RECONSTITUTION OF CMV-SPECIFIC T-CELLS FOLLOWING ADOPTIVE T-CELL IMMUNOTHERAPY AND HAEMATOPOIETIC STEM CELL TRANSPLANTATION

by

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A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

Institute of Immunology and Immunotherapy

College of Medicine and Dental Sciences

University of Birmingham

September 2016

Abstract

The human immune system responds dynamically to persistent Cytomegalovirus (CMV) infection. Immunodeficiency following haematopoietic stem cell transplantation (HSCT) can lead to CMV viraemia which is an important cause of transplant-related morbidity and mortality. This thesis investigated the reconstitution of CMV-specific T-cells in two cohorts of HSCT patients and studied the potential role of Tumour Necrosis Factor Receptor 2 (TNFR2) in regulation of CMV-specific T-cell expansion post HSCT.

The first cohort included patients who participated in a randomized phase II clinical trial of adoptive cellular therapy for CMV-specific $CD8^+$ T-cells in HSCT patients. The primary end point of study was reconstitution of CMV-specific $CD8^+$ T-cell to $10x10^3$/ml within 2 months post T-cell depleted matched unrelated donor HSCT. Reconstitution of CMV-specific $CD8^+$ T-cells was monitored for up to 6 months in 17 patients who received therapeutic T-cells and in 11 patients who received best available antiviral treatment for CMV viraemia. Adoptive cellular therapy (ACT) resulted in earlier and greater expansion of CMV-specific $CD8^+$ T cells. Within one month post cellular therapy, CMV-specific $CD8^+$ T-cells had greater expansion as compared to the control arm. Additionally, immunotherapy led to greater reconstitution of CMV-specific $CD4^+$ and non-infused CMV-specific $CD8^+$ T-cells. The number of infused therapeutic T-cells and circulating levels of Alemtuzumab were found as important factors influencing T-cell recovery after immunotherapy.

In the second cohort of patients, reconstitution of CMV-specific $CD4^+$ T-cells was studied using a novel HLA-class II tetramer. The aims here were to identify the minimum number of $CD4^+$ T-cells conferring protection against CMV viraemia and

also, to characterise their phenotype by direct visualisation. An absolute CMV-specific $CD4^+$ T-cell count of $>0.7x10^3$/ml was found to protect from recurrent CMV reactivation. Approximately, one third of specific $CD4^+$ T-cells were perforin and granzyme-B positive indicating cytotoxic potential, whilst the majority expressed T-bet confirming their Th1 activity. These cells were IL-2Rα negative whilst some had IL-7Rα and the majority expressed TNFR2. Cell surface expression of CD57 molecule on $CD4^+$ T-cells was demonstrated to be a potential universal biomarker of immune response to CMV infection which could be used for prediction of recurrent CMV viraemia in clinical practice.

In the final part of this PhD, the profile of cytokine receptor expression in T-cell subsets of healthy individuals and HSCT patients were studied. The results showed distinct cytokine receptor expression patterns in naïve versus memory T-cells. The most significant observations were: rapid decrease in expression of CD130 (gp130) (IL-6R signalling chain) and increase in expression of CD120b (TNFR2) after T-cell differentiation from naïve to effector cells. TNFR2 was present in the majority of CMV-specific T-cells and its expression per cell increased during CMV-specific T-cell expansion post HSCT. Engagement of TNFR2 with a specific ligand led to the apoptosis of CMV-specific T-cells. This phenomenon may indicate a potential role of TNF/TFR2 interaction in the contraction phase of T-cell response to CMV reactivation.

Acknowledgements

This thesis would not have been possible without the help and support of number of individuals. It is difficult to overstate my gratitude to my PhD supervisor, Dr Frederick Chen for his great insights and unwavering encouragement. His in-depth knowledge, dedication, and kind consideration, resulted in this work. I express my warmest thanks to Professor Paul Moss who strongly supported me in my research job as well as helping me to do PhD study with his great expertise and valuable criticism and allowing access to the stem cell transplant patients'samples.

I thank my PhD examiners Professors Antonio Pgliuca and Mark Drayson for their extremely valuable advice and comments. I would also like to thank Dr. Annette Pachnio for her great help in providing me with vital reagents for this research. I am deeply grateful to my colleague, Mr Guido Frumento for his wonderful technical guidance and help in processing of the clinical trial samples. Without his assistance, it was not possible to run all the samples based upon the clinical trial scheduled program. I also wish to thank Dr Heather Beard for her extremely valuable suggestions in performing experiments according to the GCLP standards. I would like to give my special thanks to the members of Stem Cell and Immunotherapy laboratory, NHS Blood and Transplant, particularly Mr Martin Cartwright, who smoothed the way for me to conduct most of my experiments in their GMP grade facilities. I also would like to thank Mr Gordon Ryan and Mrs Jasunara Begum, the research staff in the School of Cancer Sciences for their kind support and thank Professor Herald Wajant for providing me with TNFR ligand. I am deeply grateful to the Leukaemia and Lymphoma Research for their support and funding.

Words can not express how grateful I am to my parents. Their prayer for me was what sustained me thus far. I owe a great deal of loving appreciation to my wife, Dr Akram Hosseini for all of her unlimited support and consideration during my research work. A very special thanks to my beloved children, Ryan and Delara for their patience and understanding.

Finally I thank God (Allah). You are the one who let me finish my degree. I will keep on trusting You for my future. Thank you.

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Quantitative Polymerase Chain Reaction	QPCR
Regulated on Activation, Normal T-Cell Expressed and Secreted	RANTES
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Transforming Growth Factor-b	TGF-b
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Tumour Necrosis Factor-receptor	TNFR
TNF-Related Apoptosis Inducing Ligand	TRAIL
Unique Long Region	ULR
Unique Short Region	USR

Chapter 1

INTRODUCTION

A large body of evidence exists in support of immunotherapy at both research and clinical levels. Much of this is focused on the clinical outcome of immunotherapy, but immune monitoring data is equally important as they allow us to understand the behaviour of infused therapeutic cells and the host's response. The eventual success or failure of a particular immunotherapy approach can therefore be analysed and the underlying causes explored. The findings from such studies can then be used to refine or improve immunotherapy outcome and also lead to novel diagnostic technologies. This work is modelled on CMV as a target of immunotherapy, and investigated the reconstitution of CMV-specific T-cells in patients enrolled in a clinical trial of adoptive T-cell immunotherapy and in patients who received routine stem cell transplants. The first chapter of this thesis briefly discusses the virology and immunology of CMV infection and presents a summary of previously reported findings on adoptive cellular therapy with CMV-specific T-cells.

1.1 Cytomegalovirus

1.1.1 CMV genome and life cycle

The virion of CMV is composed of a nucleocapsid surrounded by the tegument which is a protein field region with the phosphoprotein 65 (pp65) as the most abundant protein. The viral envelope, derived from host cell endoplasmic reticulum-Golgi intermediate compartment, cover the tegument and contains virus encoded glycoproteins such as glycoprotein B (gB), gH and gL (Figure 1.1). The nucleocapsid encloses a 235 kbp double stranded DNA with the capacity to encode in excess of 166 gene products (Mocarski and Pass, 2007). The genome has two regions: UL – Unique long, US – Unique short which are bounded by repeat regions ; TRL (Terminal Repeat Long), IRL (Internal Repeat Long), inverted repeat of TRL, TRS –Terminal Repeat Short and IRS

– Internal Repeat Short; inverted repeat of TRS. The repeats allow rearrangements of the unique regions which are sequentially transcribed in a cascade fashion (Figure 1.2).

CMV can replicate in many differentiated cell types which are described as permissive cells for viral replication. The entry to human cells is either through direct cell fusion or through endocytic pathway and utilises some of CMV glycoproteins such as gB (Figure 1.3). Expression of CMV genes occurs sequentially. Intermediate-early (IE) genes are expressed immediately following viral entry without need for expression of other viral genes. These genes have vital roles in controlling viral and cellular gene expression in order to prepare the conditions for production of viral DNA. IE-1 and IE-2 genes regulate viral and host cell gene expression at transcriptional level and other IE gene products suppress cell death. Delayed Early (DE) genes expression depends on the expression of IE genes and their functional products are involved in viral DNA replication. Finally, Late (L) genes are expressed which encode virus structural proteins.

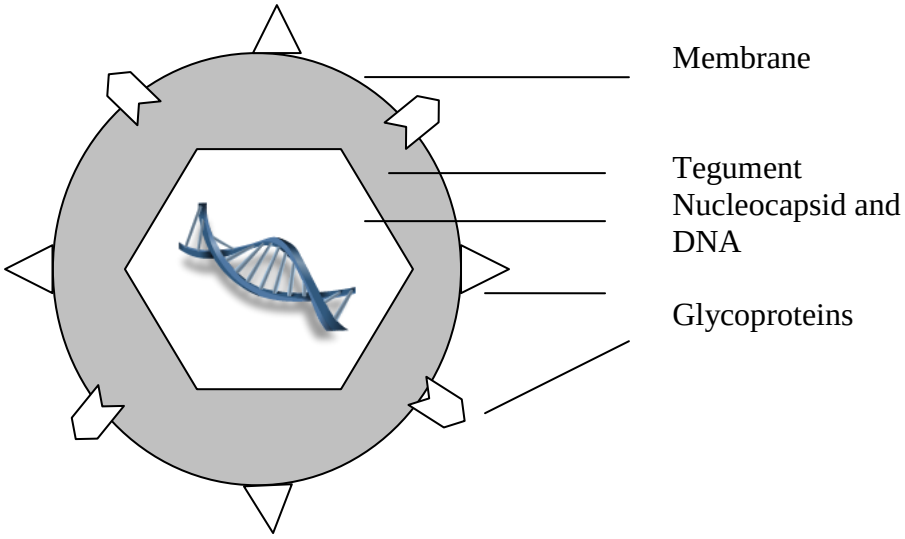


Figure 1.1 CMV virion. The virion is composed of envelope containing glycoproteins, tegument, nucleocapsid and double stranded DNA.

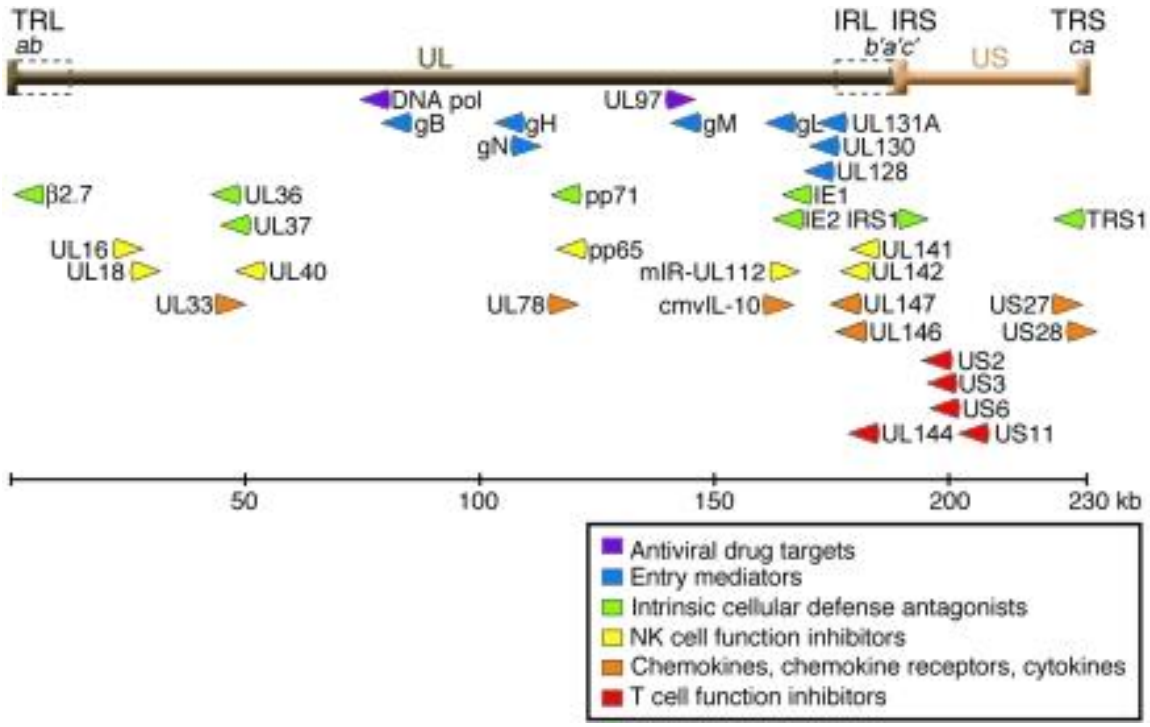


Figure 1.2 Schematic map of the CMV genome.

The CMV genome is around 230Kbp and includes two regions of unique sequences, unique long (UL) and unique short (US), bounded by two sets of inverted repeats (TRL/IRL) and (IRS/TRS). The positions of some important ORFs alongside orientation of their transcript are shown. The functional activity of CMV proteins are grouped and shown as well. (Boeckh and Geballe, 2011)

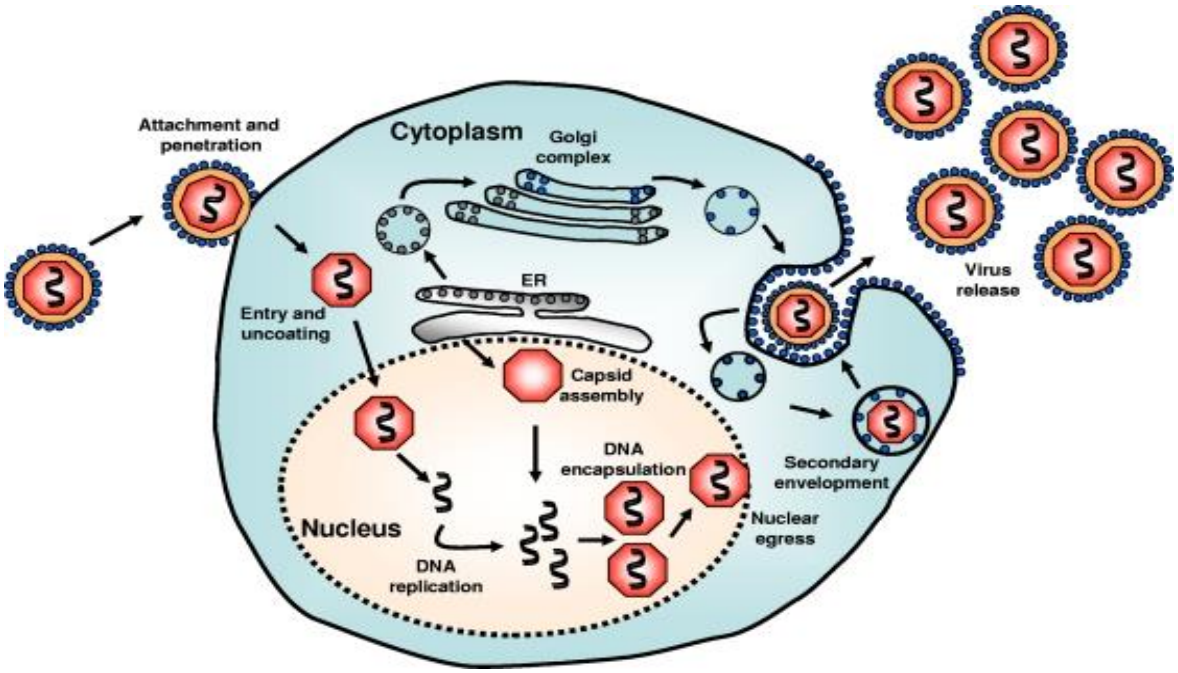


Figure 1.3 CMV life cycle in a host cell.

Viral membrane glycoproteins (e.g., gB and gH) through binding to the specific surface receptors (e.g., platelet-derived growth factor α) facilitate attachment of virus to the cell which is followed by fusion of the envelope with the cellular membrane and release of nucleocapsid into the cytoplasm. The nucleocapsids are transferred into the nucleus before release of viral DNA. These events result in the expression of IE-1/IE-2 genes which are involved in viral replication. The synthesized DNA is encapsulated and transported to the cytoplasm. The endoplasmic reticulum-Golgi intermediate compartment is the place where the secondary envelopment occurs and finally new virion is released by exocytosis at the plasma membrane. (Crough and Khanna, 2009)

1.1.2 CMV encoded proteins

1.1.2A Function

The CMV genome contains 213 open reading frames (ORF) (Sylwester et al., 2005). An ORF is a portion of genome which includes a sequence of bases that could potentially encode protein. ORFs in CMV are numbered based up on their position in UL and US, where most of them present (Figure 1.2). Many encoded proteins of CMV ORFs have been characterised and their functional properties and antigenicity studied in depth. There are around 166 proteins encoded by ORFs, some of them are membrane, tegument proteins or proteins involved in gene expression. CMV produces many different proteins that interfere with the immune system effector functions such as inhibitors of NK cell activity (UL16/UL18 encoded membrane glycoprotein).In total, there are 7 different CMV derived proteins that suppress NK cell activity. US2/3/6 family proteins degrade MHC class I and possibly class II molecules, inhibit their processing and transport and delay trafficking of MHC class I. These functions interfere with processing of antigens to T-cells and consequently, have negative impact on $CD8^+$ and $CD4^+$ T-cell activation. CMV produces proteins which are similar to cytokines and chemokines. For example, cmvIL-10 is 27% identical to human IL-10 but binds to the same receptor and has suppressive effects such as inhibition of peripheral blood mononuclear cells and their cytokine production.

1.1.2B Antigenicity

CMV is a highly immunogenic complex with many different proteins capable of stimulating both humoral and cellular immune responses (Figure 1.4). As an example, the presence of neutralising Abs against glycoproteins B and H(gB, gH) have been

shown and gB used as a vaccine to stimulate both B cells and $CD4^+$ T-cells (Sabbaj et al., 2011). Some other known targets of humoral immunity are proteins derived from UL129, UL130, and UL131A. These proteins mediate CMV entry to epithelial and endothelial cells.

Picker and his team have shown that there are 151 CMV immunogenic ORFs for $CD4^+$ and/or $CD8^+$ T-cells, and that ORF immunogenicity is influenced only slightly by ORF expression kinetics and function (Sylwester et al., 2005). They have found that total CMV-specific T-cell responses in sero-positive individuals are large, comprising on average approximately 10% of both the $CD4^+$ and $CD8^+$ memory T-cells in peripheral blood. Figure 1.5A shows the distribution of T-cell responses against proteins encoded by CMV ORFs. T-cell responses are diversely distributed against a variety of structural, early, late and CMV derived immunomodulator molecules including pp28, pp50, gH and gB. Figure 1.5B shows the strength of T-cell responses toward 10 most frequently recognized antigens including UL123 (IE-1), UL122 (IE-2) and UL83 (pp65).

In the current research, $CD8^+$ and $CD4^+$ T-cell responses against some of the well known antigenic peptides of CMV were studied (Table 1.1).

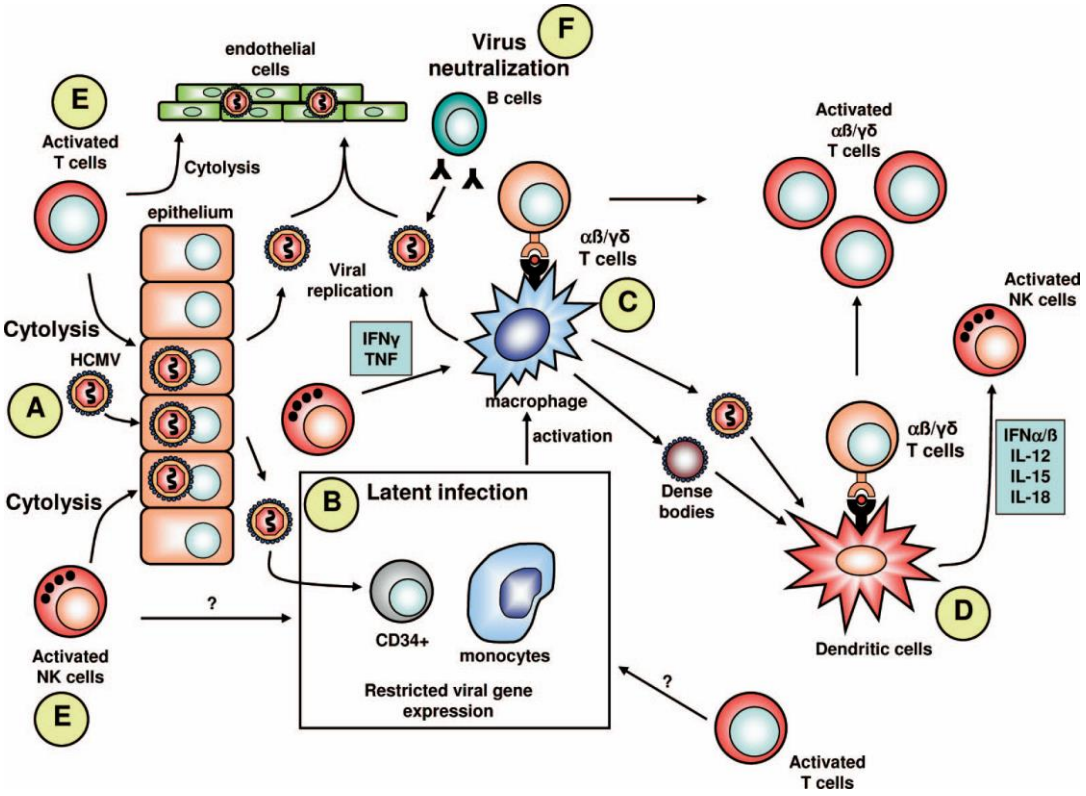


Figure 1.4 Immune response to CMV.

A) Primary CMV infection in immunocompetent individuals frequently starts with CMV replication in mucosal epithelium. B) Produced virions disseminate and infect $CD34^+$ cells and monocytes. This is the first step in the establishment of latent infection and it is likely that some CMV genes are expressed and induce T-cell activation continuously. C) Differentiation of monocytes to activated macrophages leads to production of virions or virus-associated dense bodies which can be processed by professional APCs (e.g., DCs) and presented to CMV-specific T-cells. D) In addition, these DCs activated through TLRs can also secrete a range of cytokines/chemokines, which stimulate the innate arm of the immune system. E) Activated T-cells and NK cells can lyse infected cells or produce IFN-γ or TNF-α in order to block virus replication. F) Another important arm of adaptive immunity is the B cells that produce Abs which block virus dissemination (Crough and Khanna, 2009).

