.6% and 7.4% respectively. For the process validation of clinical scale enrichment of CD154+ CMV-T, the decision was taken to use anti-biotin microbeads rather than anti-PE microbeads, due to their availability at GMP-grade. Small scale experiments using anti-biotin microbeads were performed to assess this option. Results showed that mean purity was increased using anti-biotin microbeads compared with anti-PE microbeads (71.13% ± 12.64 vs. 48.94% ± 12.80 respectively; p=0.29), but mean yield decreased (20.87% ± 9.59 vs. 30.80% ± 7.95; p=0.46). However, neither difference was statistically significant and therefore the decision to use anti-biotin microbeads in a clinical scale experiement was deemed to be an optimal approach.

Clinical scale enrichment of CD154+ CMV-T was performed with previously cryopreserved material, which had a detrimental impact on both total cell number and viability throughout the course of the experiment. The target cell number for loading onto the CliniMACS for magnetic enrichment was deemed to be 1 x 10^9 PBMCs (Feuchtinger *et al*, 2010; Peggs *et al*, 2011). Approximately 0.7 x 10^9 PBMCs were available for CMVpp65 peptide stimulation, which represented a loss of 54% from a total of 1.3 x 10^9 PBMCs left in culture overnight post MNC separation.. Following antibody and magnetic bead labelling only approximately 0.3 x 10^9 PBMCs were loaded onto the CliniMACS for enrichment, equating to a loss of approximately 40% from 0.7

x 10^9 PBMCs that were stimulated. The cumulative loss of PBMCs over the course of the experiment resulted in a total CD3+ cell number of 0.39 x 10^6 in the CD154+ fraction. Assuming an average recipient weight of 75kg, this would equate to a therapeutic dose of 5.2 x 10^4 CD3+/kg of which 54.55% were CD154+ (2.8 x 10^4/kg). Even considering such significant cell loss throughout the process, 5.2 x 10^4 CD3+/kg is still comparable to the cell numbers infused after direct selection using IFN-γ secretion (Feuchtinger *et al*, 2010; Peggs *et al*, 2011). In comparison, the CD3+ T cell dose achieved using CD154+ selection of CMV-T was greater than that achieved using HLA-peptide tetramers (Cobbold *et al*, 2005) where cells were returned at doses of 1.23-1.33 x 10^3/kg. It should be noted, however, that purity was very high using this approach, with a reported median T cell purity of 95.6%. In the present study, CD154+ purity after CD154 selection was 54.55% which is comparable to the purity achieved after IFN-γ selection in the studies of both Fuechtinger (65% ± 31) and Peggs (43.9%; range 1.4%−81.8%).

In a similar clinical study utilising the CliniMACS IFN-γ capture system, CD8+ CMV-T were generated after stimulation with the HLA-A*0201-restricted peptide NLVPMVATV (NLV) and HLA-B*0702-resticted peptide TPRVTGGGAM (TPR) (Meij *et al*, 2012). In this study, results showed cell numbers ranging from 0.25 to 14.4 x 10^6 cells and contained between 3% and 45% IFN-γ+ CD8+ T cells. Although only CD8+ T cells were isolated in the study of Meij and colleagues, these cell numbers compare favourably with the results shown here using CD154-based isolation of CMV-T. Furthermore, isolated CD8+ CMV-T using IFN-γ catch technology were subsequently cultured and although no further increase in cell numbers was observed, further enrichment of CMV-T was observed with T cell lines containing 54% to 96% NLV-specific and/or TPR-specific T cells. This approach of short-term culture to further enrich isolated CMV-T was demonstrated in Chapter 5.

The low cell viability following enrichment can be attributed firstly to the quality of the cryopreserved cells being used, as previously discussed, and secondly to the CliniMACS device itself, that generally yields a higher proportion of apoptotic cells in selected fractions (Watts *et al*, 2002) compared with other magnetic selection devices. Our experience when using the IFN-γ catch method for enrichment of CMV-T by

CliniMACS has shown yields of as much as 90% dead cells following selection, as determined by Propidium Iodide (PI) staining using flow cytometry (unpublished data).

Although the CMV-T purity in this clinical scale experiment failed to reach the levels reported by Cobbold and colleagues (Cobbold *et al*, 2005), it should be noted that as a release criterion for clinical use, the purity of isolated cells would not be required to be >90%. Indeed, in the studies of Fuechtinger and colleagues (Feuchtinger *et al*, 2010), CMV-T products containing ≥10% IFN-γ+ cells were approved for clinical use. Furthermore, a Phase III randomised study (NCT01077908) to evaluate the clinical benefit of prophylactic CMV-T therapy following T-cell depleted allogeneic HSCT for reducing recurrent CMV reactivation has a similar release criterion of 1×10^3 IFN-γ+ cells/kg. The total CD154+ cell numbers generated with the approach described here would therefore support the feasibility of adoptive transfer in a Phase I/II trial to assess the safety and efficacy of CD154+ CMV-T therapy for the prophylactic treatment of CMV disease.