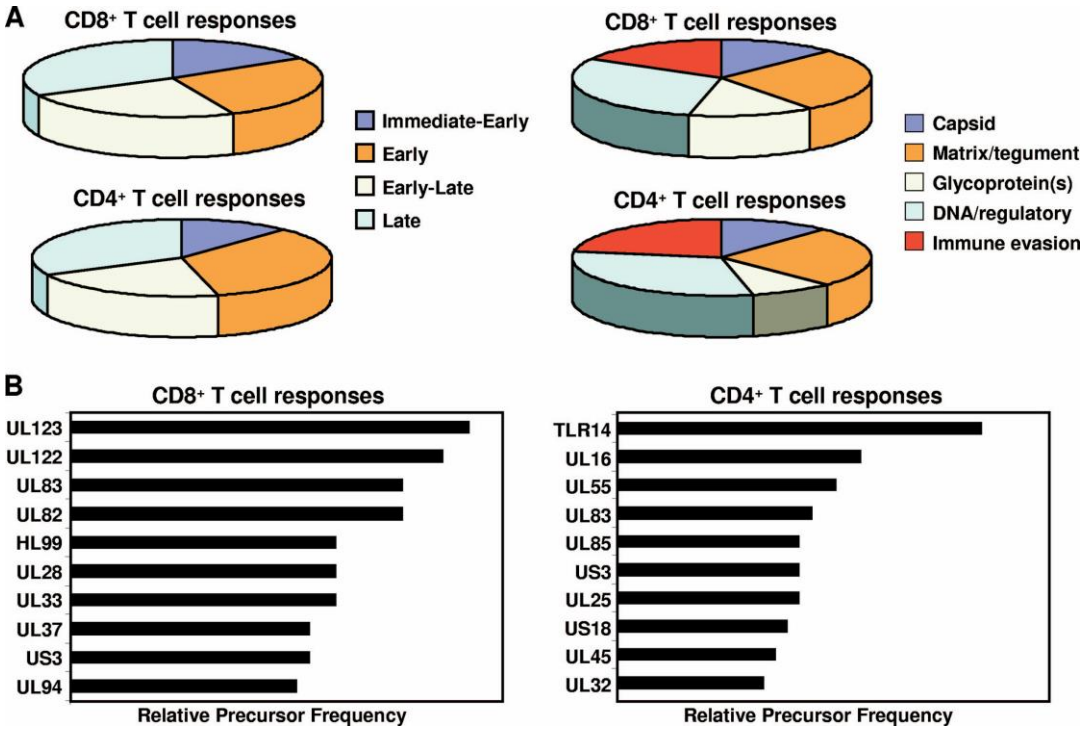


Figure 1.5 CD4$^+$ and CD8$^+$ T-cell responses to CMV proteins.

A) Relative level of T-cell responses with regard to the expression kinetics (left) or gene function (right). B) Bars are schematic representative of the magnitude of CD4$^+$ and CD8$^+$ T-cell responses to ORFs of CMV (Crough and Khanna, 2009)

Table 1.1 List of CMV derived peptides and associated HLA molecules studied in the current project.

CMV genome region	**CMV protein**	**Peptide sequence**	**HLA presenting Peptide**	**Recognised by T-cells**
UL44	pp50	VTEHDTLLY	A0101	CD8
UL83	pp65	NLVPMVATV	A0201	CD8
UL83	pp65	GPISSGHVLK	A1101	CD8
UL83	pp65	QYDPVAALF	A2402	CD8
UL83	pp65	TPRVTGGGAM	B0702	CD8
UL83	pp65	IPSINVHHY	B3501	CD8
UL123	IE-1	VLEETSVML	A0201	CD8
UL123	IE1	QIKVRVDMV	B0801	CD8
UL55	gB	DYSNTHSTRYV	DRB1*0701	CD4

1.2 CMV infection

Primary CMV infection leads to lifelong latent infection which is accompanied by periodical reactivation. During active infection, CMV is able to replicate itself in many cell types such as; endothelial cells, epithelial cells, fibroblasts, neuronal cells, smooth muscle cells, hepatocytes, trophoblasts, monocytes and dendritic cells (Plachter, Sinzger and Jahn, 1996). In the latency phase of infection, virus persists in specific cells without detectable production of infectious virus. A few number of research groups have been able to find the CMV DNA in the peripheral blood of healthy CMV sero-positive people (Taylor-Wiedeman et al., 1991; Larsson et al., 1998). Their results show that the number of cells with latent CMV infection is less than 1 in 10,000 peripheral blood mononuclear cells. Peripheral monocytes are the major carriage site of CMV DNA in healthy individuals. CMV DNA also presents in the monocyte progenitor $CD34^+$ stem cells in bone marrow. Although $CD34^+$ stem cells give rise to lymphocytes and polymorphonuclear leukocytes, CMV genome has not been found in these cell types. It should be pointed out that infected monocytes in the latent phase of infection do not express CMV IE genes. It has been suggested that CMV may exist in certain cells with a low production level of virions and no visible host cell damage or lysis. Endothelial cells are another candidate of cells with low level of virus production since CMV has been linked to atherosclerosis (Zhou et al., 1996) and CMV-specific $CD8^+$ T-cells exclusively express a chemokine receptor (CX3CR1) (Bolovan-Fritts and Spector, 2008) which binds to an endothelial cell surface molecule called fractalkine. There are also other findings supporting the role of infected endothelial cells in the activation of CMV-specific $CD4^+$ T-cells (Walker et.al, 2009). Currently, scientific evidence shows

that myeloid cells are the true site of latency in CMV sero-positive immunocompetent carriers, with little or no CMV gene expression. However, fully differentiated macrophages and dendritic cells are another possible site of continual, subclinical reactivation of virus with production of CMV virions (Reeves and Sinclair, 2008).

1.2.1 CMV reactivation

CMV reactivation from latency results in serious clinical complications in immunocompromised individuals. The factors behind CMV reactivation have not been fully understood. It has been suggested that cell host differentiation causes CMV reactivation. Differentiation of monocyte to macrophages leads to changes in the ability of these cells to support viral IE gene expression (Taylor-Wiedeman et al., 1994; Reeves et al., 2005). Furthermore, cytokines produced following allogeneic stimulation of T-cells are needed for complete reactivation of CMV in differentiated monocytes (Söderberg-Nauclér et al.,1997). Soderberg-Naucler has shown that Tumor Necrosis Factor-α (TNF-α) and Interferon-γ (IFN-γ) can induce differentiation of non-permissive monocytes into permissive macrophages. Reactivation of latent CMV through the allogeneic stimulation of peripheral blood mononuclear, derived from CMV carriers, depends upon IE gene expression. This gene expression is controlled by promoter/enhancer genes which have multiple binding sites for the transcription factor NF-κB as well as other transcription factors such as AP-1 (Sambucetti et al.,1989). Activation of these transcription factors has been documented in tissues transplanted into allogeneic recipients. Interlukin-1(IL-1) or TNF-α signaling induces phosphorylation and degradation of an inhibitory factor named IκB. Following this event, IκB is dissociated from NF-κB and allows translocation of NF-κB to the nucleus

and binding to responsive genes. Some researchers have found that TNF- α alone can substitute for allogeneic transplantation in the induction of NF-κB activity and consequently CMV-IE gene expression (Hummel et al., 2001). Immune response to an allogeneic stimulus mimics closely an inflammatory immune response. Production of inflammatory cytokines such as TNF-a, Interleukin 2 (IL-2) and IFN-γ are hallmarks of immune system activity in an allogeneic setting or response to an infection. In both settings, Interleukin 8 (IL-8), MIP-1a (Macrophage Inflammatory Protein-1a), RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), and MCP-1 (Monocyte Chemoattractant Protein-1) are produced and induce expression of endothelial cell adhesion molecules such as E-selectin and ICAM-1 (Intercellular Adhesion Molecule-1). These events facilitate migration of circulating lymphocytes and infiltration of inflammatory cells. TNF-α and IFN-γ induce expression of CD40 on antigen presenting cells (APC) which plays an important role in interaction between $CD4^+$ T-cells and APCs such as B cells and endothelial cells. Activation of T-cells by APCs leads to the production of IL-2 which in turn induces strong T-cell proliferation and differentiation to cytotoxic T-cells. All of these events provide the stage for infiltration of immune cells to the graft and recognition of foreign cells.

Similar events happen in a classic immune response to an infection and it is likely that an inflammatory response is the natural cause of CMV activation. Some investigators believe that CMV uses host immune system in order to reactivate its genes from latency (Hummel and Abecassis, 2002).

1.3 CMV immunity

CMV is the biggest known antigenic complex for humans and immune system invests considerably in both innate and acquired immunity in order to control this virus (Figure 1.4). As discussed earlier, CMV establishes a latent infection with possible frequent reactivations which constantly pressurises the immune system in the immunocompetent individual. This means that CMV is under constant immunesurveillance and CMV dissemination and clinical disease occurs when this immunity is compromised in conditions such as allogeneic transplantation, immunodeficiency in HIV infection or strong inflammatory response in the septic shock.

1.3.1A Innate immune response, production of Interferon

Type I interferons (IFN-α/β) have important roles in the control of mouse CMV during acute infection and the viral spread period (Loewendorf & Benedict, 2010). MCMV induction of IFN-αβ is the starting point of immune response to this virus. The source of this interferon is splenic stromal cells which binds to B-cells expressing lymphotoxin (LT) α/β. In a model of CMV infection of cultured fibroblasts, LT-αβ signalling induced IFN-β production. In this process, NF-κB was a key component of the IFN-β gene transcription. The first wave of IFN-α/β production is 8 hrs post MCMV infection and is followed by the second wave at 36 hrs post infection. In this time point, the majority of interferon comes from dendritic cells activated through toll like receptor 9 (TLR9). Both MCMV and CMV utilise different mechanisms to suppress interferon production. In human, TLR2 interacts with gB/gH which triggers production of inflammatory cytokines through NF-κB signalling pathway (Juckem et al., 2008).

1.3.1B NK cell response

Type I interferon has positive effect on NK cell cytotoxicity, and this is a primary mechanism by which MCMV replication is controlled at the second day of MCMV infection. NK-cells also produce IFN-β which restricts MCMV replication.

In human, NK-cell activity increases in both primary and reactivated CMV infection. A recent study showed that 30-70% of NK-cells were proliferating cells during acute CMV reactivation. This study also found that a unique subset of $CD57^{+}$ $NKG2C^{high}$ induced in that period and de-granulated after engagement of NKG2C molecules with its ligand (Foley et al., 2012). It is important to mention an interesting finding showing that the prevention of CMV reactivation post HSCT relates to one group of NK cell receptors called activating-killer immunoglobulin like receptor (KIR) (Cook et al., 2006).

1.3.2 Acquired immune response

The establishment of sustained CMV immunity with the ability to form memory responses needs strong immune responses from all components of adaptive immunity. Innate immunity, through production of interferons and TNF-α which up-regulates HLA molecule expression on APCs, triggers the first signals to the adaptive immunity. NK-cells and activated monocytes/ macrophages produce a wide range of cytokines and chemokines that are involved in the recruitment of $CD8^{+}$ T-cells from blood. Among these mediators, CXCL9 and CXCL10 bind to $CXCR3^{+}$ $CD8^{+}$ T-cells and mediate their recruitment to the infection site.

1.3.2A B-cell mediated response

Current evidence indicates important roles of antibodies (Ab) in restricting viral spread and limiting the severity of CMV disease. These neutralizing Abs are mainly against CMV-gB which is involved in cell attachment. Another target of Abs is gH which facilitates integration of viral envelope with the host cell membrane.

In humans, the transfer of antibodies from a CMV-seropositive mother protects newborn infant from CMV infection (Fowler et al., 1992). The avidity of these neutralizing Abs are not the same and transfer of low avidity Abs increases significantly the probability of the transmission of viral infection from mother to foetus (Boppana and Britt, 1995). There are documented data on vaccine development for CMV using gB as an immunogen which decreases incident cases of maternal and congenital CMV infection (Pass et al., 2009).

1.3.2B T-cell mediated immune response, role of $CD8^+$ T-cells

T-cells play a profound central role in the control of CMV reactivation. Deficiency of cellular immunity is strongly related to CMV disease with the exception of congenital CMV related disease.

$CD8^+$ T-cells are in the front-line of cellular immunity against intracellular pathogens. Antigen presentation by HLA-class I molecules with the help of cytokines and cell surface co-stimulatory molecules, induce $CD8^+$ T-cell activation. The main function of activated $CD8^+$ T-cells is destroying target cells (harbouring pathogen) through their cytotoxic mediators such as perforin and granzyme. $CD8^+$ T-cells also produce high levels of inflammatory cytokines mainly TNF-α, IFN-γ and MIP-10. These cytokines can restrict CMV proliferation and make a cytokine milieu that supports recruitment

and activation of other effector cells e.g.; macrophages and NK cells. In the peripheral blood of healthy CMV-seropositive carriers, frequency of viral specific $CD8^+$ T-cells is surprisingly high and it can even rise to 23% of all peripheral $CD8^+$ T-cells (Khan et al., 2002). These cells are oligoclonal with restricted TCR usage and can be toward different CMV peptides.

Quantitative changes in $CD8^+$ population. CMV infection has a major impact on $CD8^+$ T-cell repertoire in the peripheral blood. The number of CMV-specific $CD8^+$ T-cells increases gradually and can lead to low or even inverted CD4/CD8 ratio in the peripheral blood. The possible mechanism behind this change in the peripheral T-cells is persistent stimulation of $CD8^+$ T-cell repertoire by subclinical reactivation of CMV during latency. It has been found that $CD8^+$ T-cells block progression of MCMV gene expression during reactivation (Kurz et al., 1999; Balthesen et al., 1994). These data indicate that MCMV establishes a latent infection with frequent reactivations but the full process of virion production is aborted or blocked by T-cell surveillance.

The increase in the number of CMV or MCMV-specific T-cells over time has been referred to as memory inflation (Snyder et al., 2009). The result of inflationary memory is accumulation of effector T-cells (discussed below). Acute infection leads to the generation of T-cells specific for a wide range of antigens, followed by disappearance of most of these cells in the latency phase. The surviving cells are non-inflationary T-cells with a memory phenotype which is mainly characterised by the expression of IL-7 receptor (van Leeuwen et al., 2005). The current understanding is that non-inflationary cells have a strong proliferation capacity. It has been documented that the presence of non-inflationary T-cells in the stem cell graft, identified by low CD57 expression, confers better CMV immunity post HSCT (Scheinberg et al., 2009). One of the

controversies surrounding accumulation of inflationary T-cells is their proliferative capacity. Some recent findings suggest absence of substantial proliferation within these cells, in contrast to non-inflationary T-cells (Hertoghs et al., 2010). There are two possible mechanisms, one describes CMV-specific inflationary T-cells as long lived, resting effector cells with an estimated half life of 55 days in human (Wallace et al., 2010). The alternative explanation is that these cells are dynamic, comprising mostly short lived cells that are replaced as they die. Mice model studies support the second explanation since transfer of inflationary MCMV-specific T-cells lead to the rapid disappearance of them even in the presence of virus. These cells have low level Bcl-2 expression (anti apoptotic molecule). The source of these cells might be more memory like T-cells (IL-7R^{+} T-cells) and not naïve cells. It has been shown that T-cell clones derived from memory like T-cells but not more differentiated effector memory subsets persist after adoptive immunotherapy in non-human primate model of CMV infection (Berger et al., 2008). Another important fact is the functional ability of inflationary T-cells in terms of cytotoxicity and strong production of inflammatory cytokines. It has been suggested that encounter with CMV antigens does not drive inflationary T-cells to cell division but is enough to stimulate their functional ability (Snyder et al., 2011).

In regard to the proliferation ability of inflationary T-cells, it should be noted that there are important data obtained from *in vitro* studies showing proliferative capacity of this type of T-cells. Wills and his team studied proliferation requirement of effector cells with the phenotype of CD45RA^{+} CD28^{-} (see below). They found that these cells were able to proliferate if appropriate co-stimulatory signals are provided. The fully differentiated effector cells are CD28 negative but this team showed that another co-

stimulatory molecule named 4-1BB could induce proliferation signals to $CD8^+$ T-cells stimulated with CMV antigens (Waller et al., 2007).

Qualitative changes in $CD8^+$ T-cell population. CMV infection does dramatically change the phenotype of CMV-specific T-cells toward effector/highly differentiated cells which is unique to CMV infection amongst other viral infections (Appay et al., 2002). Peripheral blood T-cells have been classified based upon expression of some cell surface molecules. The most appreciated classification is based upon the presence or absence of a chemokine receptor called CCR7 and CD45RA molecule (Sallusto et al., 2000). T-cells with expression of both CCR7 and CD45RA are called 'naïve' T-cells. $CCR7^+$ $CD45RA^-$ cells are central memory (CM) cells. $CCR7^-$ $CD45RA^-$ and $CCR7^-$ $CD45RA^+$ T-cells are effector memory (EM) and effector/memory $CD45RA^+$ (EMRA) T-cells, respectively. Antigenic stimulation of T-cells, drive them toward a memory phenotype and response to CMV pushes these changes further. CMV-specific $CD4^+$ or $CD8^+$ T-cells have mainly effector memory and effector RA^+ phenotype, respectively. Central memory T-cells migrate to lymph nodes and after encounter with antigen, become effector T-cells with profound cytotoxicity and cytokine production ability. The phenotype of inflationary T-cells is mainly differentiated effector T-cells expressing CD45RA. CD45 molecule has two isoforms; CD45RO and CD45RA. CD45RO isoform is expressed following antigenic stimulation of naïve T-cells and presents mainly in memory T-cells, but effector cells down regulate CD45RO and re-express CD45RA isoform. Among other widely studied membrane markers are co-stimulatory CD27 and CD28 molecules which are expressed by naïve T-cells. Cell stimulation leads to the gradual down regulation of these molecules, resulting in the appearance of T-cells with $CCR7^-$ $CD45RA^+$ $CD27^-$ $CD28^-$ phenotype which is the signature of inflationary T-cells.

CD57 is another well discussed molecule in CMV infection. This surface glycoprotein is not present in naïve and nearly all of CM cells but appears in EM cells and its expression increases in EMRA cells. In this regard, CD57 positive cells are representative of fully differentiated EMRA cells with strong cytotoxic ability (Chattopadhyay et al., 2009). T-cell response following primary CMV infection has been summarized in Figure 1.6.

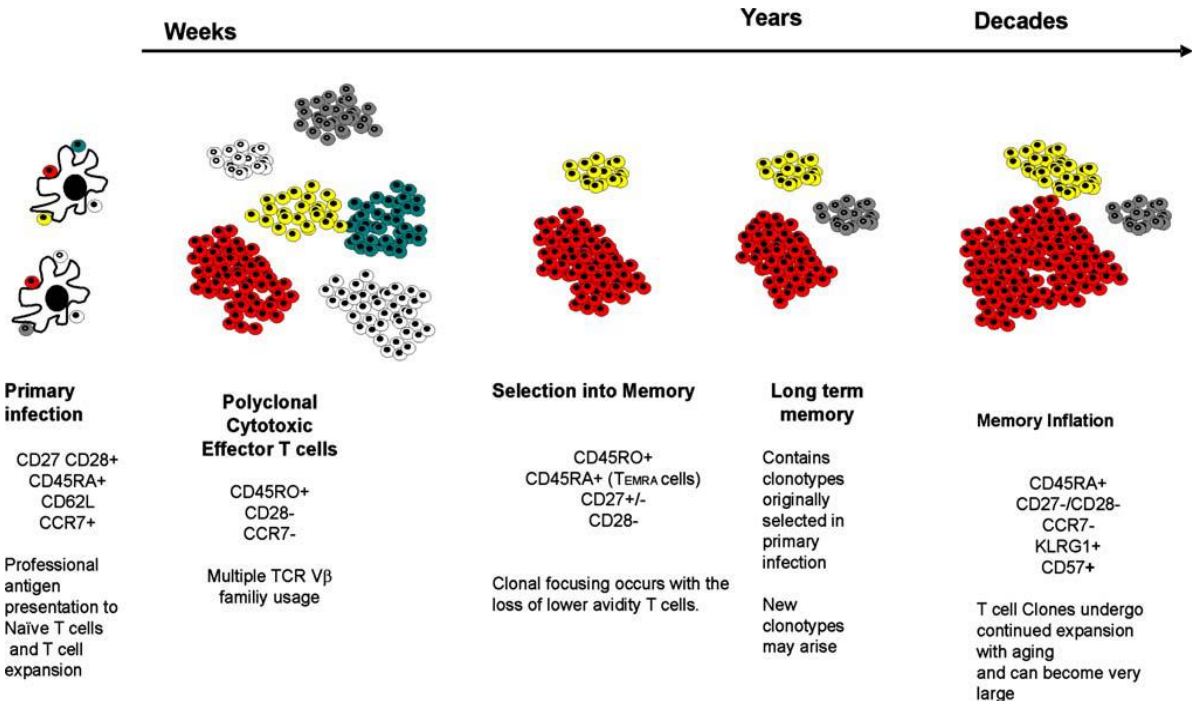


Figure 1.6 T-cell responses following primary CMV infection.

Polyclonal cytotoxic T-cells are generated following primary infection. CMV virion production and spread are controlled within a few weeks and many clones of specific T-cells disappear. An oligoclonal population of cells is selected to memory with re-expression of CD45RA molecule and losing CD27 and CD28. The selected clones into memory pool are very stable and stay for years but their frequency increases with time (memory inflation) (Crough and Khanna, 2009).

1.3.2C T-cell mediated immune response, $CD4^+$ T-cells

$CD4^+$ T-cell functional activity against viruses is less characterized. $CD4^+$ T-cells are usually considered as T-cells providing help for B-cell and $CD8^+$ T-cell responses to pathogens. New evidence has uncovered additional roles for $CD4^+$ T-cells which includes direct antiviral roles and helping innate immune cells (Swain et al., 2012). Virus specific $CD4^+$ T-cells have mainly a Th1 phenotype which is characterised by the expression of transcription factor; T-bet which regulates IFN-γ production. Type I interferon and IL-12 have major impacts on the generation of Th1 cells. IFN-γ not only augments cytotoxic activity and cell division of T-cells but induces immunoglobulin class switching and production of neutralizing IgG2a Abs. CD40L in $CD4^+$ T-cells, is one of the well known co-stimulatory molecules which binds to CD40 molecule in B and $CD8^+$ cells and sends stimulatory signals to them. $CD4^+$ T-cells also down regulate expression of two inhibitory cell surface molecules called; programmed cell death-1 (PD-1) and TNF-related apoptosis-inducing ligand (TRAIL). These activities make $CD8^+$ T-cells less prone to apoptosis and increase their functional activity. $CD4^+$ T-cells promote the generation of effector $CD8^+$ T-cells through different mechanisms including production of IL-2, IL-15 and TNF (Oh et al., 2008). Figure 1.7, illustrates $CD4^+$ T-cell helper roles in survival, activation and differentiation of B and $CD8^+$ T-cells.

$CD4^+$ T-cells have also direct engagement with virally infected cells. Following contact with professional APCs, $CD4^+$ T-cells can act as effector cells by producing a wide range of pro-inflammatory cytokines such as IFN-γ and TNF-α (Figure 1.8A, B). $CD4^+$ T-cells also produce anti-inflammatory cytokine IL-10 which may be involved in the control of inflammatory response to viruses. Recent findings indicate the presence of

cytotoxic molecules such as perforin and granzyme B and also FAS in $CD4^+$ T-cells which enable them to directly kill target cells (Figure 1.8C). It is important to mention that cytotoxic activity of $CD4^+$ T-cells does not depend upon the presence of transcription factor T-bet which is the main known transcription factor for Th1 cells. The vital transcription factor for cytotoxic activity of $CD4^+$ T-cells is EOMES (Eomesodermin) which regulates IFN-γ and granzyme B gene expression in T and NK cells (Qui et al., 2011).

Figure 1.7 Helper functions of CD4$^+$ T-cells.

A) Follicular helper T-cells (TFH) are a subset of CD4$^+$ T-cells that interact with B-cells through CD40L-CD40 binding and other co-stimulatory molecules and release of cytokines. CD4$^+$ T -cell response leads to production of neutralizing antibodies which is the ultimate goal of many vaccines. B) CD4$^+$ T-cells provide help to CD8 $^+$ T-cells directly by release of cytokines such as IL-2 and indirectly by interaction with DCs. CD4$^+$ T-cell's help generate cytotoxic T-cells and down regulation of TNF-related apoptosis-inducing ligand (TRAIL).These activity facilitates robust response of CD8$^+$ T-cells during secondary infection (Swain et al., 2012)

Figure 1.8 Antiviral activities of CD4$^+$ T-cells that involve direct contact with target infected cells.