CD154-based selection revealed a yield of 46.6%, which illustrates an improvement when compared to small scale enrichment using both anti-biotin microbeads (20.8%) and PE-microbeads (30.8%). Furthermore, this result is comparable to CMV-T yield using IFN-γ secretion (41.7%) (Peggs *et al*, 2011). Clearly, the products generated in the studies of Cobbold, Fuechtinger and Peggs differ greatly in terms of cellular composition to the product generated through CD154+ CMV-T, where T cells were majority CD4+. However, these studies have shown that similar doses, in terms of cell numbers, to that achieved here with CD154-based selection, were capable of achieving *in vivo* expansion of CMV-T. Doses of CMV+ CD4+ T cell lines ranging between 1 to 5×10^6 resulted in a rapid anti-viral effect, cleared the virus in the majority of patients (5 of 7) and persisted *in vivo* at numbers comparable to healthy individuals (Einsele *et al*, 2002). The future use of fresh G-CSF-mobilised PBMCs in favour of cryopreserved PBMCs for CD154+ CMV-T generation could significantly improve both the viability and overall cell number, which would result in higher cell numbers for adoptive transfer, in line with that of Einsele and colleagues. Therefore, the manufacture of escalating doses of CMV-T generated through CD154+ selection remains feasible.

Large scale studies investigating enrichment of CMV-T using activation markers have yet to be carried out. However, comparison of CD154-based clinical grade enrichment of CMV-T with one such study using selection of CD25+ multi-virus specific T cells at clinical grade using the CliniMACS (Lugthart *et al*, 2012) was comparable in terms of purity (55% vs. 44% respectively). The median total CD25+ cell number was 6.8 x 10^6 which represents a significantly higher yield when compared with CD154-based enrichment, although the CD25 study was targeted towards multiple viruses and cultured 3 days prior to enrichment. Experiments in Chapters 3 and 4 of this thesis showed increased expression of CD25 compared with CD154 after antigenic stimulation. CD154-based isolation was favoured over CD25 selection for the isolation of CMV-T in this clinical scale experiment due to the high frequency of Tregs present after CD25 selection, as reported in Chapter 4. Lugthart and colleagues report a median of 21% of CD4+ Treg cells in the enriched CD25+ fraction, in agreement with the CD25 selection data presented in Chapter 4. CD25-based enrichment resulted in a median yield of 50% (Lugthart *et al*, 2012) which is comparable with the 46.6% yield obtained using CD154-based enrichment.

One of the main obstacles for process validation of new cellular therapy techniques is the procurement of sufficient starting material. Unfortunately, we were not able to obtain ethical approval to procure fresh G-CSF-mobilised PBMCs at a quantity sufficient for clinical scale-up. Without ethical approval to take a large enough sample for process validation from G-CSF-mobilised apheresis, the only remaining option was to use cryopreserved cells. Donor cells, whose intended recipient was deceased, were procured after the donor cells in storage had been released for discard and subsequently approved for use in research and development under ethical approval. Although G-CSF-mobilised PBMCs are commercially available (Key Biologics; Hemacare) this is an expensive option and also requires shipping from overseas, which impacts on the quality of cells and in our experience renders them unsuitable.

The development of a CD154+ CMV-T cell therapy product clearly requires multiple process validation runs in order to quantify the feasibility of adopting this approach. Several published studies quote numbers of 2 (Powell *et al*, 2005), 5 (Albon *et al*, 2013), 6 (Otto *et al*, 2005), and 7 (Lugthart *et al*, 2012) process validation runs with

leukapheresis collections in order to demonstrate feasibility at a clinical scale. Increasing the number of process runs would therefore be a requirement for CD154-based CMV-T generation, if future plans for a small clinical trial were to be realised.

To optimise detection of functional antiviral responses, CD154+ cells were rested for 6 days after selection and before re-challenge. Immediately after the selection, the majority of cells were CD4+ (83% of CD3+) with a small proportion of CD8+ (17% of CD3+) T cells. During this rest period, the proportion of CD8+ T cells reduced dramatically (0.57% of CD3+). Although this observation is only in one clinical scale experiment, it is reflective of the small scale experiments discussed in Chapter 5 and suggestive that CD154+ CD8+ T enriched T cells do not survive in culture where the overwhelming subset is CD4+ and perhaps indicative of the elevated levels of IL-4 secreted during culture as reported in Chapter 5 that would favour CD4+ T cells over CD8+ cells (Kienzle *et al*, 2005). However, the supernatant from the re-challenge of CD154+ CMV-T after clinical scale enrichment showed minimal secretion of IL-4, arguing against this idea. This minimal secretion could, however, be attributed to the short culture period of 6 days compared with 21–23 days in the small scale experiments. One limitation of this clinical scale experiment is the failure to extend the culture period to 21 days in order to allow re-challenge experiments to be performed that could have revealed a more pronounced anti-CMV response. CD154+ CMV-T from clinical scale enrichment also revealed low-level IFN-γ secretion and low to undetectable levels of TNF-α and IL-2, both associated with a cytotoxic function. However, Granzyme B secretion was detected at significant levels (44.8% of CD3+) after CMVpp65 peptide re-challenge demonstrating a functional anti-viral response.