A) CD4$^+$ T-cells recognize viral Ags presented by APCs and produce an array of effector cytokines. Some products of activated effector cells such as IL-10 dampen inflammation. B) CD4$^+$ T-cells produce IFN-γ and TNF-α that make an antiviral state in tissues. C) Cytotoxic CD4$^+$ T-cells can directly kill infected cells through perforin and granzyme release or FAS dependent cell lysis (Swain et al., 2012)

1.3.2D CMV-specific CD4$^+$ T-cells

CD4$^+$ T-cell responses to CMV have not been as well studied as CD8$^+$ T-cell responses, due to the lack of appropriate experimental tools. Picker's team showed that in healthy CMV carriers, immune system devotes a median of 9% of its CD4$^+$ repertoire to this virus and that CD4$^+$ T-cells recognize a median of 21 different ORFs (Figure 1.5) (Sylwester et al., 2005). There are ORFs that induce potent CD8$^+$ and CD4$^+$ T-cell responses, although CD8$^+$ T-cells recognize more CMV-ORFs. Glycoprotein B is the

most frequently detected antigens for $CD4^+$ T-cells but in around 5% of healthy individuals, high frequency of $CD4^+$ specific for TRL14 and UL16 exist. As mentioned in the above section, the role of $CD4^+$ T-cells is not only restricted to providing help to the other immune cells. gB specific peripheral blood $CD4^+$ T-cells with cytotoxic function have been expanded *in vitro* (Hopkins et al., 1996). Researchers have been able to isolate gB specific $CD4^+$ CTL with the ability to release granzyme from peripheral blood (Hegde et al., 2005). These CTLs recognize an immune dominant epitope DYSNTHSTRYV from gB which is restricted through HLA $DRB1^*$ 0701 (Elkington et al., 2004). It has been shown that $CD4^+$ T-cells in large granular lymphocytosis are oligoclonal expansion of HLA-DR7 restricted DYS specific T-cells (Crompton et al., 2008). Similar to CMV-specific $CD8^+$ T-cells, $CD4^+$ T-cells specific for CMV proteins such as pp65 are oligoclonal and use restricted and stable T-cell receptors. Gamadia and colleagues (2004) showed that CMV-specific $CD4^+$ T-cells had effector-memory phenotype in healthy individuals and renal transplant recipients and produced Th1 related cytokines. These cells were granzyme B positive and their response occurred before $CD8^+$ T-cells in the peripheral blood of asymptomatic renal transplant patients. They also found that during latency, $CD4^+$ T-cells were $CCR7^-$ $CD45RA^-$ ($CD45RO^+$) $CD27^-$. During both latency and primary infection, CMV-specific $CD4^+$ T-cells expressed CD40L and produced IFN-γ, TNF-α but not IL-2 and IL-4 (Gamadia et al., 2004). Other groups have shown that $CD4^+$ $CD28^-$ T-cells exist almost exclusively in CMV infected individuals (van Leeuwen et al., 2004).

1.4 CMV and clinical complications

CMV causes severe disease when the immune system is immature or impaired. CMV infected patients during active infection, display signs of immune dysfunction such as immunosuppression and autoimmunity. CMV infection has been linked to the allograft rejection and graft-versus-host disease in solid organ and bone marrow transplant recipients, respectively. Active viral infection takes place in several autoimmune diseases (Varani and Landini, 2011). Active forms of CMV infection are: a) primary infection of a CMV- naïve host; b) endogenous infection in CMV positive individuals who suffers reactivation from latency; c) exogenous re-infection by a different strain. Active and latent infection induces Th1 type inflammatory cytokines (van de Berg et al., 2010). As discussed above, initiation of viral replication can be triggered by inflammatory conditions. For example, TNF-α binds to TNF receptor on latently infected cells, inducing signals that activate NF-κB. The activated NF-κB trans-locates into the nucleus and binds to the IE enhancer region of CMV, which leads to the viral replication (Hummel et al., 2002). There are published data that correlate high serum level of TNF and CMV reactivation and clinical complications such as atopic dermatitis, sepsis and acute graft-versus-host disease in the bone marrow transplant patients (Cantoni et al., 2010).

1.4.1 Autoimmunity

Anti-phospholipids and anti-CD13 auto-antibodies have been detected in CMV infected bone marrow transplant recipients and anti-CD13 has been linked to the development of chronic GVHD in these patients (Larsson et al., 1998). Auto-antibodies against

endothelial cell, smooth muscle cell and anti-nucleus are associated with CMV infection in solid organ transplant recipients. It is likely that these Abs increase the risk for humoral and chronic allograft rejection. In addition, hyper-gammaglobulinemia, cryoglobulinemia and autoantibody production are features of CMV related mononucleosis (Varani et al., 2002). Active CMV infection has been observed in cutaneous vasculitis and SLE associated vasculitis (Pérez-Mercado et al., 2010). CMV infection and CMV disease are risk factors for early-onset diabetes mellitus in the renal transplant patients (Hjelmesaeth et al., 2004) and incidence of this disease has declined following the introduction of pre-emptive anti viral treatments. CMV infection can damage β-cells directly or indirectly through the cytotoxic effects of activated lymphocytes or production of pro-inflammatory cytokines (Kamalkumar et al.,2009).

There are some autoimmune conditions that may lead to CMV reactivation from latency. As it was mentioned before, CMV reactivation in monocytes can happen during differentiation of monocytes to macrophages after stimulation with IFN-γ or TNF-α. Thus it is likely that chronic inflammation in autoimmune diseases provide the ideal microenvironment in which latent CMV can be reactivated in macrophages. One example of chronic inflammation is inflammatory bowel disease (IBD) that has attracted attention of different research groups to study possible involvement of CMV in IBD. CMV antigens have been detected in biopsies from patients with IBD. Patients with inactive or mild ulcerative colitis (UC) rarely suffer from CMV replication, whereas active CMV infection exists in 20% to 40% of steroid refractory UC, indicating the possible role of the virus in severity of inflammation. Active CMV infection occurs in colon where CMV-DNA has been detected by PCR, whereas blood analysis shows low or no viral load. All patients with steroid refractory UC and active CMV infection

have been CMV-seropositive, suggesting reactivation of latent virus at the site of inflammation (Domènech et al., 2008). CMV has also been related to the vascular damage in systemic sclerosis (Lunardi et al., 2006). There are auto-antibodies that are specific for systemic sclerosis and bind the late CMV protein UL94. These Abs induce apoptosis in endothelial cells and dermal fibroblasts *in vitro*. In systemic sclerosis, vascular damage and fibrosis are two main clinical complications. CMV-DNA can be detected in gastrointestinal tract of CVID patients (Common Variable Immunodeficiency) who suffer from inflammatory granulomatosis. This inflammation has been related to an exaggerated T-cell response to CMV (Marashi et al., 2011 and 2012).

1.4.2 Graft rejection

Several studies have shown that CMV infection is associated with an increased risk of graft rejection in liver, lung and renal transplantation (Varani and Landini, 2011). Randomized trials of antiviral prophylaxis and pre-emptive therapy have shown that antivirals significantly provide protection against CMV related graft rejection (Slifkin. et al., 2005). In bone marrow transplant patients, graft versus host disease and its treatment increase the risk of CMV replication (Cantoni et al., 2010). The role of CMV replication as a causative agent in development of GVHD is controversial since randomized studies of prophylaxis with antiviral drugs have not shown any impact on the risk for GVHD.

1.4.3 Mechanisms of CMV- induced pathogenesis

1.4.3A Humoral autoimmunity

Some of CMV genes are homologous to cellular genes and it may lead to cross reaction between viral and host proteins. Humoral autoimmunity can also be triggered by non-specific B-cell activation induced by CMV. CMV is a polyclonal B cell activator *in vitro* and in addition, CMV interacts with toll-like receptor (TLR) 7 and 9 in human plasmocytoid dendritic cells leading to IFN-α production and B-cell proliferation (Figure 1.9A). In clinical settings, hyper-activation of B-cells is important since auto-antibodies contribute to the development of GVHD in CMV-infected HSCT patients and graft rejection in solid organ recipients.

1.4.3B Inflammation

Active and latent CMV infections induce systemic type 1 inflammatory response (Van de Berg et al., 2010).This condition can augment alloimmune responses by enhancing the expansion of alloreactive T-cells. High level of IFN-γ can increase major histocompatibility complex (MHC) expression on graft T-cells and as a result, recognition of these cells by alloreactive T-cells is enhanced. Association of Th1 related molecules: IFN-γ, T-bet and granzyme B with transplant glomerulopathy, a risk factor for chronic graft rejection, has been shown (Homs et al., 2009). In autoimmune diseases including RA, Wegener's granulomatosis, dermatomyositis, polymyositis, multiple sclerosis and IBD, $CD4^+$ $CD28^-$ T-cells expand. These cells are Th1 cells and produce inflammatory cytokines in lesions in Wegener's graulomatosis (Komocsi et al., 2002). The predominant T-cells infiltrate inflamed muscles in patients with dermatomyositis and polymyositis, are $CD4^+$ $CD28^-$ and $CD8^+$ $CD28^-$ T-cells which secrets IFN- γ after

CMV stimulation. In healthy controls and RA patients, $CD4^+$ $CD28^-$ T-cells respond to several CMV antigens (Figure 1.9B) (Thewissen et al., 2007). CMV can stimulate the translocation of NF-κB into the nucleus, which then up-regulates TNF-α production. This leads to the further activation of latent CMV and inflammatory responses.

1.4.3C Vascular damage

CMV can infect endothelial cells *in vitro* and infected cells are dysfunctional. These cells produce more IL-8, Il-1 and some adhesion molecules. IFN-γ and TNF-α up-regulate the expression of fractalkine in the endothelial cells. CMV-specific T-cells exclusively express CX3CR1 which binds to fractalkine. It seems that CMV induced endothelial cell inflammation and damage results from chemokine-mediated pathogenesis (Figure 1.9C).

1.4.3D Immunosuppression

CMV has evolved different mechanisms to suppress immune response and persist in the host. CMV infection *in vivo* and *in vitro* reduces proliferative ability of T-cells, inhibits NK cell cytotoxicity, suppresses myelopoiesis, and impairs APC function and maturation of DC. The secretion of CMV encoded IL-10 might help the virus in the inhibition of DCs (Figure 1.9D). Immune suppression by CMV may cause a generalised immune defect which might be responsible for increased risk of secondary bacterial and fungal infection post stem cell transplantation.

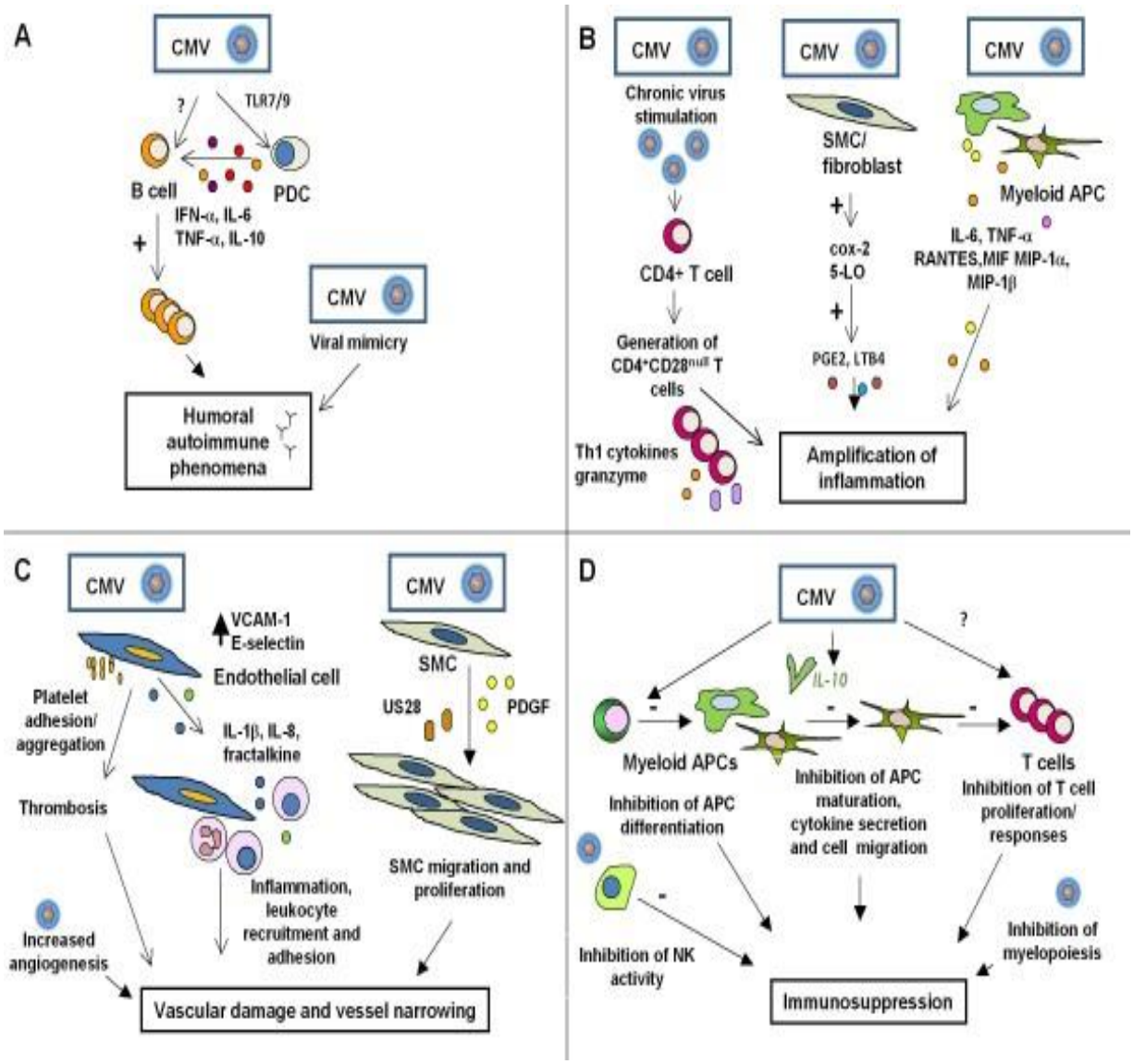


Figure 1.9 Mechanisms of CMV induced immunopathology.

A) CMV induce auto-Ab production. B) Inflammation caused by the virus. C) CMV-induced vascular damage. D) CMV and immunosuppression. PDC; plasmacytoid dendritic cells, SMC; smooth muscle cells, 5-LO: 5-lypooxygenase, cox-2, cyclooxygenase2, PGE2; prostaglandinE2, LTB4; leukotriene B4, MIF; macrophage migration inhibitory factor, MIP-1α; macrophage inflammatory protein 1-α, MIP-1β; macrophage inflammatory protein 1-β, VCAM-1; vascular cell adhesion molecule-1, PDGF; platelet-derived-growth factor, vIL-10; virally encoded IL-10. (Varani and Landini ,2011)

1.5 CMV and hematopoietic stem cell transplantation

Following hematopoietic stem cell transplantation, uncontrolled CMV reactivation can cause gastroenteritis, interstitial pneumonitis, hepatitis, retinitis and encephalitis. CMV reactivation and associated diseases post HSCT can occur early or late after transplantation (Boeckh et al., 2003). Many research studies have shown vital role of immune cell reconstitution in the control of reactivated CMV post HSCT.

HSCT is the transplantation of multi-potent hematopoietic stem cells, usually derived from bone marrow, peripheral blood, or umbilical cord blood. Prior to the transplant procedure, conditioning chemotherapy and/or irradiation treatment is given to the transplant recipients in order to eradicate their tumor cells and to suppress immune reactions. These types of conditioning therapies are classified in two groups; myeloablative which ablates bone marrow cells and non-myeloablative which is less aggressive and does not eradicate all the bone marrow cells. Among drugs used in non-myloablative treatments, is Alemtuzumab (marketed as Campath-H), a monoclonal Ab which binds to pan lymphocyte marker CD52.

The mechanism(s) of Alemtuzumab action is not fully understood and recent evidence from a human CD52 transgenic mouse model suggests that neutrophils and NK cells through ADCC deplete lymphocytes attached to anti-CD52 Ab (Hu et al., 2009).

Following the HSCT procedure and engraftment of stem cells in the bone marrow, blood cells are generated and normalise the blood cell count. Post HSCT, there is the aplastic phase (neutropenia) until recovery of neutrophils at 14 days post peripheral blood stem cell transplantation. This is followed by recovery of other cells of innate immunity including NK cells. Adaptive immune cells recovery starts within 100 days

post HSCT and $CD8^+$ T-cells appear before $CD4^+$ T-cells. Homeostatic expansion of T-cells results from peripheral memory T-cell expansion in response to cytokines plus alloantigens and from production of naïve T-cells which happens later and in the thymus. $CD4^+$ T-cell recovery is more dependant upon thymic production of naïve T-cells and as a result, CD4/CD8 ratio is inverted early post HSCT. The lack of enough T-cells with broad antigen specificity causes susceptibility to viral and fungal infections. The reconstitution of B-cells takes place much later, up to 2 years after HSCT. The low frequency of memory B-cells and impaired immunoglobulin class switching lead to susceptibility to the encapsulated bacteria.

HSCT is associated with significant changes in the levels of a variety of cytokines in the peripheral blood. This so called 'cytokine storm' is caused by different events such as conditioning regimen, donor engraftment, infections and GVHD. Results from animal models and human studies show the production of inflammatory cytokines such as IL-1 and TNF-α following myeloablative conditioning regimen which lead to a cycle of lymphocyte activation and contribution to the development of GVHD. There are also reports on increase in the levels of non-inflammatory cytokines such as G-CSF, IL-15, and IL-7 during period of cytopenia and GVHD. A recent study on the levels of 33 cytokines pre and post HSCT in patients receiving T-cell depleted $CD34^+$ cells following a myeloablative treatment, showed two cytokine storms (Melenhorst et al., 2012). The first happened during pancytopenia and peaked in week 1 post HSCT then declined as granulocytes returned (14 days post HSCT). This cytokine storm included hematopoietic and lymphopoietic cytokines such as IL-6 and G-CSF and IL-15. The second storm coincided with leukocyte reconstitution and was made up of

inflammatory, anti-inflammatory and chemokines such as IP-10, IL-1β, LIF, IL-17, IFN-γ and TNF-α.

1.5.1 Reconstitution of CMV-specific T-cells post HSCT

CMV reactivation is one of the major obstacles after HSCT due to the lack of appropriate T-cell mediated immune surveillance. Joe Meyers (1980) and his team studied for the first time, cellular immune response to CMV in the bone marrow of transplant patients. Using a proliferation assay in response to CMV stimulation, they found that the presence of proliferative response protected patients from CMV disease. The fundamental role of cytotoxic cells was unravelled by Quinnan and his colleagues (1982) who showed HLA-restricted T-cells and non-T-cell cytotoxicity correlated with recovery from CMV infection in both bone marrow and kidney transplantation. Later, it was documented that $CD8^+$ T-cells were the main component of cytotoxic T-cells (Reusser et al., 1991) and for the first time, adoptive transfer of CMV-specific $CD8^+$ T-cell clones in 3 patients was performed successfully (Riddell et al., 1992). Evaluation of immune response to CMV revealed that restoration of cytotoxic response was related to $CD4^+$ T-cell recovery (Li et al., 1994; Walter et al., 1995). These original studies indicate that reconstitution of CMV-specific $CD4^+$ and $CD8^+$ T-cells is essential for controlling CMV reactivation post HSCT. Since then, our knowledge of CMV-specific T-cells has advanced rapidly following development of 3 new experimental techniques; intracellular staining of cytokines, detection of released cytokines and cell staining with HLA-peptide multimer/tetramer complex. Using HLA-peptide tetramer reagents, it was shown that recovery of CMV-specific $CD8^+$ T-cells to levels greater than $10x10^3$/ml provided protection from CMV reactivation (Cwynarski et al., 2001). Later, another

research group showed that functional ability of CMV-specific $CD4^+$ and $CD8^+$ T-cells were also important in controlling CMV reactivation following HSCT (Ozdemir et al., 2002). Using TNF-α production as read out of cell activity, HSCT patients with or without CMV reactivation were compared. This group found that patients suffering from CMV reactivation, had higher numbers of CMV-specific T-cells but the percentage of functional cells was lower compared to the patients with no reactivation.

Another research group studied 137 patients who received non-T-cell-depleted allogeneic peripheral blood stem cell or bone marrow transplantation and found that high dose steroids and $CD4^+$ T-cell count less than $100x10^9$ /L strongly predicted impaired functional activity of $CD4^+$ T-cells (Hakki et al., 2003). This study also found that total peripheral blood $CD8^+$ T-cell number should be around $50x10^9$ /L to provide immune protection. In an attempt to clarify the importance of quality versus quantity of CMV-specific T-cells, a study investigated simultaneously intracellular cytokine production by specific T-cells and the number of tetramer binding cells (Morita-Hoshi et al., 2008). This study demonstrated that 67% of patients had more than $10x10^3$ /ml CMV-specific $CD8^+$ T-cells at the onset of disease. Intracellular staining upon stimulation by CMV lysate and peptide in patients with CMV colitis showed that the number of IFN-γ producing $CD4^+$ and $CD8^+$ T-cells were smaller at the time of colitis but increased with recovery of the disease. At the end, the investigators suggested that prediction of viral reactivation solely based upon quantification of CMV-specific T-cell number was difficult and additional functional analysis was needed. In the recent years, other research groups have focused on functional behaviour of T-cells and studied multiple aspect of their function (Stuchly et al., 2011; Costa et al., 2011; Zhou et al., 2009; Lilleri et al., 2009; Pourgheysari et al., 2008). These groups investigated

production of cytokines; IFN-γ, TNF-α and IL-2 plus up-regulation of co-stimulatory molecule; CD40L and de-granulation marker; CD107**.** One of these studies showed that CMV-specific $CD8^+$ T-cells producing both IFN-γ and IL-2 were present in patients controlling viral reactivation and cells producing only IFN-γ were non-protective T-cells. $CD4^+$ $CD40L^+$ IFN-γ^+ T-cells were the most frequent $CD4^+$ cells in both patients controlling or not controlling CMV reactivation, therefore, the authors did not find a subset representing protective $CD4^+$ T-cells (Stuchly et al, 2011). Lilleri (2009) reported a protective role for IFN-γ^+ IL-2^+ $CD8^+$ T-cell population. These findings are in accordance with results of studies on Human Immunodeficiency Virus (HIV) and Leishmania major vaccine showing importance of multifunctional compare to monofunctional T-cells in the control of infection (Zimmerli et al., 2005; Darrah et al., 2007) . It should be mentioned that most of studies on the quality of CMV-specific T-cells post HSCT have not analysed quantity of these cells and vice versa, so there is still need for detailed studies on large cohort of patients looking for both quality and quantity aspects of CMV-specific T-cells. These studies will be beneficial for clinicians to predict viral reactivation and more importantly decide on management of patients during reconstitution of immunity to CMV.

There are also multiple studies on CMV-specific T-cells post solid organ transplantation. Results of those studies are similar to data obtained from stem cell transplant patients and in addition provide information on immune response in primary CMV infection. Molecular profiling of CMV-specific $CD8^+$ T-cells post primary infection in kidney transplant patients has shown that very early after infection, $CD8^+$ T-cells expressed the T1 related transcription factors T-bet and *EOMES* in parallel with continuous expression of IFNG mRNA and IFN-γ–regulated genes (Hertoghs et al.,

2010). During acute and latency phase, CMV-specific T-cells expressed perforin, granzyme B and CX3CR1 and down regulated IL-7R with no changes in expression of IL2Rα (CD25) but increased IL2Rβ (CD122).