In conclusion, the results presented in this Chapter illustrate the successful development of a rapid and simple methodology for the enrichment of CMV-T from G-CSF-mobilised PBMCs using CD154 activation-induced expression, to a clinical grade scale. The protocol is yet to achieve full GMP-compliance due to the non-availability of GMP-grade anti-CD154 and anti-CD40 antibodies, although the future use of non-GMP compliant reagents could be overcome by a comprehensive risk assessment of these reagents (Lowdell *et al*, 2012a). The selection method used (CliniMACS) is already in

widespread clinical use, which makes this approach of CMV-T isolation broadly applicable to many transplant centres.

Chapter 7

General Discussion

The generation of CMV-T for adoptive transfer from freshly isolated, non-G-CSF-mobilised blood or apheresates has been successfully demonstrated using a variety of methods, resulting in products containing CD4+ T cells, CD8+ T cells or a mixture of both (Peggs, 2009). The widespread application of some of these methods has been limited by the logistics and costs of the culture techniques together with the timeframe of 2-3 weeks required to manufacture them. More recently, direct selection techniques such as IFN-γ capture (Feuchtinger *et al*, 2010; Peggs *et al*, 2011) and HLA-peptide tetramers/streptamers (Cobbold *et al*, 2005; Schmitt *et al*, 2011) have demonstrated that CMV-T manufacture can be simple, fast and relatively inexpensive, with the generation of clinically relevant cell numbers for adoptive transfer. Both approaches are amenable to GMP standards, which makes these strategies attractive and more accessible to the wider cellular therapy community. However, the availability of GMP-grade tetramers/streptamers is limited to a small number of HLA Class I specificities and therefore prevents a sizeable number of HSCT patients from receiving this treatment option and restricts the cellular therapy product to a CD8 phenotype.

The principle aim of the work described in this thesis was the detection, isolation and expansion of CMV-T from G-CSF-mobilised PBMCs, and the subsequent design and implementation of a large-scale protocol to allow this methodology to be translated into routine clinical practice.

7.1 Optimisation of CD154+ CMV-T manufacture from G-CSF-mobilised donors

To date, the methods for generating CMV-T have focussed primarily on using PBMCs collected by leukapheresis from the original HSCT donor. In the case of related donors, procuring a second apheresate for CMV-T generation is inconvenient to the donor and is associated with some level of pain and discomfort. Obtaining a second apheresate

from unrelated donors has proven more difficult, either due to donor refusal, registry refusal or simply scheduling difficulties. Using an aliquot of the original apheresis collection avoids duplication of donor assessment, infectious disease marker screening, and product collection and shipping, all of which are often impractical in unrelated donors. Therefore, the prospect of manufacturing antigen-specific T cells from an aliquot of the original HSCT sample, following G-CSF mobilisation, is attractive but has yet to be fully investigated.

The identification and isolation of CMV-T from G-CSF-mobilised PBMCs described in this thesis initially used the IFN-γ catch method due to the commercially available reagents and relative simplicity of enrichment using the CliniMACS device. However, upon identification of CMV-T determined by IFN-γ secretion, a reduction was observed in G-CSF-mobilised compared with non-mobilised PBMCs. Furthermore, following enrichment of CMV-T, a decrease in both purity and yield of IFN-γ secreting CD3+ T cells was observed compared with non-mobilised PBMCs. Although there was no significant difference in the intensity of IFN-γ staining after CMVpp65 stimulation, the reduction in purity and yield after IFN-γ enrichment is most likely a consequence of a reduction in the frequency of IFN-γ secreting cells. It remains unclear whether the use of individual CMVpp65 peptides based upon donor HLA type or the use of CMV IE-1 peptides would result in a more efficient system for the identification and isolation of CMV-T (Gratama *et al*, 2008; Zandvliet *et al*, 2010; Slezak *et al*, 2007) as the kinetics for IFN-γ secretion in G-CSF-mobilised PBMCs may differ between epitopes at different peptide concentrations. But nonetheless, such a tailored donor-specific process would not lend itself to widespread adoption.

The data presented Chapters 3 and 4 of this thesis reported on the investigation of activation markers as a potential route for the isolation of CMV-T following CMVpp65 peptide stimulation; and subsequently focussed on CD25 and CD154 expression as two optimal targets. The successful expansion of CMV-T from G-CSF-mobilised PBMCs was demonstrated, and upon re-challenge expanded cells showed a high level of specificity for CMVpp65 and secreted inflammatory cytokines demonstrating functionality. Furthermore, CMV-T cell lines were capable of lysing CMVpp65 PHA target cells in a Calcein-AM cytotoxicity assay (Neri *et al*, 2001). These results,

presented in Chapter 5, have been published (Samuel *et al*, 2013). Following the novel finding described here of the feasibility of identifying, isolating and expanding functional CMV-T from G-CSF-mobilised donors, the first phase I/II clinical trial using CMV-T expanded from G-CSF-mobilised PBMCs has also been reported (Clancy *et al*, 2013). The study of Clancy and colleagues supports the data presented in this thesis and demonstrates the proof-of-principle of using only a small aliquot of the original HSC apheresis (1.5–4ml) without any detrimental impact on the quality of the graft itself. However, the Clancy study raises two important questions that potentially compromise the widespread application of this manufacturing process. First CMV-T were expanded in culture over 21 days in order to generate sufficient numbers for adoptive transfer. This required the generation of monocyte-derived DCs transfected with an adenovirus vector (Ad5f35pp65) encoding the entire CMVpp65 protein, which are both time- and labour-intensive and difficult to establish under current GMP regulations (Fuji *et al*, 2011). In comparison, the advantages of direct selection, including that of CD154+ CMV-T generation, are the reduced level of manufacturing required and the potential for a much more rapid production, which allows for a more cost-effective ad-hoc production. Second, there are considerable regulatory compliance issues with using a transfected cell line in the manufacture process, with regards to gene therapy vs. somatic cell therapy licenses. Effective demonstration of containment of transfected cells, to prevent cross contamination through viral integration, would inevitably need to be provided to national regulatory bodies for this system to become globally applicable. These complicated regulatory issues might impede the effective translation of this strategy into widespread clinical use.

7.2   Alternative strategies for anti-viral T cell culture and expansion

New technology is emerging to overcome many of the limitations associated with scale-up when using conventional cultureware. A number of bioreactors, for example the WAVE bioreactor (GE Healthcare Life Sciences) have been explored for the expansion of T cells in suspension, (Tran *et al*, 2007) that could be easily transferred for the manufacture of CMV-T. However, this culture system cannot be accommodated in a conventional incubator and requires accessory devices for heating, rocking and medium change which makes the process more complex logistically and also expensive. The

optimisation of cell culture has led to new cultureware on the market, such as gas-permeable bioreactors (G-Rex, Wilson Wolf Manufacturing Corporation). In this system O_2 and CO_2 are exchanged across a silicone membrane at the base of the flask, resulting in reduced medium change and increased final cell numbers (Lapteva & Vera, 2011). A recent study has demonstrated that the expansion of EBV-CTLs using this system results in a decreased frequency of culture manipulation, increased cell output (3-20-fold) and a shortening of culture time (Vera *et al*, 2010).

In circumstances where an aliquot of G-CSF-mobilised PBMCs is not sufficient for the manufacture of a clinically relevant CMV-T dose after direct CD154 selection, the G-Rex culture system offers a GMP-ready solution that could enable CD154+ CMV-T translation into clinical production with a high level of simplicity. Furthermore, the manufacture of multi-virus specific T cells using CD154 selection still requires a short-term culture period (Khanna *et al*, 2011), where the G-Rex system would offer an alternative to current culture limitations. The new technology now available offers the exciting potential to build on the work reported in this thesis, in order to achieve the clinical scale bioprocess optimisation of a CD154+ CMV-T isolation strategy from G-CSF-mobilised PBMCs.

7.3   CMV-seronegative donors

The direct isolation approach of both CD154 and CD25 activation-induced expression reported in this thesis, as well as IFN-γ catch and streptamer/pentamer technology, could be seen as having a limited application due to its requirement for CMV-seropositive donors. CMV-seropositive patients receiving a HSCT from a CMV-seronegative donor cannot presently benefit from such adoptive cellular therapy treatment strategies. Current investigations to overcome this limitation have included the use of third-party T cell donors for the manufacture of anti-viral immunotherapies. This strategy was first successfully tested using partially HLA-matched EBV-specific T cells in a phase II multicentre clinical trial to treat post-transplant lymphoproliferative disease (Haque *et al*, 2007; Haque *et al*, 2010). Following adoptive transfer, cells were highly effective and did not induce any GvHD. Third party CMV-T generated using the IFN-γ catch system has also been successfully demonstrated, albeit in only two patients,

with *in vivo* expansion of CMV-T and clearance of CMV infection observed in one patient (Feuchtinger *et al*, 2010;Schottker *et al*, 2008). The effectiveness of third-party donor-derived T cells in treating CMV is now being investigated in a phase II trial (NCT01646645) with an estimated study completion date of July 2015 (Eiz-Vesper *et al*, 2012). The strategy of using third party donors to overcome the limitations associated with only manufacturing from CMV-seropositive donors represents a potential translation of the approach of utilising CD154-selected CMV-T. Whether CD154 CMV-T manufactured from G-CSF-mobilised third-party donors or whether it would be restricted to non-mobilised donors would have to be assessed in future clinical trials.