1.6 Adoptive cellular therapy for the prevention and treatment of CMV disease

The vital role of protective CMV-specific T-cells in HSCT has led to several attempts to passively restore CMV cellular immunity by transferring cells to immune compromised patients. Different strategies have been developed to obtain enough number of CMV-specific cells which some of them are presented in Figure 1.10. Riddell and colleagues (1992) pioneered the adoptive cellular therapy for the prevention of CMV disease by cloning CMV-specific $CD8^+$ T-cells derived from bone marrow donors. They transferred $CD8^+$ T-cells to 14 patients in 4 intravenous infusions 30 to 40 days after marrow transplantation and found that infused cells were able to expand in all the patients and provided protection against CMV reactivation and disease without any toxic effects. They also found that CMV-specific $CD4^+$ T-cells were required to maintain long term $CD8^+$ T-cell response (Table 1.2). In other study, K.Peggs and his team (2003) generated autologous CMV-specific T-cells by stimulation with DCs presenting CMV antigens and treated 16 patients with those cells. They observed massive expansion of transferred T-cells and there was no need for antiviral drugs in 8 patients. In another study, 10^7 /m^2 CMV-specific T-cell lines (mainly $CD4^+$ T-cells) were transferred into HSCT patients at a median of 120 days after transplantation (Einsele et al., 2002). In the absence of antiviral drugs, CMV viral load dropped at a

median of 20 days and cell therapy was successful in 5 out of 7 patients. Interestingly, they recorded an increase in CMV-specific $CD4^+$ T-cells in 5 patients in addition to increase in $CD8^+$ T-cells. In 2 out of 7 patients who were under strong immune suppression at the time or after cell infusion, only transient decrease in viral load occurred. In this study, CMV-specific T-cells with broad antigen specificity were produced since the cells had been stimulated with CMV or CMV lysate. In order to reduce the risk of live virus, Micklethwaite and colleagues (2008) used DCs sensitized with HLA- class I restricted epitopes to stimulate T-cells. This method increased the precursor frequency of CMV-specific T-cells but it limited its use only to patients who carry that particular HLA. M. Cobbold and his team (2005) used a completely different strategy to obtain viral specific T-cells. They isolated CMV-specific T-cells from peripheral blood using MHC-peptide multimer complexes and without any *in vitro* manipulation, isolated cells were transferred into patients which resulted in clearance of viraemia in 8 of 9 patients. Some groups have successfully generated and clinically used multi-specific T-cell lines targeting CMV, EBV and adenovirus (Leen et al., 2006).

Table 1.2 lists the published reports and their important findings on adoptive cellular therapy for CMV. In addition to the listed papers, a recent study showed the possibility of generating CMV specific T –cells from the naïve T-cells of CMV seronegative individuals or from cord blood. These cells were able to protect 2 out of 3 patients received cord blood transplants from CMV reactivation and helped the third patients in the control of reactivation (Hanley et al., 2015). Taken together, these results demonstrate the ability of T-cell therapy in restoring CMV-specific immunity without significant toxicity and with a low risk of GVHD induction.

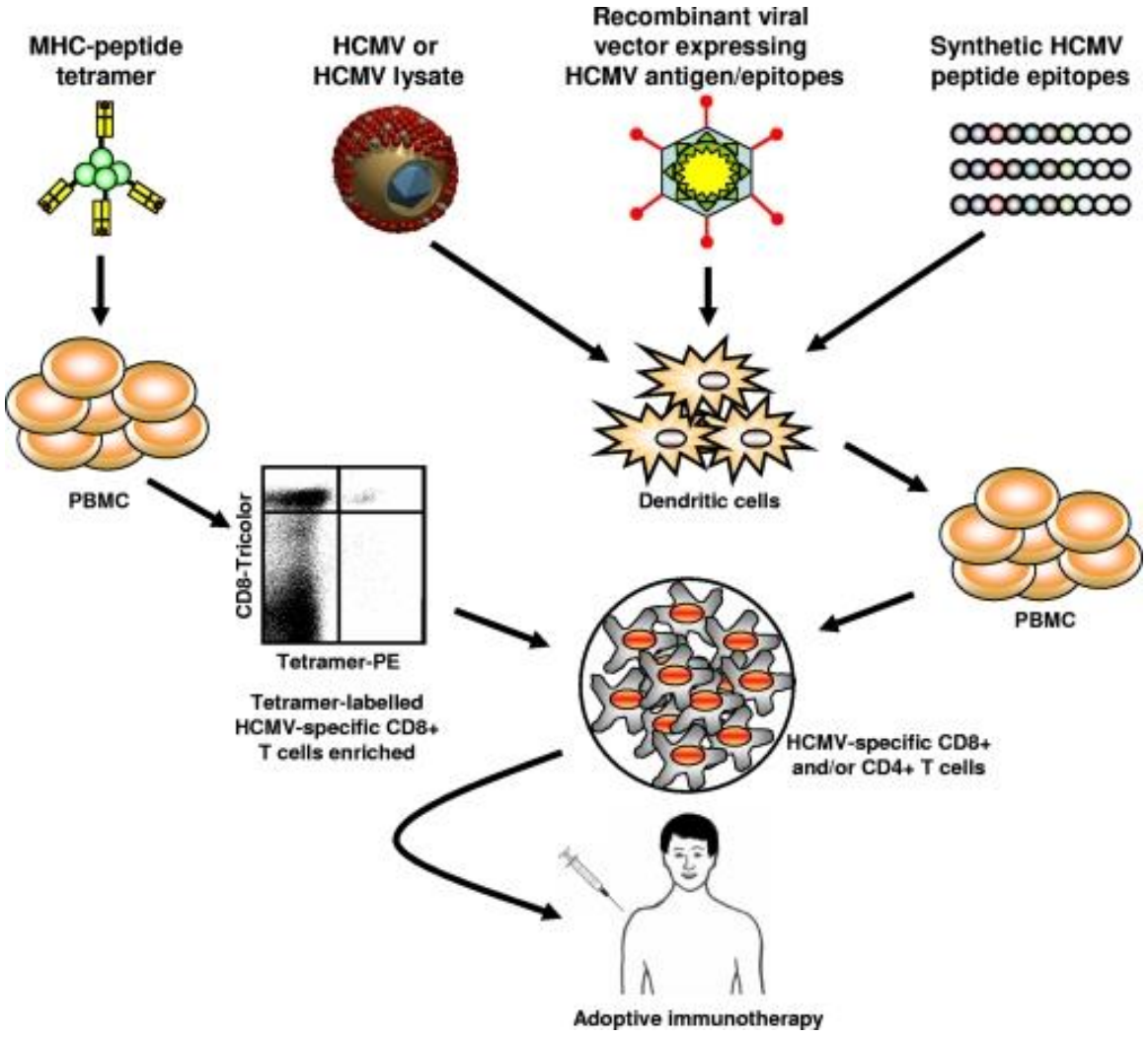


Figure 1.10 Strategies for adoptive cellular therapy of CMV-specific T-cells.

Different methods have been explored to obtain enough number of CMV-specific T-cells which includes enrichment of CMV-specific T-cells using MHC-peptide multimer/tetramer and *in vitro* T-cell stimulation with DCs sensitized with a range of CMV derived antigens. (Crough and Khanna, 2009)

Table 1.2 Summary of published reports on adoptive cellular therapy of CMV-specific T-cells post-HSCT.

Study / Year	Number of patients	Pre-emptive therapy or Prophylaxis	T-cell source / Antigenic Stim.	Number of Infused cells / Days post HSCT	Method of Immune-monitoring	Peak CMV-CD4/ CD8 counts post cell infusion	Results
Walter, 1995	14	Prophylaxis	Allogeneic (donor-derived) CD8 T clones stimulated with CMV infected fibroblasts	Cells given in 4 weeks as $33,100,330,1000x10^3/m^2$ first infusion at day: 0	Cytotoxic activity of cells against CMV	NA	Reconstitution of CMV immunity in all patients
Einsele, 2002	8	Pre-emptive therapy	Allogeneic CTL stimulated with CMV lystae	$10x10^7/m^2$ median infusion day: 120	Analysis of IFN-γ response or multimer staining	CD4: $7.9x10^3$ /ml CD8: $42X10^3$ /ml	Clearance of infection in 5/7 evaluable patients
Peggs, 2003	16	Pre-emptive therapy	Allogeneic CMV-CTL	$1x10^5$/kg, median infusion day: 36	Multimer staining	CD4:NA CD8: $570X10^3$/ml	Reconstitution of viral immunity
Cobbold 2005	9	Pre-emptive therapy	Allogeneic CMV tetramer selected T-cells	$8.6X10^3$/kg	Multimer staining	CD4:NA CD8: $156x10^3$ /ml	8/9 cleared infection
Mickleth -waite, 2008	12	Prophylaxis	Allogeneic CMV-CTL generated by stimulation of DC with pp65 vector	$2X10^7/m^2$ infusion at day: 56	Analysis of IFN-γ response, Multimer staining	CD4:NA CD8: $280x10^3$ /ml	Increase in NLV-tetramer binding T cells in 6 recipients, reduced antiviral therapy
Peggs, 2009	30	Pre-emptive therapy & Prophylaxis	T- cell lines generated by CMV lystae stimulation	$1x10^5$/Kg cultured cells(78% CD4, 28% CD8) Infusion at day: 28	Analysis of IFN-γ response and multimer staining	CD4:NA CD8: $110x10^3$/ml	CMV-specific T cell therapy effectively prevents the requirement for recurrent treatment and the occurrence of late CMV disease

Feuchtin-ger 2010	18	Pre-emptive therapy	IFN-γ capture selection of pp65 specific cells	$50x10^3$/kg T-cells : $21x10^3$/kg CD4 and $6.7x10^3$/kg CD8 Infusion at day: 118	IFN-γ response to pp65	NA	Clearance or > 1 log reduction in viral load in 15 patients
Schmitt, 2011	2	Therapy/ Recurrent viraemia	Streptam-er selection	$2.2x10^5$/kg isolated T-cells with purity of 97%	Multimer staining	CD4:NA CD8: $400x10^3$ /ml	Discontinued antiviral in recurrent reactivations without further high titre viraemia
Peggs, 2011	18	Therapy (11 patient) Prophylax.-is (7 patients)	IFN-γ capture selection of pp65 specific T-cells	$10x10^3$/kg T-cells: $2.8x10^3$/kg CD4,$0.63x10^3$/kg CD8 infusion: d42	IFN-γ response to pp65	CD4: 5x 10^3 /ml CD8: 17 $x10^3$ /ml	No patient treated prophylactica-lly required antivirals; 2 patients treated pre-emptively required no antivirals and 7 had a single episode
Blyth, 2013	50	Prophylax-is	T-cell lines after 21 days stimulati-on by dendritic cells loaded with CMV antigens	$2x10^7/m^2$ CTL including : mean CD4:26% mean CD8:68% Infusion: day 45	IFN-γ response to pp65	NA	There was no significant difference in the incidences of CMV viraemia, but the CMV peak titre, the number of patients needed anti-CMV drugs ,the number of treatment days per patient and the number of patients with late CMV viraemia were significantly lower in ACT patients
Koehne/ 2015	17	Pre-emptive therapy	p65 stimulated allogeneic T-cells	$0.5\text{-}2x10^6$/kg including mainly CD8 and some CD4 T-cells	-	-	15 cleared viraemia including 3 out of 5 with overt disease

Project aims

This thesis studied the reconstitution of CMV-specific T-cell responses following HSCT and investigated cytokine receptor expression in these cells. The reconstitution of T-cell immunity against CMV viraemia was studied in two HSCT patient cohorts: a) patients enrolled in a phase II randomised controlled study of adoptive immunotherapy for CMV reactivation using ex-vivo HLA-multimer selected donor CMV-specific $CD8^+$ T-cells, and b) non-trial CMV sero-positive HLA-DRB1*0701 HSCT patients.

The objectives of the clinical trial study were: i) to analyse the reconstitution of CMV-specific $CD8^+$ T cells post immunotherapy and compare with the reconstitution of these cells in patients who did not receive cell therapy (control group). ii) to evaluate the trial end point achievement which defined as reconstitution of $10x10^4$/ml CMV-specific $CD8^+$ T-cells within a period of 2 months post-cellular therapy and iii) to study the reconstitution of non-infused CMV-specific $CD8^+$ and $CD4^+$ T-cells in patients who received cell therapy in comparison with the control group.

In the second cohort of HSCT patients, the objective was to explore CMV-specific $CD4^+$ T-cell reconstitution following HSCT. For the first time, a novel HLA-class II tetramer was used i) to analyse the kinetics of CMV-specific $CD4^+$ T-cell reconstitution and their phenotypes in relation to CMV-specific $CD8^+$ T-cell reconstitution, and ii) to assess the clinical utility of immune monitoring using both HLA-class I and II multimers for risk stratification of patients. A further objective of the study was to introduce a novel putative HLA-independent marker for monitoring CD4 immunity against CMV.

In the final part of this thesis, the expression pattern of receptors to the cytokines involved in the cell expansion following HSCT was studied in normal healthy

individuals and HSCT patients. Because of the unusual pattern of expression, TNF receptor was studied in CMV-specific T cells to explore the functional activity of this receptor and its potential role in the contraction of expanded CMV-specific T-cells.

Chapter 2

MATERIALS AND METHODS

2.1 Study populations

2.1.1 ACE-ASPECT trial patients

The clinical trial ACE-ASPECT (Adoptive CMV immunotherapy-Alternate Donor Study of Pre-Emptive Cellular Therapy), sponsored by Cell Medica Ltd with ClinicalTrials.gov Identifier NCT01220895, was a multi-centre phase II randomised controlled trial. The trial had two arms with a randomisation ratio of 2:1 of CMV-specific $CD8^+$ T-cell therapy with best available therapy (BAT) versus BAT only. Nine centres recruited patients to the trial (University College London Hospital, Queen Elizabeth Hospital, Birmingham, Churchill Hospital, Oxford, City Hospital, Nottingham, Manchester Royal Infirmary and the Christie Hospital, Manchester, King's College Hospital, London, Royal Free Hospital, London and University Hospitals Bristol NHS Trust).

The CMV-specific $CD8^+$ T-cells used for ACT were manufactured under the sponsorship of Cell Medica by the GMP facilities of the Royal Free Hospital, London and NHS Blood and Transplant, Birmingham.

Eligibility

The trial was designed for patients undergoing allogeneic stem cell transplant who were CMV sero-positive and who received HSCT from HLA-matched CMV sero-positive unrelated donors. Patients were 16 years or older and were T-cell depleted in vivo with Alemtuzumab incorporated into the conditioning regimen. All patients and donors consented to the trial. Patients had also given consent to additional study procedures as per study schedule. Donors consented to the use of their harvested stem cells that were in excess to what was required for transplantation for selection of CMV-specific T cells. Other inclusion criteria included donor engraftment (neutrophils $>0.5x10^9$/ml) prior to

the cell therapy. Recipients and donors must had to have negative serology for Human immunodeficiency virus (HIV), Hepatitis B and C, HTLV 1 and 2, and syphilis. Patients and transplant donors shared one or more of the following HLA-class I alleles: A^*0101, A^*0201, A^*2402, B^*0702 and B^*0801. The following patients were excluded: pregnant or lactating women, co-existing medical problems that would place the patient at significant risk of death due to GVHD or its sequela, active GVHD > grade I prior to CMV-specific T-cell infusion, concurrent use of systemic corticosteroids prior to CMV-specific T- cell infusion, organ dysfunction to (prior to cell infusion) as measured by: creatinine > 200 μmol/l, bilirubin > 50 μmol/l alanine transferase > 3x upper limit of normal, and donors with less than $50x10^9$/L platelets.

All patients received Alemtuzumab (Campath-1H) for in vivo T-cell depletion, a humanized IgG1 anti CD52 monoclonal antibody that targets lymphocytes and other immune cells. Therapeutic CMV-specific $CD8^+$ T- cells were infused at the time of CMV reactivation but beyond 28 days post HSCT, to minimise the anti-lymphocyte activity of Alemtuzumab which has been shown to remain in the recipient's circulation for at least 28 days post transplant (Morris et al., 2003).

2.1.2 Patients studied for the characterization of CMV-specific $CD4^+$ T-cell reconstitution post HSCT through the use of HLA-class II tetramers

Detailed descriptions of 20 CMV sero-positive HLA-DRB1*0701 HSCT patients participated in this study are documented in chapter 4. This chapter also contains demographics data on studied patients.

2.1.3 Healthy blood donors and patients analysed for cytokine receptor expression in T-cell subsets

In order to study the normal pattern of cytokine receptor expression in the peripheral blood T-cell subsets and CMV-specific cells, 10 ml heparinised bloods from 27 healthy platelet donors (mean age +/- SD: 43 +/- 11.9) were studied following peripheral blood mononuclear cells (PBMC) isolation by Ficoll density gradient centrifugation. Freshly isolated cells were used to study cytokine receptor expression in T-cell subsets and analyse the effect of TNF-receptor ligation. In addition, fresh or frozen PBMC from 5 non-clinical trial HSCT patients were used to study TNFR in CMV-specific T-cells. The patients were under care of transplant unit of Haematology department, Queen Elizabeth Hospital, Birmingham and all the patients and healthy blood donors had agreed to participate in the study and signed the consent form.

2.2 Methods used for immune monitoring of ACE-ASPECT trial patients

2.2.1 Peripheral blood processing

For immune monitoring of CMV-specific immunity after CMV reactivation, peripheral blood samples were obtained from 13 patient visits. The first peripheral blood sample was taken before the cellular therapy or at the beginning of CMV reactivation in the control patients. This was followed by weekly peripheral blood collection for 8 weeks and then monthly for 4 months. In total, 13 longitudinal blood sample collections were planned for each patient. The blood samples included 16-20 ml blood in heparin and 4-5

ml in EDTA tubes. Royal Mail's first class delivery service was used for the posting of blood samples which in most cases, reached to the centralised research laboratory (Stem Cell and Immunotherapy, NHSBT Birmingham) for processing and immune monitoring within 24 hrs of venesection.

Heparinised whole blood samples were first centrifuged at 2000 rpm (MSE-Harrier 18/80R) for 5 min. in order to collect up to 3 ml plasma which was frozen immediately at -20 C. The remaining blood was resuspended and mixed at ratio of 1:1 in CliniMACS EDTA/PBS buffer (Miltenyi Biotec Ltd) containing 0.5% human serum albumin (HAS). This mixture was carefully overlaid onto Lymphoprep (Axis-Shield) at a ratio of 2:1 in 50 ml sterile Falcon® tubes and centrifuged at 2000 rpm for 20 min at room temperature. Following centrifugation, the mononuclear layer was collected with a Pasteur pipette and transferred into a fresh 50 ml Falcon® tube and the volume topped-up to 50 ml with 0.5% HAS-CliniMACS buffer and centrifuged at 1500 rpm for 10 min (RT) in order to remove any residual Lymphoprep. The resultant PBMC pellet was re-suspended in 30 ml 0.5% HAS-CliniMACS and centrifuged at 1200 rpm for another 5 min. Following the final wash, the PBMC pellet was re-suspended in 1 ml 0.5% HAS-CliniMACS buffer. Afterward, the cells were counted using a ABX Pentra (Horiba Medical) cell counter. The cells were then placed on ice for subsequent staining. To determine the total whole blood count, blood from the EDTA tube was diluted 1:10 with PBS and analysed with the ABX Pentra cell counter.

2.2.2 Enumeration of CMV-specific $CD8^+$ T-cells

In order to quantify *ex vivo* CMV-specific $CD8^+$ T-cells in the peripheral blood, a dual platform flow cytometry assay was used. In the first staining platform, whole blood

was transferred into a Trucount tube (BD Biosciences) in order to enumerate the absolute $CD3^+$, $CD4^+$ and $CD8^+$ T-cell numbers, and for the second platform, ficoll gradient-separated PBMCs were stained with PE-conjugated Streptamers® (IBA, GmbH) and anti-CD3 and CD8 fluorochrome-conjugated antibodies in order to determine the frequency of CMV-specific $CD8^+$ T-cells. The number of CMV-specific $CD8^+$ T-cells was then calculated by multiplying the frequency of these cells with the absolute count of $CD8^+$ T-cells.

2.2.2A Determination of T-cell counts using Trucount tube

The number of T-cells was determined by using Trucount tubes. 100 µl of blood from the EDTA tube was taken by reverse pipetting and transferred into a Trucount tube. Next, the following antibodies were added at 5 µl each: anti-CD45-FITC, anti-CD3-APCH7, anti-CD8-APC and anti-CD4-PECy7 (all from BD Biosciences) (Table 2.1). To exclude dead cells, 5 µl of 7-AAD was also added. This mixture was then incubated for 30 min at RT in the dark. This was a no wash staining method and prior to FACS analysis, RBCs were lysed by 10 min incubation with 1 ml of diluted Pharmlyse (1 to 10 dilution with DDW) (BD Biosciences). The stained cells were analysed by FACS-Canto II (BD) within 45 min.

2.2.2B Detection of CMV-specific $CD8^+$ T-cells

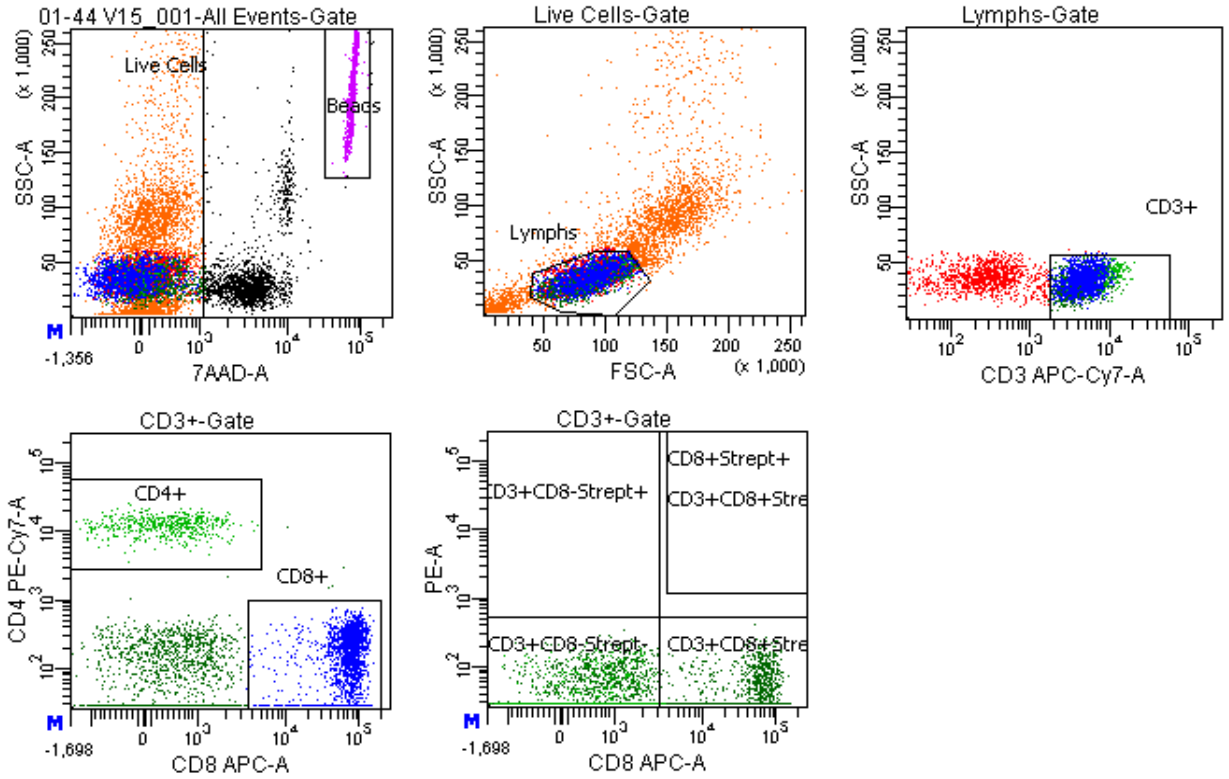
CMV-specific $CD8^+$ T-cells were identified using HLA-class I restricted Streptamers® (Table 2.2). This was a 2 step procedure and the manufacturer's recommendation was followed. In the first step, 4 µl HLA-class I Streptamer® was mixed with 5 µl Streptactin PE (IBA, GmbH) and 41 ml cold 0.5% HAS–CliniMACS buffer. This

mixture incubated 45 min on ice in the dark. Following the incubation, PE-conjugated Streptamer® was added to up to $5x10^6$/ml PBMCs (kept already on ice) and incubated for 45 min on ice in the dark. It was followed by 2 cell washes with 2 ml 0.5% HAS-CliniMACS buffer (1500 rpm, 5min). Then, cells were stained with the same antibodies used for the enumeration of T-cell counts in Trucount tube. SOP (standard operating procedure) 2144/3.1, prepared in NHS Blood and Transplant, Birmingham, was the SOP for the analysis of CMV-specific $CD8^+$ T-cells.