7.4   Future strategies for anti-viral treatment of CMV Infection

The promise that cell therapies hold for the reconstitution of CMV-T responses after allogeneic HSCT as an alternative strategy to anti-viral drugs is more profound when dose-related toxicities and the emergence of resistance and late-onset CMV disease associated with drugs such as ganciclovir, foscarnet and cidovir (Harter & Michel, 2012) are considered. Several clinical trials are ongoing for the assessment of alternative compounds for future CMV treatment including CMX-001 (Harter & Michel, 2012) and letermovir (known as AIC246) which interact with the viral UL56 subunit (Kaul *et al*, 2011). The next 5 years is expected to reveal potential new CMV management strategies with these new substances. However, even anti-viral drugs that have shown great promise in early clinical trials, such as maribavir, a competitive inhibitor of the UL97 protein, have shown limitations in recent phase III clinical trials (Marty *et al*, 2011) and the first patient with maribavir-resistance during therapy has already been reported (Strasfeld *et al*, 2010). A common theme that has emerged from studies that have reported on the clinical application of CMV-T adoptive transfer has been the reconstitution of CMV immunity in the majority of cases (Peggs, 2009). This is a critical factor in assessing the long-term success of HSCT and, therefore, represents compelling evidence that the adoptive transfer of CMV-T is the optimal approach to the treatment of CMV disease.

7.5   Translation of a GMP-compliant process for CD154+ CMV-T manufacture from G-CSF-mobilised PBMCs

The final results Chapter of this thesis focussed on performing a large-scale CD154+ CMV-T isolation to assess the feasibility of translating the process into clinical practice. One of the main issues concerning the potential translation of this process is the availability of GMP-grade reagents for CD154 labelling and subsequent isolation. Even with the introduction of GMP-grade anti-biotin beads for selection, the process still requires non-GMP grade anti-CD154 biotin and anti-CD40 antibodies. Whilst the production of both antibodies to GMP-grade remains a viable option through industry GMP biopharmaceutical manufacturing, it is likely to be very expensive. Two Phase I studies using an anti-CD40 humanised monoclonal antibody, lucatumumab (HCD122, Novartis) have recently been reported for clinical use in relapsed CLL (Byrd *et al*, 2012) and multiple myeloma (Bensinger *et al*, 2012), which holds promise for its possible use in clinical grade manufacture of CD154+ CMV-T. However, its assessment for use in the manufacturing process described in this thesis would need to assessed, and it would also need to be determined whether it could be provided at a significantly smaller size (~100µg) for clinical scale-up. A clinical trial investigating the use of an anti-CD40L (CD154) antibody (BG9588, Biogen Inc.), in patients with proliferative lupus glomerulonephritis (Boumpas *et al*, 2003) and an ongoing Phase I/II trial for use in systemic lupus erythematosus (NCT00001789) have also been published. This is suggestive that a commercially available anti-CD154 antibody for clinical use could be realised in the immediate future. However, the same questions that arise with an anti-CD40 antibody apply equally to an anti-CD154 antibody, and the possibility of its use in clinical scale manufacture of CD154+ CMV-T would require further extensive studies.

Future experiments investigating the isolation of CMV-T from G-CSF-mobilised PBMCs could focus on targeting of CD137 activation-induced expression (Watanabe *et al*, 2008; Wehler *et al*, 2008). Isolation of multi-virus specific CD4+ and CD8+ T cells through CD137 expression has been demonstrated for application in anti-viral immune reconstitution after allogeneic HSCT (Zandvliet *et al*, 2011). Initial experiments reported in this thesis revealed optimal CD137 expression at 24 hours in support of

Wehler and colleagues (Wehler *et al*, 2008), with a similar level of expression between G-CSF-mobilised and non-mobilised PBMCs (0.88% ± 0.45; 0.56% ± 0.16, respectively). A 24-hour stimulation might be considered to be sub-optimal in terms of the logistical operation of manufacture compared to a 6 hour or 16 hour overnight stimulation. However, Watanabe and colleagues (Watanabe *et al*, 2008) suggest 16 hours to be the optimal time of expression for CD137. Investigating CD137-based isolation has become an attractive option with the introduction of a commercially available, fully GMP-compliant CD137/anti-biotin reagent (Miletnyi Biotec) for use with the CliniMACS system. Furthermore, the isolation of both CD4+ and CD8+ T cells directed against CMV could also be deemed as a more attractive option than CD4+ alone using CD154+ isolation; however, it still remains unclear whether co-transfer is likely to be more successful than transfer of either population alone. Further investigation of CD137-based enrichment from G-CSF-mobilised PBMCs is warranted due to its relative ease of GMP-translation with the availability of clinical grade reagents and a widely applicable method.

7.6   Alloreactivity of CMV-T isolated from G-CSF-Mobilised PBMCs

The assessment of alloreactivity of CD154+ and CD25+ CMV-T following direct selection and after short-term expansion in culture is an area of future work that would expand on the results reported in this thesis. Future experiments would look to include ethical approval for the collection of recipient blood samples for use in a modified mixed lymphocyte reaction (MMLR) to assess alloreactivity (Bishara *et al*, 1999) of donor CD154+ CMV-T. The use of third party allogeneic PBMCs as effectors in a MLR is a possible strategy to bypass the need for collecting recipient blood. However, this strategy would mean conducting experiments in a HLA-mismatched setting, where a degree of alloreactivity of CMV-T would be expected. Given that HLA disparity in the HSCT field is associated with graft failure, GvHD and non-relapse mortality (Fuchs, 2012), most immunologists would consider this to be a non-productive experimental approach. Other functional assays that would form the basis of future experiements to measure alloreactivity include, limiting diltution analysis to monitor the frequency of donor anti-recipient cytotoxic and helper T lymphocyte precursors (CTL-p and HTL-p), which have been shown to predict the occurrence of aGvHD after BMT (Kaminski *et al*,

1989; Schwarer *et al*, 1993). Perhaps more difficult to achieve logisitically would be the use of a skin expalant model for predicting GvHD (Vogelsang *et al*, 1985). Although the skin expant model has been shown to be a clear predictor of GvHD and a more accurate indicator of aGvHD than CTL-p and HTL-p frequency analysis (Dickinson *et al*, 1994; Dickinson *et al*, 1998), it would require patient consent for the procurement of punch biopsies of skin, which could be difficult to achieve from an ethics perspective.

The mean purity of CD154+ and CD25+ cells amongst the CD3+ T cell population was 48.9% (9.6–78.7) and 89.9% (85.1–96.5) respectively, whilst CD154+ purity after selection was 48.9% (9.6-78.7) and 54.6% after clinical scale enrichment. The variable purity in CD154-selection from donor to donor could have a potentially detrimental effect on both the safety and efficacy of CD154+ CMV-T, in the context of those cells that are not CMV-specific and may be alloreactive and capable of inducing GvHD. It has been reported, however, that no correlation was observed between GvHD and IFN-γ purity of cellular therapy products infused in the Phase I/II clinical trial conducted by Peggs and colleagues (Peggs *et al*, 2011).

Khanna and colleagues (Khanna *et al*, 2011) demonstrated that alloreactivity of ex-vivo generated CD154+ T cell lines to third party DCs is nearly abrogated. Autologous PBMCs exposed to third party DCs showed a strong proliferative response, whereas proliferation of antigen-specific T cell lines was nearly abrogated. However, CMV-T lines were not analysed in isolation, but were included in a multi-virus-specific T cell line; nor were they analysed immediately after direct selection when purity was only 8–15%. The assessment of alloreactivity of CD25+ CMV-T was confounded by the high frequency of Tregs present following CD25 enrichment in G-CSF-mobilised PBMCs, as reported in Chapter 4. CD25+ CMV-T were shown to be capable of suppressing proliferation of autologous PBMCs after polyclonal T cell activation. In agreement with this finding, CD25-enriched CTL have been reported to show a marked reduction in proliferation in response to culture with allogeneic stimulators, compared to unmanipulated PBMCs, and to be capable of suppressing alloreactive proliferative responses in a dose-dependent manner (Lugthart *et al*, 2012).

The functional population of Tregs present after CD25-selection makes the true alloreactivity of CD25+ CMV-T difficult to evaluate. As with the studies of Khanna, Lugthart's studies isolated multi-virus-specific T cells, which do not allow for the assessment of CMV-T alone, and therefore make it difficult to a make a meaningful comparison with the CD154+ and CD25+ CMV-T data described in this thesis. The statistically significant higher background expression of CD25 (mean, $4.55 \pm 0.59\%$) on G-CSF-mobilised PBMCs compared to CD154+ (mean, $1.14 \pm 0.27\%$; $p=0.0008$) expression would also suggest that CD25-selected CMV-T may contain a significant proportion of non-CMV-T and non-Tregs potentially capable of inducing alloreactivity. Indeed, the role of CD25 in driving alloreactivity has been assessed in three clinical trials using CD25 expression as a tool for allodepletion of donor T cells (Solomon *et al*, 2005; Amrolia *et al*, 2006; Andre-Schmutz *et al*, 2002). Allodepletion in these studies was performed using a CD25-immunotoxin in order to reduce GvHD after adoptive transfer, and demonstrated feasibility and safety, whilst retaining a population of functional CMV-T.