2.2.2C Flow cytometry analysis of T-cell counts and gating strategy

For determination of blue and red lasers voltages and compensation values, first a compensation set up was established by using PBMCs. BD CompBeads were not used since the flow cytometry panel included 7-AAD. Therefore, cells were used and to obtain dead cells (7-AAD positive), cells were killed by ethanol. Since Trucount tubes contained very small beads labelled with fluorochromes, it was necessary to set a threshold on one of fluorescent channels instead of forward scatter (FSC) channel. In this regard, threshold of 1200 FITC was chosen for acquiring stained cells. The gating steps included: plotting 7-AAD versus side scatter (SSC) in order to exclude dead cells, then plotted FSC versus SSC for lymphocyte gating which then followed by plotting CD3-APCH7 against SSC which allowed detection of $CD3^+$ T-cells and then by selecting this population, a plot for CD4-PECy7 versus CD8-APC was made in order to identify $CD4^+$ and $CD8^+$ populations (Figure 2.1A). To detect the population of counting beads, APC or APCH7 against SSC channel was plotted and a very bright population of events with high FCS and SSC classified as beads. The number of events in the beads population was used in the following equation to obtain cell counts:

CD8^{+}cell number/µl= number of cell events X beads count/ number of beads event X test volume(100µl)

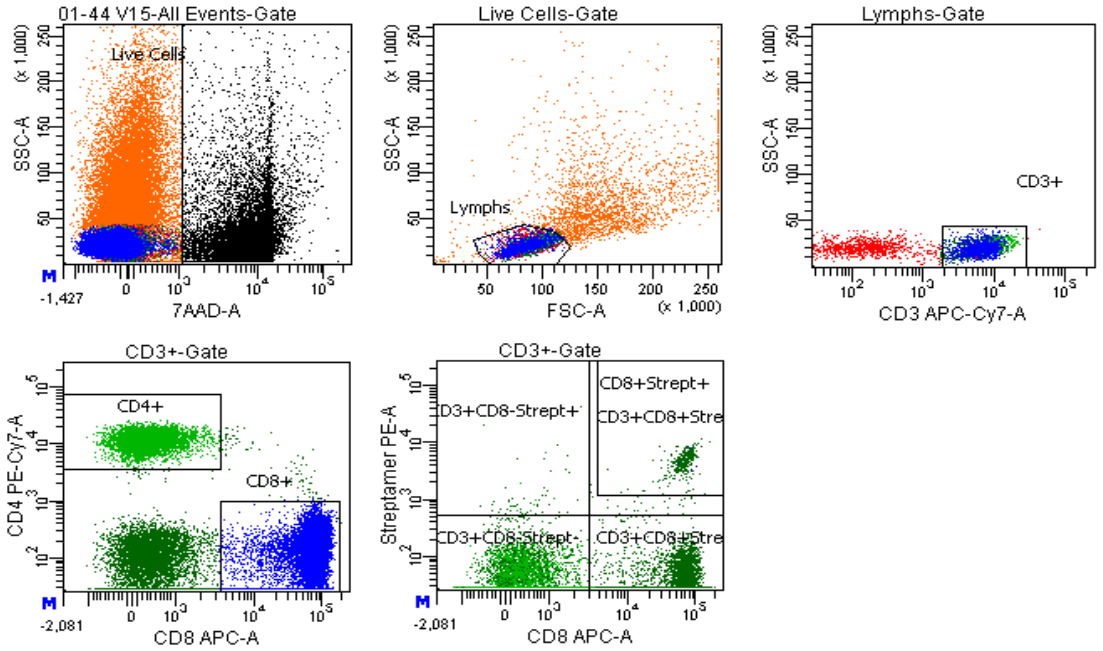


Specimen Name: 01-44 V15_001
Tube Name: 2_001
Record Date: Jun 25, 2013 4:09:37 PM

Population	#Events	%Parent	%Grand Parent
Lymphs	####	57.09	40.91
CD3+	####	51.99	29.68
CD4+	2,466	11.59	6.03
CD8+	11,279	53.03	27.57
CD8+Strept+	0	0.00	0.00
CD3+CD8-Strept+	0	0.00	0.00
CD3+CD8+Strept+	0	0.00	0.00
CD3+CD8-Strept-	9,884	46.47	24.16
CD3+CD8+Strept-	11,385	53.53	27.83
Beads	10,768	10.77	####

Figure 2.1A Flow cytometry gating strategy for identification of T-cells and counting beads.

In the first staining platform, whole blood cells were stained in BD-Trucount tubes in order to calculate the number of $CD8^+$ T-cells in the peripheral blood. Gating strategy included identification of the beads region, viable lymphocytes and their T-cell subsets. This tube did not include Streptamers®.



Specimen Name: 01-44 V15
Tube Name: QIK
Record Date: Jun 25, 2013 4:18:22 PM

Population	#Events	%Parent	%Grand Parent
Lymphs	####	60.65	48.53
CD3+	####	41.93	25.43
CD4+	26,770	16.80	7.05
CD8+	94,236	59.15	24.80
CD8+Strept+	720	0.76	0.45
CD3+CD8-Strept+	99	0.06	0.03
CD3+CD8+Strept+	780	0.49	0.21
CD3+CD8-Strept-	64,309	40.36	16.92
CD3+CD8+Strept-	94,139	59.09	24.77

Figure 2.1B Flow cytometry gating strategy for identification of T-cells and counting beads.

In the second platform, Streptamer® staining was performed to obtain the frequency of viable CMV-specific $CD3^+$ $CD8^+$ T-cells. This staining tube did not include the counting beads, but the FACS voltages and compensation settings were the same as the first staining tube.

In order to have reliable results, at least 100,000 events were collected in Trucount tubes and if the patient was lymphopenic, all of the lysed blood sample was acquired. Due to very low lymphocyte count in some cases, up to 400 µl blood (instead of routine 100 µl blood) was added to Trucount tubes and the volume of Pharmlyse adjusted accordingly. The flow cytometry gating strategy was the same for both platforms (tubes) with the exception that the second tube with Streptamer® had no counting beads and cells stained with Streptamer® were gated out from $CD8^+$ T-cells to obtain their frequency (Figure 2.1B) and consequently their number using the following formula:

CMV-specific $CD8^+$ T-cell number/ µl = percent of CMV-specific cells in $CD8^+$ population X $CD8^+$ T-cell number in 1µl /100

The detection limit of CMV-specific T-cells was set up as 0.10% of $CD8^+$ T-cells following a series of preliminary experiments and previous findings (Yao et al., 2008). This was due to non-specific staining of cells by Streptamer® in some samples which could be up to 0.07% of $CD8^+$ T-cells. In addition, only clustered and not scattered dots were accepted as positive Streptamer®-PE stained cells.

2.2.3 Functional behaviour of CMV-specific T-cells in ACE-ASPECT trial patients

2.2.3A Phenotypic analysis of CMV-specific $CD8^+$ T-cells

Phenotypic analysis of CMV-specific $CD8^+$ T-cells was carried out in some patients who were not as lymphopenic and from whom we had extra cells for this study. Following Streptamer® staining, PBMCs were stained with anti-CCR7-FITC (10 µl) (R&D Systems) and 5 µl of the following antibodies: anti-CD45RA-PECy7 (BD Biosciences), anti-CD57-APC (Biolegend), anti-CD8-APCeflour 870 (eBiosciences) and 7-AAD. The incubation time was 30 min at 4C which was followed by one cell wash with PBS.

2.2.3B Functional analysis of CMV-specific $CD8^+$ T-cells through flow cytometry

Functional behaviour of CMV-specific $CD8^+$ T-cells was studied using intracellular cytokine staining method (Lachmann et al, 2012). Freshly isolated PBMCs were suspended in RPMI-1640 with 10% heat inactivated human AB serum (Biosera) at density of $0.5x10^6$ cells/0.5 ml in sterile capped 12x75 mm propylene tubes (Table 2.4). Cells were stimulated with 1 µg/ml of viral peptides (Table 2.5) or stimulated with peptide dissolvent as a negative control in the presence of 5 µg/ml anti-CD28/CD49d antibody (BD Biosciences) as a co-stimulatory molecule. PBMCs stimulated with SEB (Sigma) (0.5 µg/ml) served as positive control for this assay. Following one hour incubation of cells in a humidified incubator at 37C with 5% CO_2, freshly thawed Brefeldin A (Sigma) at concentration of 5 µg/ml was added to the cell culture and cells were incubated for an extra 14 hours at 37C, 5% CO_2. After the incubation period, tubes were vortexed to mix, centrifuged at 1500 rpm for 5 min at RT and the cell pellet re-

suspended in 1 ml PBS before cell wash (1500 rpm, 5min). The supernatant was completely discarded and 100 µl fixation solution (Caltag A medium, Invitrogen) was added. This mixture was incubated for 20 min at RT in the dark before addition of PBS for cell wash. After that, the supernatant was completely removed and 80 µl permeabilization solution (Caltag B medium) was added. Then the following antibodies for cell surface and intracellular staining were added at 5 µl: anti-CD8-APC-efluore780 (eBiosciences), anti-CD3-PE (R&D systems), anti-TNFα-PECy7, anti-IL2-FITC, 7-AAD and anti-INFγ-APC (2 µl) (all from BD Biosciences). Stained cells were then incubated for 25 min at RT (protected from light) and after that, washed as before and analysed by flow cytometry (FACSCanto-II) immediately after final wash. The details of antibodies and isotype controls are listed in Table 2.1 and Table 2.3.

2.2.4 Reconstitution of CMV-specific $CD4^+$ T-cells in ACE-ASPECT trial patients

Due to lack of relevant HLA-class II specific Streptamers® or tetramers, CMV-specific $CD4^+$ T-cells were detected using intracellular cytokine staining after cell stimulation with CMV lysate (provided by Professor Moss group). PBMCs at concentration of $0.5x10^6$/ml in RPMI plus 10% heat inactivated human AB serum were stimulated with 5 µl of CMV lysate and anti-CD28/CD49d antibody (5 µg/ml) (BD Biosciences) for 1 hour at 37C in 5% CO_2 incubator before adding Brefeldin A. After further 14 hours incubation, the procedure mentioned in the section 2.2.3B was followed with the exception of adding anti-CD4-APCefluor780 (5 µl) (eBiosciences) instead of adding anti-CD8 antibody.

2.2.4A Flow cytometry analysis of CMV-specific T-cell phenotype and function

Different compensation set ups were prepared for FACS analysis of different staining panels. Flow cytometry threshold was set as FSC 20000. For phenotypic analysis, up to 200,000 lymphocytes were acquired and then a plot for CCR7-FITC versus CD45RA-PECy7 was prepared. For functional studies, $CD3^+$ T-cells were gated out from the lymphocyte population and then, $CD4^+$ or $CD8^+$ T-cells were identified. To obtain final frequencies of cytokine producing cells, background subtraction was performed by deducting frequency of cytokine producing cells in unstimulated PBMC from cells producing the same cytokine after antigenic stimulation. For determination of cells producing different combination of cytokines, the Boolean gating strategy was adopted. In all cases, IFN-γ producing cells had the highest frequencies. Therefore, $CD4^+$ or $CD8^+$ T-cells producing IFN-γ were first identified and amongst them, cells producing either IL-2, TNF-α or both were then determined. This method measured the frequencies of different expression profiles corresponding to single, double and triple cytokine producing cells.

In all the flow cytometry assays, FACS-Diva software version 6.3 (BD) was used for cell acquisition and data analysis.

2.2.5 IFN-γ Elispot for analysis of CMV-specific T-cell function

Functional activity of CMV-specific T-cells was analysed by Elispot method as well. In the first day of experiment, PVDF membrane in non-sterile 96 wells plate (MAIPN45) (Milllipore) was activated by adding 15 µl 35% Ethanol in distilled water for maximum 1 min. During this step, extra care was carried in order to cover all membrane area with Ethanol. After removing Ethanol by flicking out, the plate was washed 5 times with 200 µl distilled water. It was followed by adding 50 µl anti-IFN-γ (1-D1K) (Mabtech) at 15

µg/ml (67 fold dilution) in PBS in order to coat the plate. Following overnight incubation at 4C, the plate was washed 5 times with 200 µl sterile PBS and blocked with 50 µl RPMI with 10% human serum for at least one hour at 37C. Then, the blocking solution was removed and without any wash, cell suspension at concentration of $2.5x10^5$ in 100 µl RPMI+10% human AB serum was added. PBMCs were stimulated with two CMV peptide pools; CMV pp65 and CMV IE-1 PepTivator Peptide Pool (Miltenyi Biotec). These peptide pools contained 15-mer peptides with 11 amino acid overlap. Manufacturer's recommended working concentration of these antigens was used which gave a final concentration of 1µg/ml for each peptide in the pool. In addition, PBMCs were stimulated with individual CMV derived peptides (Proimmune) (1 µg/ml) based on the HLA type of patients (Table 2.5). As positive control, cells were incubated with SEB (0.5 µg/ml) and all stimulations were performed in triplicate. In order to obtain background response, cells were stimulated with the peptide diluents and the plate was incubated overnight (37C, 5% CO_2). Following incubation, cells were removed and Elispot plate was washed 5 times with 200 µl PBS. It was followed by adding 50 µl of the secondary anti-IFN-γ antibody conjugated with biotin (7-B6-1) (Mabtech). The final concentration of this antibody was 1 µg/ml and prepared by 1000 fold dilution of original concentration in PBS+0.5% FCS. The incubation time was 2 hrs at RT which was followed by 5 times wash with 200 µl PBS. Then 50 µl Streptavidin-Alkaline Phosphatase (diluted 1:1000 in PBS with 0.5%FCS) was added for 1 hr at RT. After 4 times wash with PBS and once with distilled water, 50 µl BIO-RAD detection solution was added. For 5 ml of this solution, 200 µl AP buffer (BIORAD), 50 µl BIORAD-reagent A and 50 µl reagent B were added to 4.7 ml distilled water. This was incubated at RT until enzymatic reaction developed in the format of visible spots (30 to

60 min). When spots became visible, the front and back of plate were thoroughly washed with tap water and left at RT to be air dried. The number of spots in each well was enumerated using the AID Elispot- reader and mean numbers of dots (in triplicate wells) were reported following deduction of background dots in negative control wells.

2.3 Methods used for the analysis of CMV-specific $CD4^+$ T-cell reconstitution after HSCT by HLA-class II restricted tetramers

Characterisation of reconstituting CMV-specific $CD4^+$ T-cells in non-clinical trial HSCT patients was conducted through usage of a novel HLA-class II tetramer. The HLA-class II tetramer was consist of HLA-DRB1*0701 and CMV glycoprotein B-derived peptide DYSNTHSTRYV (DYS) and custom made by Benaroya Research Institute, WA. For detection of CMV-specific $CD8^+$ T-cells, Streptamers® were used (Table 2.1). To obtain frequencies of CMV-specific T-cells, the second flow cytometry panel described in section 2.2 was used which contained anti-CD45-FITC, anti-CD3-APCH7, anti-CD8-APC and anti-CD4-PECy7 and 7-AAD (all at 5 µl each) (Table 2.1). Total lymphocyte count reported in routine monitoring of haematological parameters (Clinical Haematology Service, Queen Elizabeth Hospital) was considered for enumeration of total and CMV-specific T-cells. This method may underestimate the actual number of cells since some of them die during PBMC isolation or cell freezing process. In this study, different flow cytometry panels were designed in order to characterise the phenotype of T-cells which included T-cell subset phenotypes, cytokine receptor expression and staining for intracellular perforin, granzyme B and T-bet. In order to detect intracellular expression of above mentioned molecules, cells were first

stained with multimers as described before and then fixed with Caltag A reagent (100 µl/RT/20 min), washed with PBS and 80 µl Caltag B reagent was added with antibodies against perforin, granzyme B and T-bet for 20 min at RT and in dark. Stained cells were analysed immediately.

Tables 2.1 and 2.3 list the detail of antibodies and isotype controls used in this study. In all flow cytometry analysis, appropriate isotype controls with the same concentrations as the main antibodies were used to discriminate positive from negative population.

2.4 Methods used for analysis of cytokine receptor expression in T-cell subsets and effects of TNFR2 on differentiated T-cells

Analysis of cytokine receptor expression and effects of TNFR2 ligation was performed on healthy blood donors and HSCT patients. In order to study the results of TNFR2 ligation in recently activated CMV-specific T-cells, PBMCs from HSCT patients were used.

Detection of cytokine receptor expressing cells was dependent on using isotype controls in order to distinguish between negative and positive cells. Detailed information on anti-cytokine receptor antibodies and their isotype controls are presented in Tables 2.6 and 2.7. In addition, the following antibodies were used: anti-CD4 and anti-CD8-APC-eFlour 780 (e-Biosciences, UK), anti-CCR7- FITC (R&D Systems, UK), anti CD45RA-PE-Cy7, anti CD3-Percp, and 7-AAD (all from BD Biosciences, UK), anti CD57-APC (Biolegend, San Diego). CMV specific $CD8^+$ T-cells were identified by using HLA-class I Streptamers®.

CMV-specific $CD4^+$ T-cells were identified through usage of HLA-DRB1*0701 restricted DYS tetramer.

2.4.1 Flow cytometry analysis

Following blocking of immunoglobulin receptors with 1ul human IgG, PBMCs in phosphate buffered saline (PBS) were incubated with 10ul of PE conjugated anti cytokine receptor antibodies for 35 min at RT. After two washes, cells were incubated for 30 min in RT with the rest of antibodies including anti-CCR7, CD45RA, either CD4 or CD8, CD3 and CD57 molecules. After final wash, cells were immediately acquired by FACS-Canto II flow cytometry (BD) and data analysed with FACS-Diva software (version 6.2, BD). In order to discriminate cytokine receptor positive cells from negative population, isotype control was used at similar concentration to anti cytokine receptor antibodies.

2.4.2 Detection of TNFR2 expression in CMV-specific $CD8^+$ T-cells

Streptamers® were incubated with up to $1x10^6$/ml PBMC for 45 min on ice. Following two washes, 3 ul of anti TNFR2–biotin (BD Pharmingen) was added to the cells (30 min at 4°C) and after wash, cells were incubated with APC-Streptavidin (BD Pharmingen) for 15 min at 4°C, washed and stained with anti-CD3 and CD8 antibodies.

2.4.3 Cell incubation with TNFR2 ligand

PBMC at concentration of $1x10^5$/ml in 200ul of RPMI with 10% FCS were incubated with 50,100 and 200 ng/ml of TNFR2 ligand (Flag-TNC-scTNF (143N/145R)) (a courtesy gift from Professor Harald Wajant, Division of Molecular Internal Medicine, Würzburg University Clinics, Wuerzburg, Germany) for 3 days. Following incubation

period, cells were washed, re-suspended in100ul of PBS and transferred to the Trucount tubes (BD). Then, anti CD3, CD4, CD8 antibodies and 7-AAD were added in order to quantify the number of viable T cells. For the measurement of CMV-specific T cells, incubated cells were first stained with the Streptamer® as mentioned before and then transferred to the Trucount tubes. The cell numbers after incubation with the ligand were compared with the number of un-stimulated cells (without ligand) in order to detect any change in the cell number. For the detection of apoptotic cells after incubation with TNFR2 ligand, Annexin V-FITC kit (Trevigen, Gaithersburg, MD) was used to discriminate apoptotic from necrotic $CD8^+$ T-cells. For this experiment, $CD8^+$ T-cells were isolated from PBMCs of a healthy blood donor by using $CD8^+$ isolation kit (Miltenyi Biotec). Then, cells were labelled with anti-TNFR2-PE and TNFR2 positive cells were separated from TNFR2 negative $CD8^+$ T-cells through usage of anti-PE MicroBeads (Miltenyi Biotec). Following manufacturing recommendations, cells from positive ($TNFR2^+$) and negative ($TNF2^-$) were collected and cultured at $1x10^5$ cells/100µl RPMI with 10%FCS. Flag-TNC-scTNF (143N/145R) at concentrations of 50, 100 and 200ng/ml were incubated with the isolated cells for 21 hrs at 37C, 5% Co2. This was followed by cell staining with Annexin V-FITC kit and analysis of stained cells by flow cytometry method.

2.5 Statistical analysis

Non-parametrical Mann-Whitney test and parametrical t-test were used for statistical comparison between two groups of data. For correlation analysis, non-parametrical Spearman's rank coefficient correlation test was used. All statistical analysis and graph preparations were made through GraphPad Prism software version 5 (La Jolla, USA).

Table2.1 List of antibodies used in detection and phenotypic analysis of CMV-specific $CD4^+$ and $CD8^+$ T-cells.

Antibody	Fluorophore	Source	Catalogue number	Isotype*	Volume (µl)
CD3	APC-H7	BD Biosciences	641397	IgG1,κ	5
CD4	PE-Cy7	BD Pharmingen	557852	IgG1, κ	5
CD8	APC	BD Pharmingen	555369	IgG1, κ	5
CD45	FITC	BD Biosciences	345808	IgG1, κ	5
CD45RA	PE-Cy7	BD Pharmingen	560675	IgG2b, κ	5
CCR7	FITC	R&D Systems	FAB197F	IgG2a	10
CD8	APC-eFluor 780	eBiosciences	47-0087-42	IgG1, κ	5
CD4	APC-eFluor 780	eBiosciences	47-0049-42	IgG1, κ	5
CD57	APC	Biolegend	322314	IgM	7
Perforin	FITC	eBiosciences	11-9994-42	IgG2b, κ	5
Granzyme b	FITC	BD Biosciences	560211	IgG1,κ	20
T-bet	PerCP-Cy5.5	BD Biosciences	561316	IgG1, κ	5
CD25	PE-Cy7	BD Biosciences	557741	IgG1, κ	5
CD127	FITC	eBiosciences	11-1278-42	IgG1, κ	5
IL-2	FITC	BD Biosciences	340448	IgG1, κ	20
IFN-γ	APC	BD Biosciences	554702	IgG1, κ	3
TNF-α	PE-Cy7	BD Biosciences	557647	IgG1, κ	5
CD3	PE	R&D systems	FAB100P	IgG1	5

*All the monoclonal antibodies are mouse immunoglobulin

Table 2.2 List of MHC class I Streptamers® (IBA, BioTagnology GmbH).