In both CD25+ and CD154+ isolated CMV-T, the predominant T cell subset was CD4 (>90%). From an alloreactivity stand point, the lack of a significant proportion of CD8+ T cells could be viewed as beneficial in terms of reducing the possible incidence of GvHD, since CD8+ T cells are the major effectors of GvHD in experimental models of BMT, whereas CD4+ T cells have a more variable role (Korngold *et al*, 2005). A study of the adoptive transfer of CD8-depleted DLI as a treatment strategy for relapse and mixed chimerism reported that no acute infusional toxicity occurred and enhanced frequencies of circulating CD4+ and CD8+ CMV-T were observed after transfer (Meyer *et al*, 2007). However, a similar trial transferring CD8-depleted DLI reported GvHD as the major toxicity, with two patients dying as a result of complication relating to GvHD (Orti *et al*, 2009). In this trial, however, CD8-depleted DLI were administered in a high-risk group of patients. Additional studies are clearly needed to further assess the safety and efficacy profile of these cells with regard to GvHD.

In this thesis, the memory phenotype of CMV-T generated from G-CSF-mobilised PBMCs consisted predominantly of effector memory and central memory. Several groups have independently reported that effector memory cells are unable to induce

GvHD in different animal models (Anderson *et al*, 2003;Chen *et al*, 2004;Xystrakis *et al*, 2004). This finding was extended in a mouse model to include central memory cells and demonstrate that the GvHD-inducing potential was exclusively contained in the naïve phenotype T cells (Chen *et al*, 2007). This would suggest that both CD25+ and CD154+ CMV-T contain a minimal population of cells capable of inducing GvHD. Contradictory to results from G-CSF-mobilised CMV-T expansions, the memory phenotyping of CD154+ CMV-T following clinical scale manufacture revealed a significant population of naïve T cells (45.5%). However, this result was only observed in 1 donor and the population of naïve cells did not survive in culture after 6 days, which questions their survival *in vivo*.

The alloreactivity of both CD154+ and CD25+ CMV-T and the memory T cell responses in GvHD are important future research questions to address that will facilitate the clinical translation of CMV-T for adoptive transfer. The phase I/II trials published to date evaluating the transfer of IFN-γ selected CMV-T have reported no apparent increase in incidence or severity of acute or of chronic GvHD above that seen in comparable untreated patients (Feuchtinger *et al*, 2010; Peggs *et al*, 2011).

7.7   CMV-specific Tregs and G-CSF-mobilised PBMCs – future development

Isolation of CD25+ CMV-T from G-CSF-mobilised PBMCs revealed a high frequency of FoxP3+ Tregs (mean, 64.24% ± 4.88) that were capable of suppressing polyclonal T cell activation in a CFSE proliferation assay. This observation contrasted with the significantly reduced frequency of FoxP3+ Tregs in non-mobilised PBMCs (mean, 33.84% ± 7.17; *p*=0.008). The high frequency of FoxP3+ Tregs in CD25+ enriched CMV-T from G-CSF-mobilised PBMCs was shown to fall markedly during expansion in short-term culture (mean, 4.28%; *p*=0.0001). However, in the same CFSE proliferation assay, CD25+ expanded CMV-T were still capable of exerting suppression. Together, these results supported the selection of CD154 as an optimal activation-induced expression marker for isolating CMV-T at a clinical scale, as opposed to CD25. However, the observation of such a significant population of Tregs in G-CSF-mobilised PBMCs following CMVpp65 stimulation opens up another research avenue for future consideration. In much the same way that CMV-T manufactured from

G-CSF-mobilised PBMCs was thought not to be advantageous due to the alteration of cytokine networks and polarisation of T cell function induced by G-CSF (Arpinati *et al*, 2000;Pan *et al*, 1999;Tayebi *et al*, 2001), major concerns also surround the clinical use of Tregs manufactured from the same starting material. The results reported in this thesis support the work of Ukena and colleagues (Ukena *et al*, 2012), which suggests that concerns surrounding the functionality of G-CSF-mobilised Tregs, in terms of suppressive capacity, are perhaps not warranted. The isolation of donor Tregs from the original HSC graft is an attractive option, as it removes the need for a second steady-state leukapheresis, thereby simplifying the current clinical application and allowing an extension of this therapeutic strategy into the unrelated donor setting.