Streptamer	Catalogue number
MHC I-Strep HLA-A*0101; CMV pp50; VTEHDTLLY	6-7024-005
MHC I Strep HLA-A*0201; CMV pp65; NLVPMVATV	6-7001-005
MHC I Strep HLA-A*0201; CMV IE1; VLEETSVML	6-7041-005
MHC I Strep HLA-A*2402; CMVpp 65; QYDPVAALFL	6-7028-005
MHC I Strep HLA-B*0702; CMVpp65; TPRVTGGGAM	6-7027-005
MHC I Strep HLA-B*0801; CMV IE1; QIKVRVDMV	6-7017-005
MHC I Strep HLA-B*3501; CMV pp65; IPSINVHHY	6-7048-005

Table 2.3 List of isotype controls used in the phenotypic analysis of CMV-specific T-cells.

Fluorophore*	Isotype	Source	Catalogue number	Volume (µl)
FITC	IgG2b,κ	eBiosciences	11-4732	0.6
FITC	IgG1,κ	BD Biosciences	551954	3
PerCP-Cy5.5	IgG1, κ	BD Biosciences	550795	1
PE-Cy7	IgG1, κ	BDBiosciences	557872	1
FITC	IgG1, κ	eBiosciences	11-4714-73	0.6

* These isotype controls were used for perforin, granzyme B, T-bet, CD25 and CD127

Table 2.4 Reagents and consumables used in the study of CMV-specific T-cells.

Reagents/Consumable	**Source**	**Catalogue number**
Trucount tube	BD Biosciences	340334
BD Pharm Lyse	BD Biosciences	555899
Lymphoprep	Axis-Sheild PoC AS	1114545
CliniMACS PBS/EDTA buffer	Miltenyi Biotec GmbH	700-25
RPMI-1640	Sigma	R8758
Human Serum Albumin (Zenalb 20)	Bio Products Laboratory	-
Centrifuge tube(50 ml)	Corning	430921
Centrifuge tube(15 ml)	Corning	430791
FALCON tubes (5 ml sterile)	FALCON	352054
Sterile water	Baxter	UKF7114
Vacutainer- 9ml (lithium Heparin)	BD	455084
Vacutainer- 4 ml (EDTA)	BD	454021

Table 2.5 Antigens and reagents used for the cell stimulation assay.

Antigens/ Reagents	**Source**	**Catalogue number**	**Concentration (µg/ml)**
CMV /peptide NLVPMVATV	Proimmune	CPD7383	1
CMV/peptide VTEHDTLLY	Proimmune	CPD7384	1
CMV/peptide TPRVTGGGAM	Proimmune	CPD7385	1
CMV/peptide QIKVRVDMV	Proimmune	CPD7386	1
CMV/peptide QYDPVAALF	Proimmune	CPD7388	1
SEB	Sigma	S0812	0.5
Brefeldin A	Sigma	37651	5
Anti CD28/CD49d	BD Biosciences	347690	5µl

Table 2.6 List of anti- cytokine receptor antibodies.

Antibody	Fluorophore/ Conjugate	Source	Catalogue number	Isotype*	Volume (µl)
CD25(IL2Ra)	PE	AbD serotec	MCA2127PE	IgG1	5
CD127(IL7Ra)	PerCP/Cy5.5	Biolegend	A019D5	IgG1	5
CD130(gp130)	PE	R&D Systems	FAB228P	IgG1	10
CD120a(TNFRI)	PE	R&D Systems	FAB225P	IgG1	10
CD120b(TNFRII)	PE	R&D Systems	FAB226P	IgG2a	10
IL15Ra	PE	R&D Systems	FAB1471P	IgG2b	10
CD120b(TNFRII)	Biotin	BD Biosciences	552477	IgG2b	3

*All monoclonal antibodies are mouse immunoglobulin with exception of anti - CD120b-biotin which is rat IgG2b

Table 2.7 Isotype controls used in the cytokine receptor study.

Fluorophore*	Isotype	Source	Catalogue number	Volume (µl)
PE	IgG1	AbD serotec	MCA928PE	5
PerCP/Cy5.5	IgG1	Biolegend	MOPC-21	2
PE	IgG1	R&D Systems	IC002P	10
PE	IgG2a	R&D systems	IC003P	10
PE	IgG2b	R&D systems	IC0041P	10

Chapter 3

RECONSTITUTION OF CMV-SPECIFIC T-CELLS FOLLOWING CELLULAR THERAPY

(Phase II trial: **ACE** (Adoptive Cellular Immunotherapy)-**ASPECT** (Alternate Donor Study of Pre-emptive Cellular Therapy)

3.1 Background

3.1.1 Adoptive cellular therapy- Phase I clinical trial

Consistent immune control of lifelong CMV infection is required for protection against overt replication of the virus. Immune suppression in the transplant setting leads to the reactivation of CMV and CMV DNAaemia, associated with considerable morbidity and mortality. CMV reactivation can cause multiorgan disease in HSCT patients, including pneumonia, hepatitis, gastroenteritis, retinitis, and encephalitis, and the disease can develop both early and late after the transplantation procedure (Boeckh and Ljungman, 2009). Adoptive cellular therapy with CMV-specific $CD8^+$ T-cells is a promising strategy for accelerating immunity against CMV post transplantation. One of the methods for the generation of CMV- reactive cells is direct T-cell isolation by means of magnetic beads. This method allows rapid access to the T-cells without need for labour-intense and prolonged in vitro culture. In addition, because *ex vivo* selection does not alter the function and identity of cells, it is considered as minimally manipulated and therefore not considered as an investigative advanced therapeutic medicinal product (ATMP). Compared to *in vitro* cell expansion, direct cell selection is easier to achieve GMP-compliance (Sellar et al., 2014). In a phase I clinical trial conducted in collaboration between the University of Birmingham and NHS Blood and Transplant, CMV-specific $CD8^+$ T-cells from stem cell donors were isolated using a GMP-grade immunomagnetic column (cliniMACS®) with HLA-class I tetramers labelled with PE fluorophore and a secondary anti-PE magnetic beads (Cobbold et al., 2005). Transfer of isolated cells with median cell dosage of $8.6x10^3$/kg (median total 0.5×10^6) into 9 HSCT patients showed persistence of therapeutic cells in the treated patients and clearance CMV infection in 8 out of 9 including one patient with a history of prolonged

CMV infection. Seven of these 9 patients, received CTLs after the first episode of viraemia and 2 patients were treated for persistent viraemia. CTLs were infused within 4 hrs of selection and had a median T-cell purity of 95.6%. CMV-specific $CD8^+$ T-cells were detectable within a median of 10 days after cell therapy and their presence was maintained during the follow-up period of up to 24 months. The number of CMV-specific $CD8^+$ T-cells reached its peak level between 12 and 30 days post T-cell infusion with a range of 1-156 cells/µl of blood. It was also shown that CTLs were functional and able to produce IFN-γ. 89% of patients controlled CMV viraemia and there was a reduction in the use of ganciclovir by 48%. Three out of nine patients in this phase I trial had received stem cell transplant from unrelated donors which is considered a high risk factor for CMV reactivation. One of these 3 patients experienced 4 months of persistent CMV reactivation with no detectable CMV-specific $CD8^+$ T-cells prior to cell therapy. Four days after infusion of therapeutic cells, CMV-specific $CD8^+$ T-cells were detectable and the CMV viraemia was cleared at 11 days after infusion. The second patient with unrelated stem cell transplant had no CMV-specific immunity before cell infusion and was able to control the viraemia after that. The third patient also cleared viraemia following cell therapy. These results and lack of significant GVHD in patients, clearly demonstrated the safety and tolerability of adoptive cell therapy, with preliminary evidence of efficacy.

3.1.2 Adoptive cellular therapy- Phase II clinical trial

Following the successful conclusion of the phase I trial, a randomised phase II clinical trial named ACE-ASPECT was conducted, a collaboration between Cell Medica, Ltd as sponsor, University of Birmingham and NHS Blood and Transplant. The primary objective of this study was to evaluate the immunological efficacy of pre-emptive CMV- specific adoptive cellular therapy following T-cell depleted allogeneic HSCT in terms of the peak number of circulating CMV-reactive $CD8^+$ T-cells within the first 2 months post-ACT. The trial had two arms: arm A enrolled patients for cell therapy plus antiviral treatment and arm B enrolled patients who received best available therapy (BAT) using standard of care antiviral drugs alone for the treatment of CMV reactivation. The CMV-specific $CD8^+$ T-cells were selected from the original donor stem cell harvest, using excess cells that were not required for transplantation and DLI storage. Selection of CMV-specific T-cells were immunomagnetically performed from these fresh harvests using a GMP grade magnetic column CliniMACS after incubating the cells with novel commercially available CMV-specific HLA-multimers (Streptamers®) that were directly linked to magnetic beads by Stage Cell Therpeutics, GmbH (Gottingen, Germany) (Figure 3.1). After isolation and further processing, cells were transferred to a cryobag or cryovial at a maximum cell number of $3x10^4$ $CD3^+$ T-cells per kg of recipient body weight. Following CMV reactivation, the cryopreserved cell product was released from the laboratory and transported to the patient's bedside, thawed rapidly at 37°C and infused intravenously.

A total of 52 CMV sero-positive patients undergoing T-cell depleted allogeneic HSCT using CMV sero-positive donors were recruited to the ACE-ASPECT. 24 patients (46%) were withdrawn from the study due to GVHD, leukaemia relapse and other

reasons and did not complete the immune assessment programme. The immune reconstitution was analysed in the remaining 28 evaluable patients. 17 patients were randomised to receive CMV-specific $CD8^+$ T-cells plus antiviral treatment (arm A). 11 patients were randomised to the BAT group to receive only antiviral drug for the treatment of CMV viraemia (arm B) and among them, 10 had evaluable immune monitoring data.

3.1.2A Study objectives and endpoints

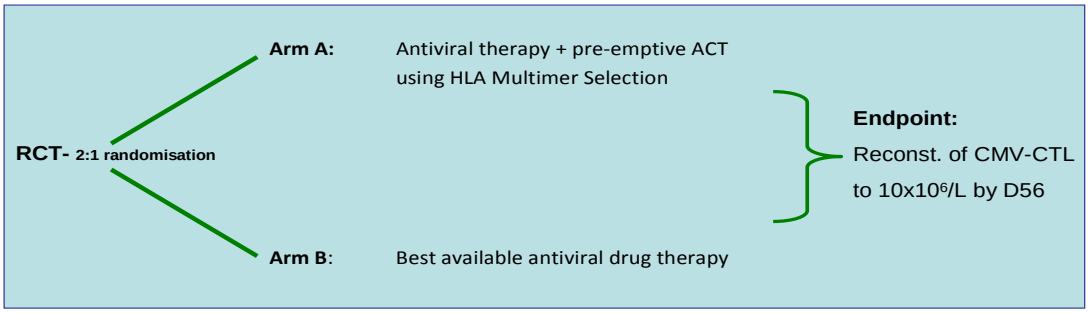
The primary objective of this study was to evaluate the efficacy of immune reconstitution of pre-emptive CMV-specific adoptive cellular therapy following T-cell depleted allogeneic HSCT in terms of the peak number of circulating CMV-reactive $CD8^+$ T-cells within the first 2 months post-ACT. The secondary objectives included: the incidence and severity of GVHD, the time to detection of CMV-reactive T-cells post ACT, evaluation of the potential clinical benefit of cell therapy following T-cell depleted allogeneic HSCT as measured by reduction in CMV anti-viral drug treatment and number of in-patient days and investigate the feasibility of use of ACT generated from G-CSF mobilised peripheral blood. The primary endpoint was the percentage of patients with a peak number of circulating CMV-reactive T-cells above $10x10^3$/ml within the first two months post single positive PCR result (or ACT infusion).

The reconstitution of CMV- specific $CD8^+$ T-cells were monitored using HLA-class I restricted Streptamers®, weekly for the first 56 days post cell therapy and then monthly for 4 months up to a total of 6 months post CTL infusion/ first viral detection (control group).

ACE – ASPECT

Adoptive **C**MV Immunmoth**E**rapy - **A**lternate Donor **S**tudy of **P**re-**E**mptive **C**ellular **T**herapy

Phase II randomised controlled trial of pre-emptive CMV adoptive CTL immunotherapy for patients receiving HSCT from unrelated donor. Total: 52 patients recruited. Study completed Feb 2014



Inclusion criteria

- **Unrelated** Donors
- **High CMV risk**: CMV D/R+/+; -/+ **Alemtuzumab T Cell Depletion**
- Selection from **Stem Cell Harvest**
- Donor >0.1% streptamer detectable CMV-CTL

Exclusion criteria

GVHD II or systemic steroids

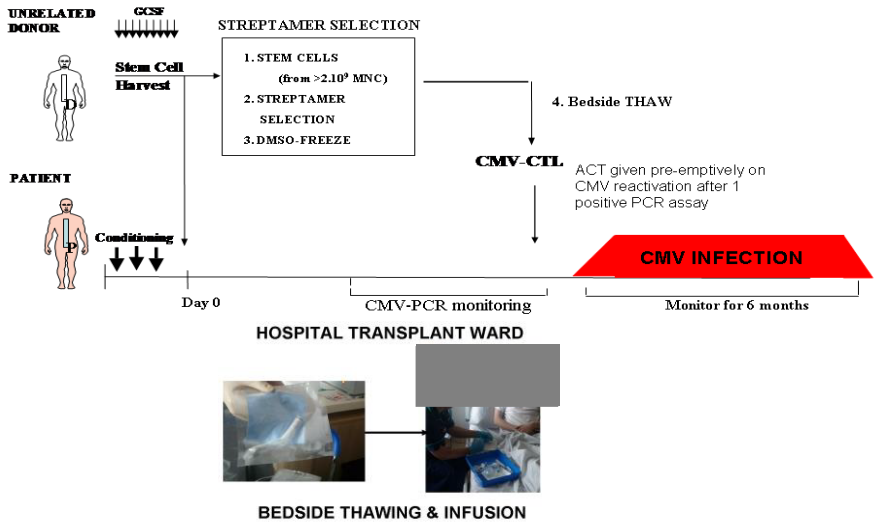


Figure 3.1 ACE Trial arms (ACT and control arms) and cell therapy plan for ACT arm

3.2 Results

3.2.1 Quality controls for the quantification of T-cells

3.2.1A Internal controls

In order to perform T-cell staining with internal controls in place, BD™ Multi-Check Control was used. This is a stabilized preparation of normal peripheral blood which is used for control of immunophenotyping by flow cytometry. The BD™ Multi-Check is a control for antibody staining, red blood cell lysis, instrument setup and performance, and data analysis. Figure 3.2 shows the $CD3^+$, $CD4^+$ and $CD8^+$ T-cell counts of BD™ Multi-Check samples obtained in our assay and reported by BD. T-cell counts obtained by our flow cytometry method were in close match with the counts given by BD. The coefficient of variations (CV) of the cell counts obtained by our method and reported by BD were less than 10% in all cases. As an example, the following cell counts were calculated by our assay: 1330/µl, 845/µl and 364/µl for $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in BD™ Multi-Check with Lot Number: BM072N. The reported cell counts by BD were: 1210/µl, 776/µl and 365/µl for $CD3^+$, $CD4^+$ and $CD8^+$ T-cells, respectively. The resulted CVs for these cell counts of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells were 7.4%, 8.9% and 0.2% , respectively.

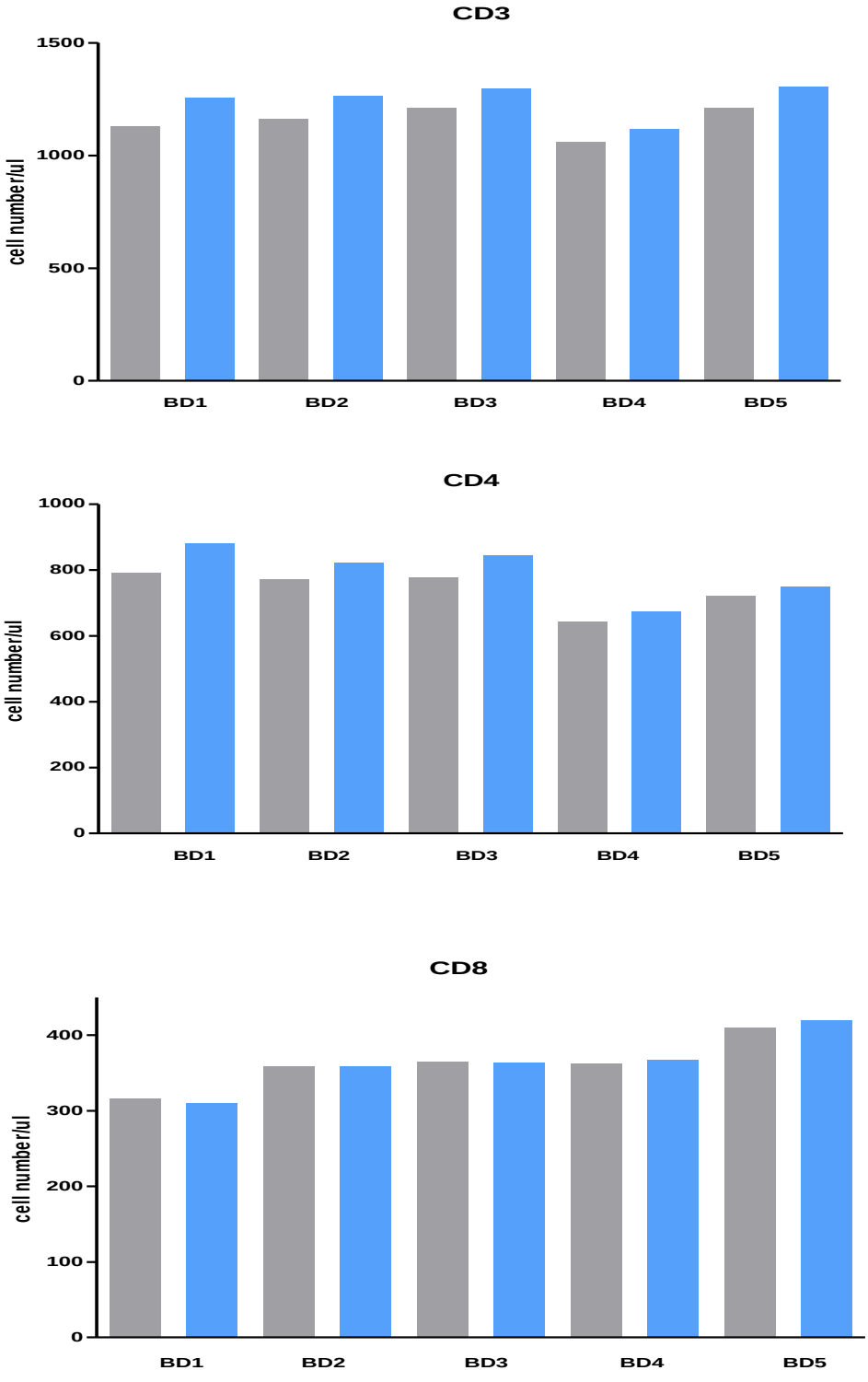


Figure 3.2 Internal quality control of flow cytometry method used for T-cell quantification.

Cell counts of BD™ Multi-Check samples, calculated by our assay (blue bars) in comparison to the cell counts reported by BD (grey bars). The X axis shows five different BD™ Multi-Check samples with the following Lot numbers: BM112N, BM082N, BM072N, BM100N and BM068.

3.2.1B External quality control

National External Quality Assurance Scheme (NEQAS) samples were used to check the quantification of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells using established flow cytometry settings and panels. The results were compared with the mean consensus results reported for NEQAS blood samples. Figure 3.2 shows the results of T-cell counts of the NEQAS blood samples calculated by our assay (bright brown bars) in comparison to the cell numbers reported by NEQAS (bright red bars). In each case, the experiments were performed in duplicate and the mean of cell counts documented. In all the tested samples, CVs between calculated $CD4^+$ and $CD8^+$ T-cell counts and cell counts reported by NEQAS were less than 10%. This was within acceptable range according to the NEQAS reports (Figure 3.3). The CVs for $CD3^+$ counts were above 10%, averaging 11.1% above mean consensus counts reported by NEQAS. This was found to be due to the degradation of fluorochrome bound to anti-CD3. This fluorochrome was a tandem dye named APC-H7 which degraded and produced non-specific signals in APC channel (Le Roy et al., 2009). In order to minimize the consequences of this problem, a tight CD3 gate in FACS plots was chosen to include only bright clustered cell population. However, it should be mentioned that the CD3 count was not a vital parameter in this study and CD3 staining was mainly used to identify $CD8^+$ $CD3^+$ T-cell population. The CD8 count determined by our flow cytometry was however, in close accordance with NEQAS counts.

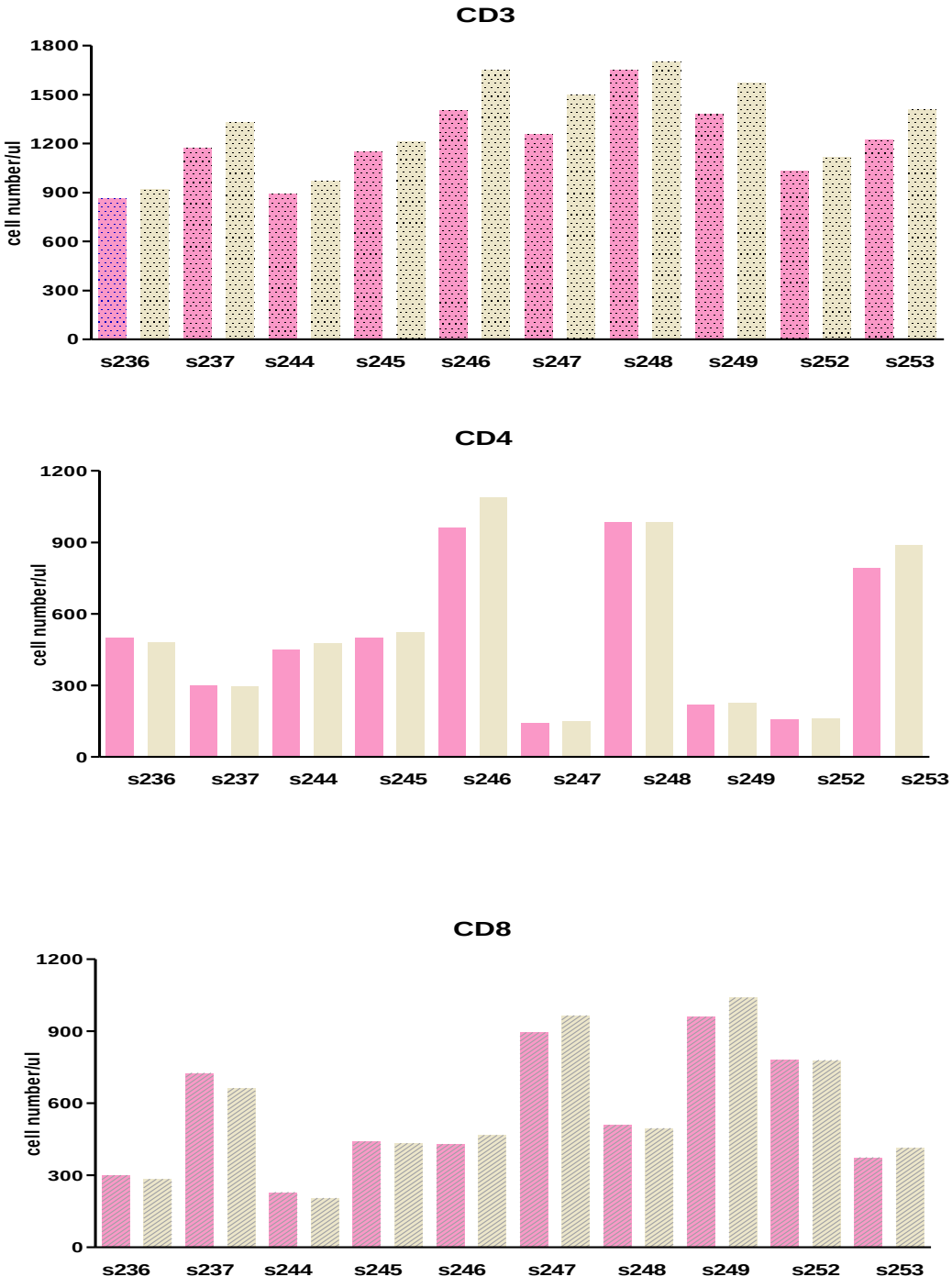


Figure 3.3 External quality control of flow cytometry method used for T-cell quantification.

The numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T–cells in the NEQAS blood samples, calculated by used method in this thesis (bright brown bars) in comparison to the cell counts reported by NEQAS (pink bars). The X axis shows identification numbers of 10 different blood samples provided by NEQAS and used for the comparison analysis.

3.2.1C Control of Streptamer® staining

CMV-specific T-cell numbers were quantified by staining of PBMCs with HLA-class I Streptamers®. Streptamers® are complexes of HLA-class I molecules, antigenic peptide and Strep-Tactin conjugated to a fluorochrome (Figure 3.4). In order to control the staining with this reagent, a series of experiments were performed. First, staining with Streptamers® was compared with HLA-class I tetramer staining. HLA-class I tetramers are the original and most used reagent for staining specific T-cells through interaction of TCR and HLA-class I molecule.

In these experiments, PBMCs from CMV sero-positive HSCT patients and healthy blood donors with different types of HLA-class I molecules were stained in parallel with Streptamers® and tetramers. In the next step and to check non-specific staining properties of Streptamers®, PBMCs of donors with HLA types different from HLA molecules of Streptamers®, used for T-cell staining. For example, HLA-A*0201/NLV Streptamer which binds TCR of HLA-A*0201 donor was used to stain PBMCs of HLA-B*0701 donor. In addition, we checked non-specific bindings of Streptamers® to $CD3^+$ $CD4^+$ T-cells in CMV sero-positive donors. The results showed that both Streptamers® and tetramers detect similar frequencies of CMV-specific T-cells and in some cases, there were a low no non-specific binding of Streptamers® to PBMCs from a donor with non-relevant HLA-types (Table 3.1). There were also low frequencies of non-specific binding to $CD4^+$ cells in some cases. In order to reduce background staining, a step including three washes after cell staining was introduced and a threshold level of 0.10% $CD3^+$ $CD8^+$ $Streptamers^{®+}$ cells was determined as cut off. Streptamer® positive cells with the frequency of less than 0.10% of $CD8^+$ T-cells were not reported as CMV-

specific T-cells. Frequencies of above this level and if the stained cells had a demarcated population (not scattered staining) were considered as positive staining.

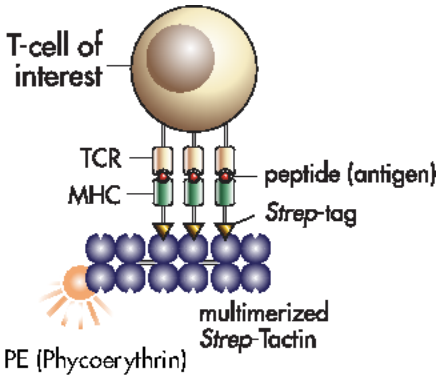


Figure 3.4 Schematic picture of Streptamer® and its binding to T-cell through engagement of TCR and MHC molecule (modified from IBA, GmbH)

Table 3.1 Frequencies of T-cells stained with different multimers for control of Streptamer® staining.

HLA restriction and Ag specificity of multimers	Patient 1* HLA:A1,A2,B8		Patient 2 HLA:A2,B7		Healthy donor 1 HLA:A1		Health donor 2 HLA:B7	
	Strept.	Tet,	Strept.	Tet,	Strept.	Tet,	Strept.	Tet,
HLA-A2/NLV	21.94**	22.00	1.56	1.48	0.00	0.00	0.00	0.00
HLA-B8/QIK	0.88	0.96	0.01	0.00	0.00	0.00	0.01	0.00
HLA-A1/VTE	1.96	2.00	0.00	0.00	0.58	0.56	0.00	0.00
HLA-B7/TPR	0.01	0.00	2.15	2.25	0.02	0.01	1.78	1.90

* HLA typing of patients and controls, ** frequencies of $CD3^+$ $CD8^+$ T-cells stained either with Streptamer® (Strept) or tetramer (Tet). Frequencies of multimer binding $CD3^+$ $CD4^+$ T-cells in these samples were 0.02, 0.00, 0.02 and 0.03 percent, respectively.

3.2.2 Absolute CMV-specific $CD8^+$ T-cell numbers following ACT and the trial end point showed no statistically significant difference between the two arms of trial

The trial end point (reconstitution of $10x10^3$/ml CMV-specific $CD8^+$T-cells in up to 2 months post-ACT) was achieved in 8 out of 17 patients who had cell therapy. In one patient, the number of CMV-specific cells was higher than the trial end point at the time of cell infusion and in the rest of patients (n=8), the frequency of these cells stayed below $10x10^3$/ml in 2 months post-ACT (Table 3.2). At 2 months post-ACT, the highest numbers for CMV-specific $CD8^+$ T-cells ranged from 0.2-$147x10^3$/ml with $mean^+$/-SD: 35^+/-47 $x10^3$/ml. In 11 out of 17 patients, cell expansion continued after 2 months and reached to the higher levels (range:5-$528x10^3$/ml, $mean^+$/-SD:91^+/-$150x10^3$/ml) (Table 3.2).

Among 10 patients of control group (arm B), two patients (02/23 and 03/43) had higher than $10x10^3$/ml CMV-specific T-cells at the beginning of CMV viraemia. In the remaining arm B patients, the trial end point was observed in 3 patients. The peak number of CMV-specific $CD8^+$ T-cells ranged from 0.003-$121x10^3$/ml with $mean^+$/-SD: 25^+/-$40x10^3$/ml within 2 months post first CMV^+ PCR and continued to rise after this period in 8 out of 10 patients (range:16-$111x10^3$/ml, $mean^+$/-SD:54^+/-$38x10^3$/ml) (Table 3.3). Therefore, after exclusion of patients who had more than$10x10^3$/ml cells at the beginning of study, the trial end point was achieved in 8 out of 16 (50%) in arm A and in 3 out of 8 patients in arm B (37.5%). This difference was not statistically significant (p=0.67). Additionally, analysis of the peak CMV-specific $CD8^+$ T-cell numbers either in the period of 2 months or after that (up to 6 months as the study scheduled program) revealed no difference between the two study arms (p=0.57 and 0.51, respectively). Figure 3.5 illustrates the overall reconstitution of CMV-specific $CD8^+$ T-cells in both arms of trial in up to 2 months post-ACT/first viral detection. Although numerically

there was no statistical difference in the reconstitution of CMV specific $CD8^+$ T-cells between arms A and B, a difference in the rate of reconstitution early post cell therapy/first CMV^+ PCR was detected (discussed later).

Table 3.2 Magnitude of CMV-specific $CD8^+$ T-cell reconstitution post ACT.

Patient code	End point at day	Peak cell number* up to 2 months at day	Peak cell number up to 2 months ($x10^3$/ml)	Peak cell number after 2 months at day	Peak cell number after 2 months ($x10^3$/ml)
01/01	7	14	109	140	112
01/02	7	21	147	-**	-**
08/04	21	21	12	170	14
02/06	NR	35	6	-	-
11/07	NR	56	6	140	34
11/08	NR	56	4	170	36
09/09	NR	56	0.2	112	37
08/13	21	21	36	-	-
01/15	0	28	101	-	-
01/16	21	28	16	-	-
08/20	14	35	18	84	528
06/29	NR	49	0.9	170	5
05/36	NR	35	7	140	27
09/41	56	56	26	-	-
01/44	NR	56	3	170	58
08/46	NR	56	2	140	16
01/49	21	28	105	112	124

The table shows the day at which the trial end point reached and days that the maximum cell numbers were recorded during and beyond the period of 2 months post ACT. The maximum cell numbers ($x10^3$/ml) also reported.*The peak cell number shows the frequency of CMV-specific $CD8^+$ T-cells with the same specificity as the infused cells. **-, There was not any cell expansion after 2 months. NR; the trial endpoint not reached.

Table 3.3 Magnitude of CMV-specific CD8$^+$ T-cell reconstitution in arm B patients.

Patient code	End point at day	Peak cell * number within 2 months at day	Peak cell number within 2 months (x10^3/ml)	Peak cell number after 2 months at day	Peak cell number after 2 months (x10^3/ml)
02/03	7	7	28	84	30
06/22	35	35	11	112	20
02/23	0	0	121	-**	-
05/25	NR	56	0.003	112	63
04/32	42	42	12	-	-
08/37	NR	49	2	170	16
05/40	NR	56	0.7	170	111
03/43	0	14	70	112	92
05/47	NR	14	4.6	112	79
09/50	NR	56	0.6	84	18

The table shows the day at which the trial end point reached and days that the maximum cell numbers were recorded during and beyond the period of 2 months post ACT. The maximum cell numbers(x10^3/ml) also reported.* The peak cell number shows the frequency of dominant CMV-specific CD8$^+$ T-cells. **-, There was no further cell expansion after 2 months. NR; the trial endpoint not reached.

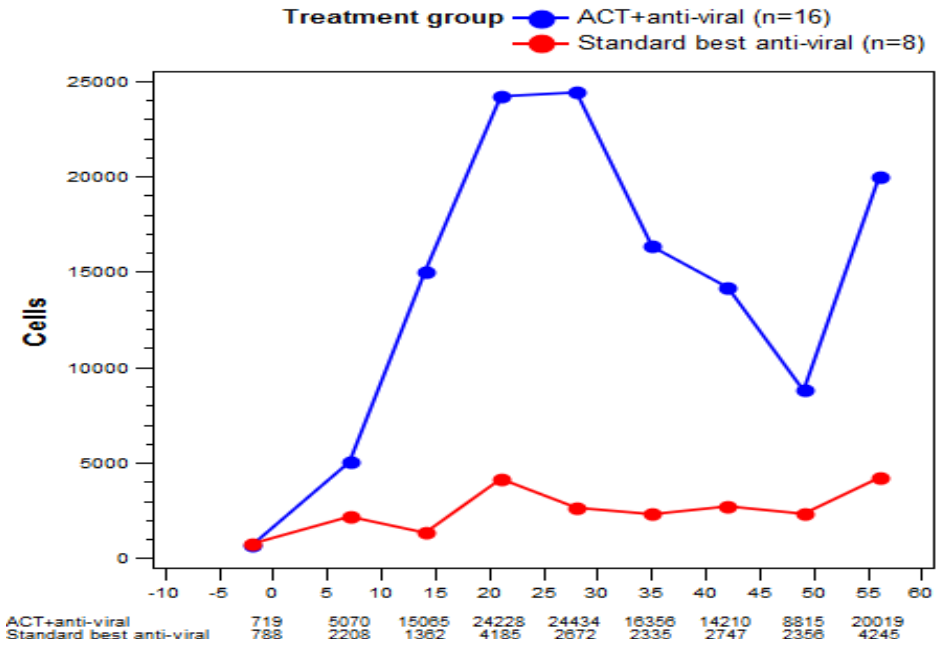


Figure 3.5 Expansion of CMV-specific CD8$^+$ T-cells up to 2 months post-ACT (arm A) or first detection of CMV viraemia (arm B).
The means of cell numbers from all the patients of arms A and B in each time point are plotted against the time to show the overall expansion patterns of CMV-specific T-cells during the study period (this figure was presented by Professor K.Peggs at BTM Tandem meeting, 2014).

3.2.3 Reconstitution of total $CD3^+$ and $CD8^+$ T- lymphocyte populations

In order to have a broader picture of T-cell reconstitution, the frequencies of total $CD3^+$ and $CD8^+$ T-cells at their peaks were analysed. There were no statistically significant differences in the peak levels of $CD3^+$ T-cells within 2 months post ACT/first viral detection or beyond that between the two arms of trial (p=0.68). This was true for the $CD8^+$ T-cells, as well (p=0.73) (Tables 3.4 and 3.5).

Table 3.4 Magnitude of $CD3^+$ and $CD8^+$ T-cell reconstitution post ACT.

Patient code	Peak $CD3^+$ number within 2 months at day	Peak $CD3^+$ number within 2 months ($x10^3$/ml)	Peak $CD3^+$ number after 2 months at day	Peak $CD3^+$ number after 2 months ($x10^3$/ml)	Peak $CD8^+$ number within 2 months at day	Peak $CD8^+$ number within 2 months ($x10^3$/ml)	Peak $CD8^+$ number after 2 months at day	Peak $CD8^+$ number after 2 months ($x10^3$/ml)
01/01	35	343	112	1164	21	266	140	594
01/02	21	1444	-*	-	21	991	-	-
08/04	56	284	170	583	21	77	170	242
02/06	56	582	-	-	49	710	-	-
11/07	56	181	170	292	56	33	170	160
11/08	56	139	170	1234	56	70	170	996
09/09	56	62	112	3538	56	14	112	2996
08/13	21	393	-	-	21	248	-	-
01/15	56	950	84	1305	21	690	84	1048
01/16	14	466	-	-	14	386	-	-
08/20	56	243	112	1640	14	160	84	1337
06/29	49	1405	-	-	49	1164	-	-
05/36	7	87	140	1050	14	75	140	909
09/41	42	92	-	-	56	34	-	-
01/44	56	130	112	541	56	114	112	284
08/46	56	350	140	747	56	139	140	503
01/49	21	185	170	927	21	122	112	500

The figures show the highest detected levels of $CD3^+$ and $CD8^+$ T-cells within and beyond 2 months post-ACT.*- There was no further cell expansion beyond 2 months.

Table 3.5 Magnitude of $CD3^+$ and $CD8^+$ T-cell reconstitution post first CMV viral detection in arm B patients.

Patient code	Peak $CD3^+$ number within 2 months at day	Peak $CD3^+$ number within 2 months ($x10^3$/ml)	Peak $CD3^+$ number after 2 months at day	Peak $CD3^+$ number after 2 months ($x10^3$/ml)	Peak $CD8^+$ number within 2 months at day	Peak $CD8^+$ number within 2 months ($x10^3$/ml)	Peak $CD8^+$ number after 2 months at day	Peak $CD8^+$ number after 2 months ($x10^3$/ml)
02/03	0	184	140	262	0	110	140	158
06/22	56	198	84	648	14	153	112	642
02/23	0	268	- *	-	0	171	-	-
05/25	56	10	140	1048	56	4	140	870
04/32	56	1024	112	2555	56	838	112	2186
08/37	56	847	-	-	56	688	-	-
05/40	56	166	170	2234	49	45	170	2024
03/43	56	471	112	1142	14	361	112	984
05/7	35	100	112	764	28	37	112	477
09/50	56	109	84	254	56	80	84	211

The figures show the highest detected levels of $CD3^+$ and $CD8^+$ T- cells within and beyond 2 months post viral detection.
*- There was no further cell expansion.

3.2.4 The expansion rate of CMV-specific $CD8^+$ T-cells was different between arms A and B patients

The expansion of CMV-specific T-cells post-HSCT is time-dependent. This relationship can be quantified by correlating CMV-specific T-cell levels with time (the period of study). By comparing the correlation lines between cell numbers and time (one month post-ACT/first viral detection) in arm A and B, a difference in the slope of the 2 arms was observed (Figure 3.6). Cell expansion in arm A patients demonstrated a considerably closer correlation with time than arm B ($r=0.91$, $p=0.01$ versus $r=0.31$, $p=0.61$, respectively). This suggests a 'faster' reconstitution amongst patients who received cell therapy which happened within 1 month post-ACT. By analysing the cell numbers in the first month post-ACT, a clear difference in the expansion rate of CMV-

specific CD8$^+$ T-cells between the two cohorts was seen (Figure 3.6), where patients in arm A experienced a considerably greater cell expansion than in arm B.

This differential effect between the two arms was further strengthened by comparing the geometric increase in the number of CMV-specific T-cells within the first 2 months. The fold increase was calculated by comparing the highest expanded level of T-cells to the baseline level (day 0 which was the day of cell infusion). As Figure 3.7A shows, the bigger fold increase of CMV-specific CD8$^+$ T-cells occurred in arm A in comparison to arm B within 2 months post-ACT (p=0.002). However, when the data was analysed over 6 months, the fold increases of CMV-specific CD8$^+$ T-cells were similar in arms A and B patients (Figure 3.7B).

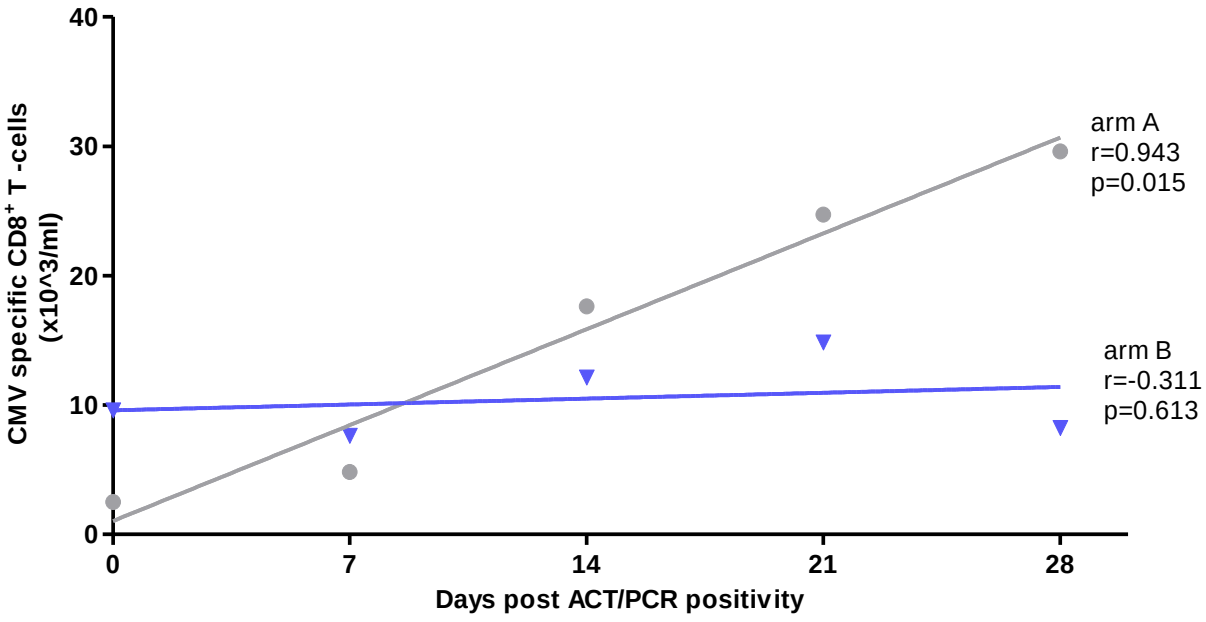


Figure 3.6 Expansion of CMV-specific CD8$^+$ T-cells within one month post ACT/first CMV viraemia detection.

There was a greater cell expansion in the first month post ACT in arm A than in arm B patients. The numbers show the mean frequencies of cells in each time point.

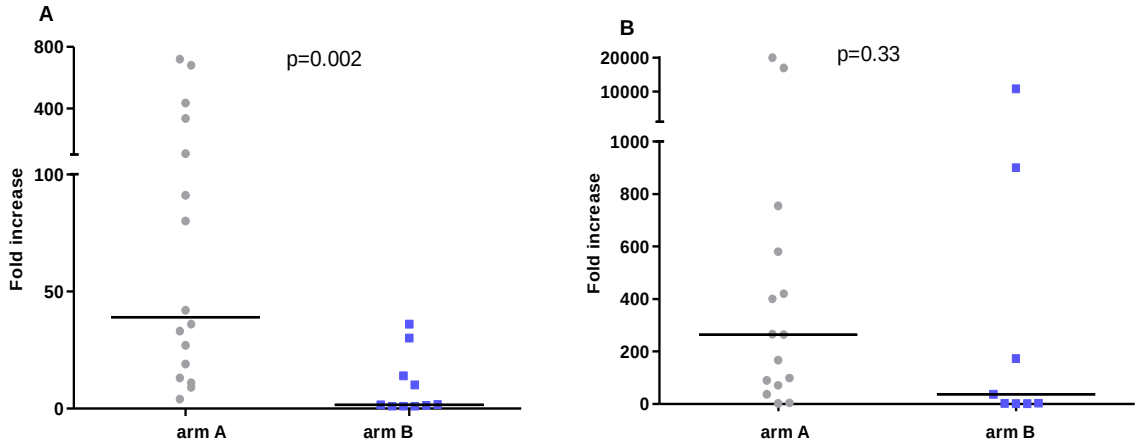


Figure 3.7 Fold increase in the number of CMV-specific CD8$^+$ T-cells.

This figure shows the higher fold increases in the frequencies of CMV-specific T-cells (A) within first 2 months post-ACT in arm A when compared to arm B patients. (B) In both groups, fold increases in the frequencies of these cells over the total period of study were similar. Bars show the median fold increases.

3.2.5 Geometric increases in CD3$^+$ and CD8$^+$ T-cell populations

In the next step, fold increases in the number of total CD3$^+$ and CD8$^+$ T-cells were analysed. Similar to the previous finding for CMV-specific CD8$^+$ T-cells, fold increases of total CD3$^+$ and CD8$^+$ T-cells within the first 2 months post ACT were also found to be higher in arm A than B (Figure 3.8) but not when analysed for the whole 6 month period post-ACT.

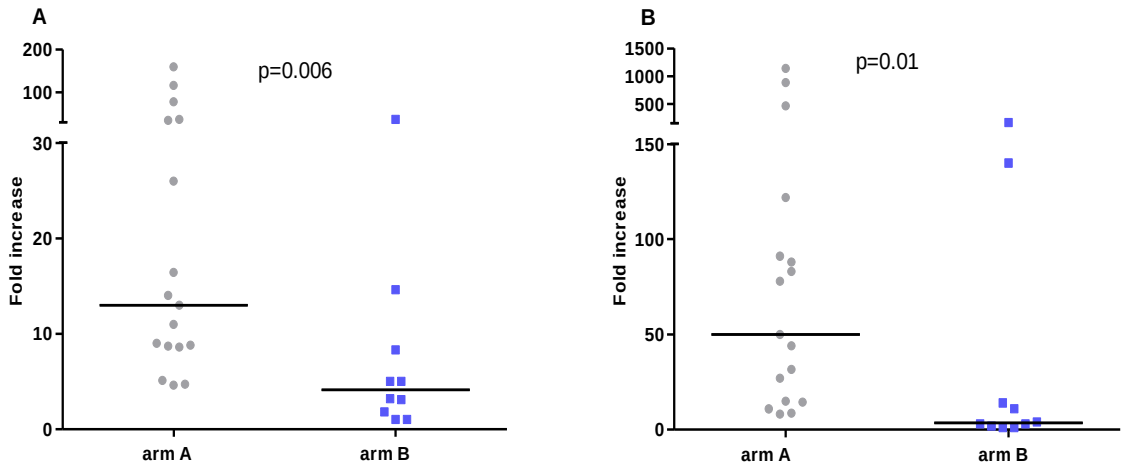


Figure 3.8 Fold increases in the numbers of CD3$^+$ and CD8$^+$ T-cells within 2 months post-ACT/first CMV viraemia.