Brincks and colleagues (Brincks *et al*, 2013) proposed that during secondary influenza virus infection, Treg activation is specific to the pathogen and results in regulation of pathogen-specific responses. As already discussed in Chapter 4, very little data exist regarding the emergence and generation of Tregs during CMVpp65 stimulation and certainly none with regard to G-CSF-mobilisation. Future experiments to build on the initial results reported in this thesis would look to test the hypotheses that adaptive Tregs arise as a consequence of CMVpp65 stimulation, and that these cells are capable of suppressing the proliferation of expanded CMV-T in response to CMVpp65 re-challenge. Furthermore, the capacity of CD25+ Tregs isolated from G-CSF-mobilised PBMCs, following CMVpp65 stimulation, to suppress alloreactivity in an MLR would provide data on their value as a potential treatment strategy for GvHD in HSCT. Fully GMP-compliant clinical scale manufacture of CD25+ multi-virus specific T cells has been successfully demonstrated (Lugthart *et al*, 2012) in non-mobilised PBMCs. CD25+ enriched cells contained FoxP3+ CD127− Tregs at a median frequency of 21% and were capable of suppressing alloreactivity. The hypothesis of Lughart and colleagues, that a cellular therapy T cell product containing a mixture of Tregs and anti-viral T cells could prevent GvHD whilst maintaining anti-viral immunity after adoptive transfer could be extended to studies in G-CSF-mobilised PBMCs.

7.8   Final concluding remarks

The strategy of identifying, isolating and expanding CMV-T from G-CSF-mobilised PBMCs has been examined, resulting in the development of a clinical scale system to demonstrate the feasibility of this approach as a therapeutic option for the treatment of CMV disease following allogeneic HSCT. Prior to the start of the research reported in this thesis, no published study had shown the feasibility of this approach. The E-publication in October 2012 of the data presented here was the first report of the novel finding that functional CMV-T can be isolated from G-CSF-mobilised PBMCs (Samuel *et al*, 2013). This work was supported by the subsequent publication demonstrating the effectiveness of utilising G-CSF-mobilised PBMCs for CMV-T manufacture and their safety following adoptive transfer in 7 patients (Clancy *et al*, 2013). The clinical application of G-CSF-mobilised PBMCs for cellular therapy has also developed to include the feasibility of Treg manufacture as a strategy for treating GvHD (Ukena *et al*, 2012). The efficacy of G-CSF-mobilised PBMCs for CMV-T manufacture will be determined with the likely publication of several clinical studies over the next 5 years, which could pave the way for this strategy to be adopted as a routine therapy with widespread application. The question remains as to whether CD154+ CMV-T are capable of conferring protection against CMV infection *in vivo* in recipients of HSCT, but this can only be addressed with future clinical trials.

A limitation of this study was the failure to procure paired donor samples to allow a comparison of the CMV response in G-CSF-mobilised and non-mobilised PBMCs to be made in the same donor. The use of paired samples could have allowed for greater statistical power when analysing the effect of G-CSF mobilisation, given the high inter-donor variability of the CMV immune response.

As a final conclusion, the work presented in this thesis represents a general approach to manufacturing anti-viral immunotherapies that does not require successive donations, thereby alleviating the many practical problems incurred with procuring cells from unrelated donors.

Appendix

Appendix 1: Ethics Approval

NHS

Health Research Authority

North West London REC 1
REC Office
Maternity, Level 7
Northwick Park Hospital
Watford Road
Harrow
HA1 3UJ

Telephone: 020 8869 5446
Facsimile: 020 8869 5222

01 December 2010

Dr Mark Lowdell

Dear Dr Lowdell

Study Title: **Investigation of the biological activities of anti-viral lymphocytes isolated from peripheral blood.**

REC reference number: **10/H0722/72**

Thank you for your letter of 16 November 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair, in consultation with one member.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as

10/H0722/72	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

On Behalf of
**Ms Stephanie Ellis
Chair**

Email:

Enclosures: "After ethical review – guidance for researchers"

Copy to: **Mr Edward Samuel**

Appendix 2: Publications

- **Samuel E**, Newton K, Mackinnon S and Lowdell MW. (2012) Successful isolation and expansion of CMV-reactive T cells from G-CSF mobilized donors that retain a strong cytotoxic effector function . *British Journal of Haematology*; 160: 87-100. PMID: 23043413

Appendix 3: Abstracts

- T regulatory cells enriched from G-CSF mobilised apheresates do not survive short term culture – Impact on adoptive immunotherapy
  International Society of Cellular Therapy, Seattle, 2012

- CMV-reactive T cells can be isolated from G-CSF mobilised peripheral blood apheresates
  American Society of Haematology, Orlando, 2010

Chapter 8

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