(A)Higher CD3+ and (B) CD8+ T-cell expansions in arm A compared to arm B patients occurred within 2 months post-ACT/first detected viraemia. Bars show the median fold increases.

Following transplantation, CD3$^+$ and CD8$^+$ T-cells expanded rapidly within the first month in patients who received cell therapy (arm A), compared to those who did not (Figure 3.9).

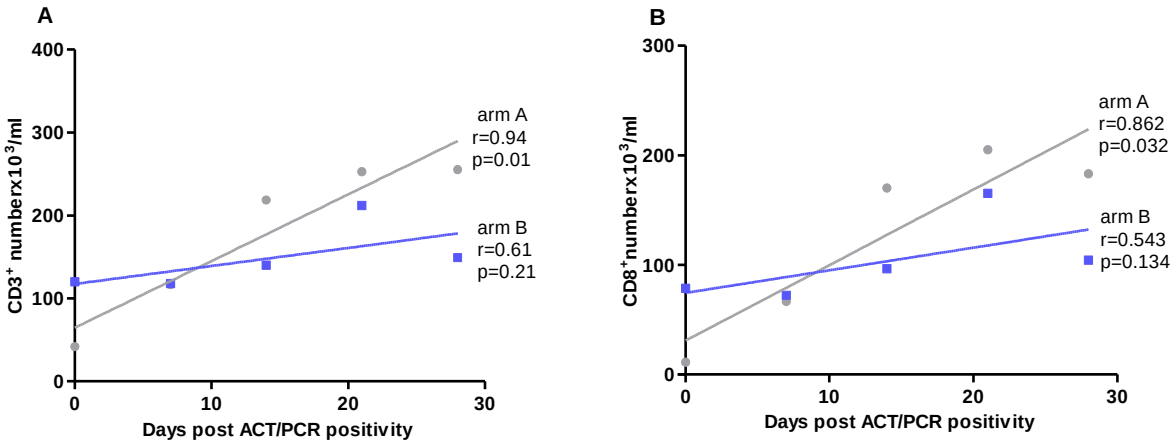


Figure 3.9 CD3$^+$ and CD8$^+$ T-cell expansions over one month post-ACT/first viraemia. There were higher cell expansions in CD3$^+$ (A) and (B) CD8$^+$ T-cells in arm A patients in one month post-ACT.

3.2.6 Expansion of non-infused CMV-specific T-cells in arm A patients

In 10 out of 17 patients in arm A, it was possible to study the reconstitution of CMV-specific $CD8^+$ T-cells targeted against CMV peptides different from the specificity of infused T-cells. Depending on the availability of Streptamer® specificities, reconstitutions of T-cell responses specific for up to three CMV derived epitopes were analysed. The results showed that in all of these patients, the dominant response belonged to the infused cells. Figure 3.10 shows the lowest and highest level of infused (dominant response) and three non-infused cells (sub-dominant) during 6 month period of study. Analysis of the second biggest response in those 10 patients revealed their parallel expansion with the infused cells which increased rapidly over 1 month. Figure 3.10 shows increase in the number of the sub-dominant non-infused CMV-specific T-cells in comparison to the infused cells in one months period post- ACT.

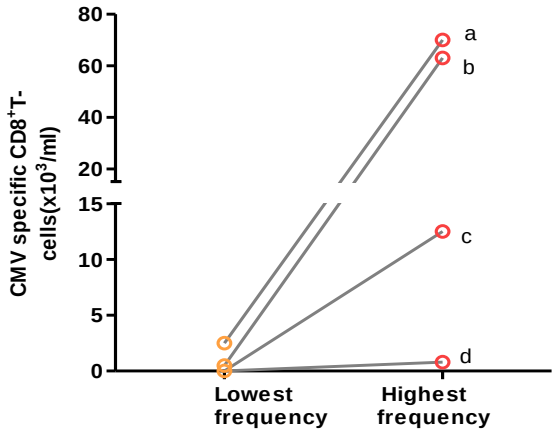


Figure 3.10 The highest and lowest median frequencies of different CMV-specific $CD8^+$ T-cells.

The figure shows the frequencies of (a) infused, (b-d) the first, second and third sub-dominant non-infused CMV-specific $CD8^+$ T-cells in arm A patients.

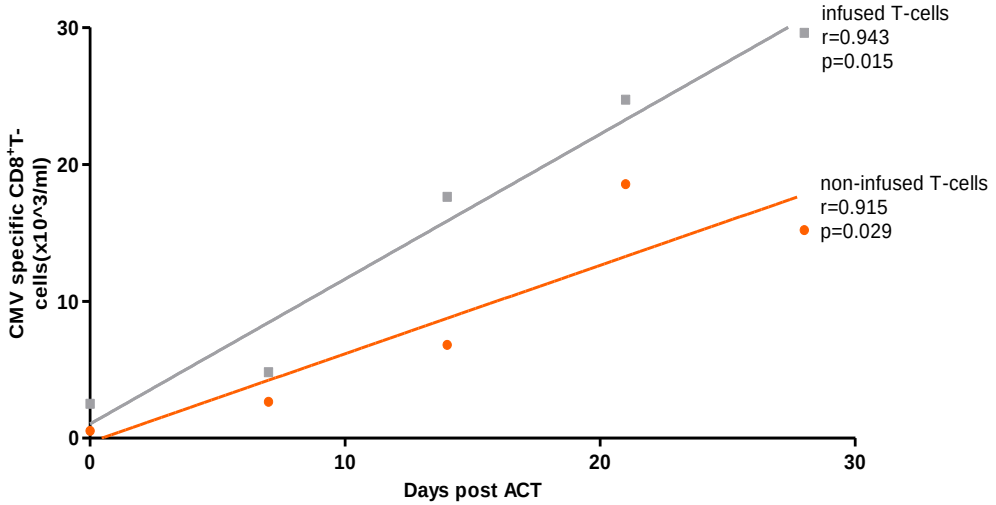


Figure 3.11 Expansion of infused and non-infused CMV-specific CD8$^+$ T-cells post-ACT.
Similar to the infused cells, non-infused CMV-specific CD8$^+$ T-cells expanded rapidly over 1 month post- ACT in arm A patients. The non-infused cell is the first sub-dominant CMV-specific T-cell response.

In 6 out of 10 arm B patients, depending on the availability of Streptamers®, up to 3 different CMV-specific CD8$^+$ T-cells were analysed. The mean frequency of dominant CMV-specific T-cell response increase from 9.6 to 40.0x10^3/ml. The sub-dominant response increased from 0 to 5.8x10^3/ml.

In both arms, patients' immune system produced T-cells with diverse epitope specificities. Therefore, the expansion of CMV-specific T-cells other than the infused cells was a natural phenomenon. The important finding was the rapid expansion of non-infused CMV-specific CD8$^+$ T-cells alongside the infused T-cells in arm A patients (Figure 3.11). This observation was not seen in arm B patients and both the dominant (Figure 3.6) and sub-dominant responses developed slowly in the first month post CMV reactivation. These results and properties of CD4$^+$ T-cell reconstitution in arm A (discussed below), show that cellular therapy had a global positive effect on T-cell reconstitution.

3.2.7 Reconstitution of $CD4^+$ T-cell population

In the next step, the reconstitution of $CD4^+$ T-cells was analysed. The data showed that the increase in the number of these cells from base line to the highest level within 2 months post-ACT was higher in arm A compared to arm B patients (p=0.008, Figure 3.12A). In addition, $CD4^+$ T-cells also expanded rapidly in arm A during the first month post ACT whilst their number in arm B declined (Figure 3.12B). For the overall period of study, there was not any statistically significant difference in median fold increases between arm A (13.1) and arm B (16.0) patients (p=0.45).

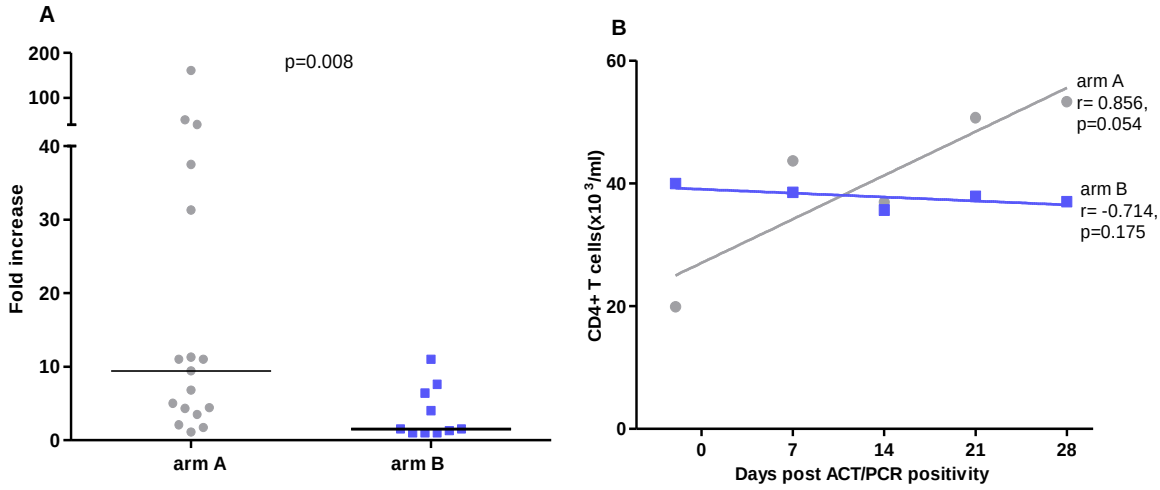


Figure 3.12 Reconstitution of $CD4^+$ T-cells in arms A and B patients.

(A) The fold increase in the number of $CD4^+$ T-cells was higher in arm A patients during the first 2 months post ACT.(B) The mean number of $CD4^+$ T-cells increased over one month post ACT in arm A whilst their number in arm B patients, experienced a decline.

3.2.8 Concomitant expansion of $CD4^+$ and $CD8^+$ T-cells

In both arm A and B patients, concomitant reconstitution of $CD4^+$ and $CD8^+$ T-cells was observed. Figure 3.13 shows a strong correlation between the frequencies of these cells post-ACT in all arm A patients. The correlation between expansion of $CD4^+$ and $CD8^+$ T-cells in arm B was also statistically significant (r=0.59, p=0.0001).

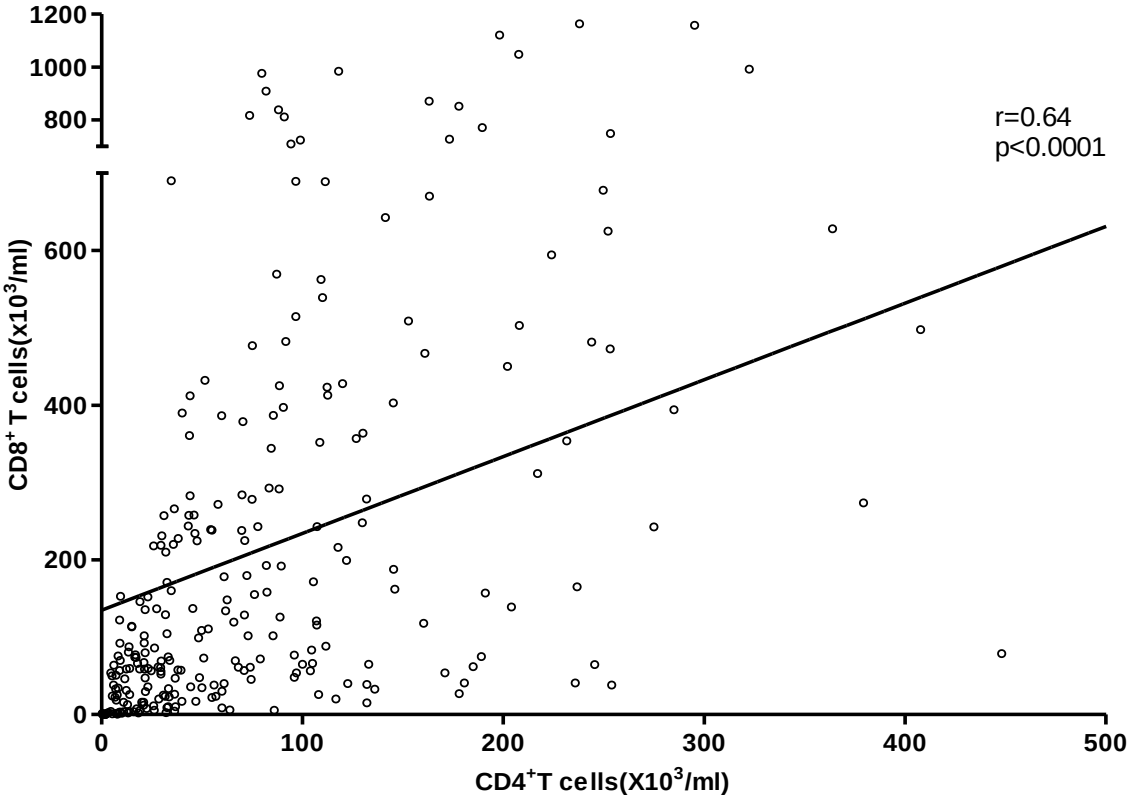


Figure 3.13 Co-expansion of $CD4^+$ and $CD8^+$ T-cells during immune reconstitution post-ACT in arm A.

There was a strong correlation between the numbers of peripheral blood $CD4^+$ and $CD8^+$ T-cells in arm A patients during the period of study.

3.2.9 CMV-specific CD4$^+$ and CD8$^+$ T-cells expanded co-ordinately

In order to study CMV-specific CD4$^+$ T-cells, and due to lack of appropriate multimers, a cell stimulation method with CMV lysate was performed to detect specific CD4$^+$ T-cell responses. After the incubation, CMV-specific CD4$^+$ T-cells were identified as IFN-γ producing cells. Analysis of IFN-γ production by CD4$^+$ T-cells in 40 samples collected from 9 arm A patients demonstrated a correlation between the number of CMV–specific CD4$^+$ T-cells (CD4$^+$ IFN-γ$^+$) and CMV-specific CD8$^+$ T-cells (detected by Streptamer® staining) (r=0.41, p=0.008) (Figure 3.14).

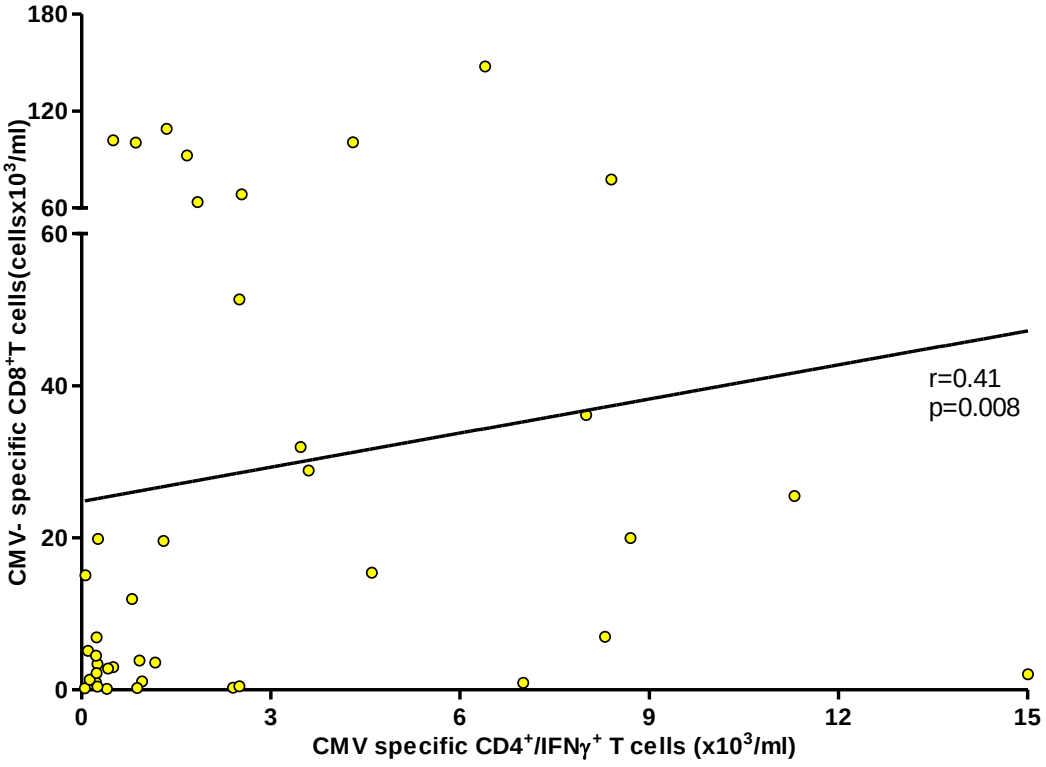


Figure 3.14 Concomitant reconstitution of CMV-specific CD4$^+$ and CD8$^+$ T-cells post-ACT.

CMV-specific T-cells expanded in parallel in 9 studied arm A patients.

Also as discussed in the next chapters, analysis of CMV-specific $CD4^+$ T-cells showed that CD57 co-expressed by most of these cells and $CD4^+$ $CD57^+$ T-cells were present only in CMV infected individuals. In this regard, reconstitution of these cells was studied in 8 arm A patients and correlated with the number of CMV-specific $CD8^+$ T-cells. Results showed a statistically significant correlation between the number of CMV-specific $CD8^+$ T-cells and the frequency of $CD4^+$ $CD57^+$ T-cells post-ACT (Figure 3.15).

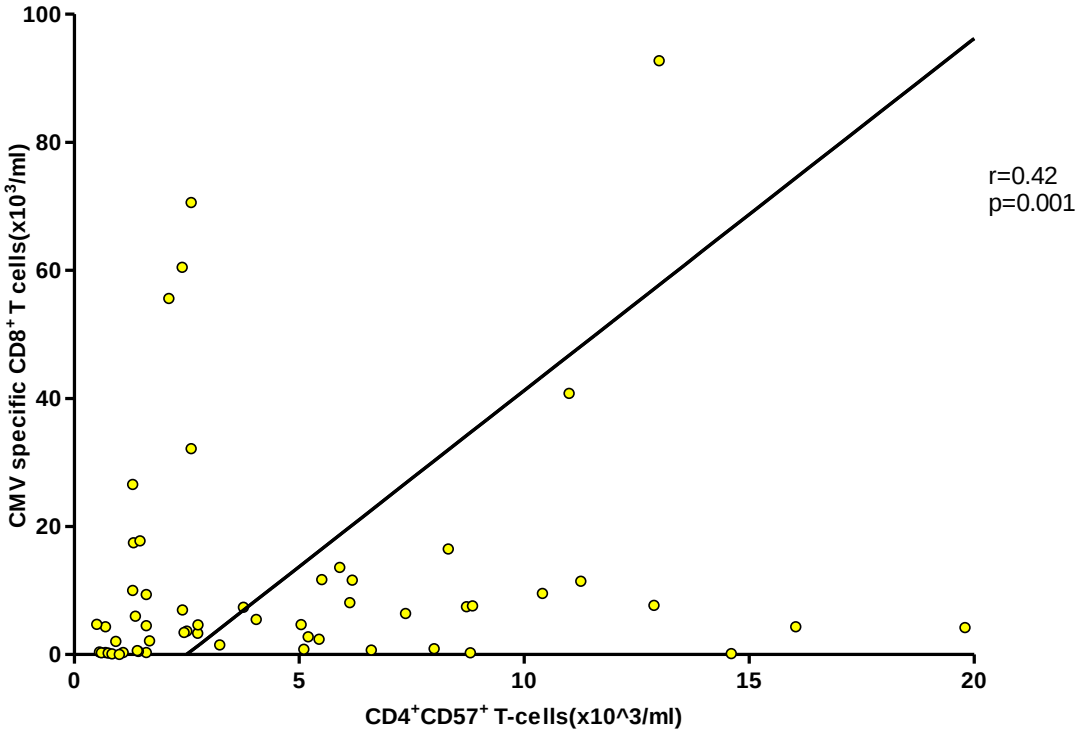


Figure 3.15 Co-expansion of CMV-specific $CD8^+$ T-cells and $CD4^+$ $CD57^+$ T-cells.

During immune response to CMV-reactivation, the frequency of $CD4^+$ $CD57^+$ T-cells increased in parallel with CMV-specific $CD8^+$ T-cells.

3.2.10 CMV specific antibody response

The IgG antibody response to CMV was measured using an in-house ELISA assay in 10 arm A and 5 arm B patients. In response to CMV reactivation, levels of anti-CMV antibody gradually increased over time in both groups. There was not any correlation between this increase and the time of study (Figure 3.16). The lowest detected Ab levels early post CMV reactivation were 176 and 226 units which increased to 309 and 406 units in arms A and B patients, respectively. This possibly reflects the fact that during the short period of this study, anti-CMV Abs were of recipient origin and synthesis of new Abs occurs late following HSCT.

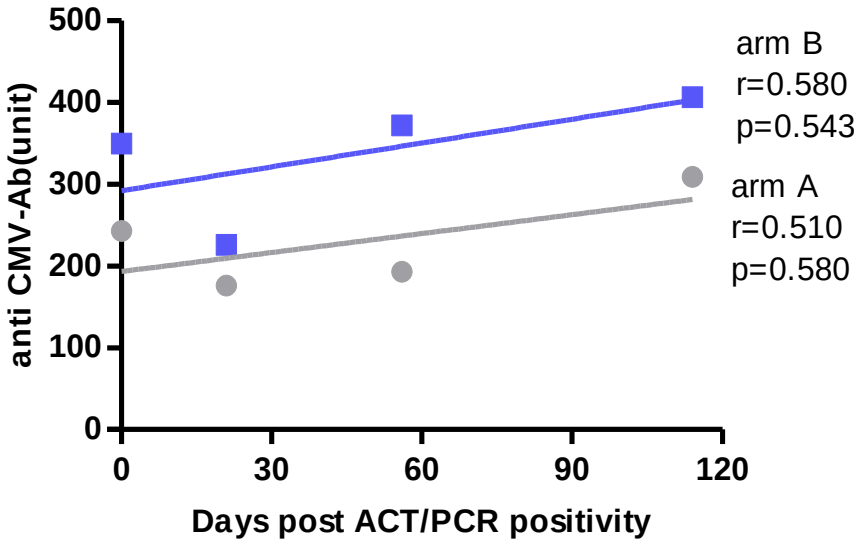


Figure 3.16 IgG response to CMV in arms A and B patients.

3.2.11 Functional behaviour of CMV-specific T-cells

3.2.11a Elispot results

In addition to the frequency of CMV-specific T-cells, assaying the functional activity of them is important for assessing the capacity of the cells to control CMV reactivation. Cytokine production following antigenic stimulation was studied using Elispot and intracellular staining methods. In Elispot, PBMCs were stimulated with CMV-derived epitopes consisting of 9 amino acids which stimulated $CD8^+$ T-cell responses. Following cell stimulation, IFN-γ response was analysed by counting the number of spots, representing individual cytokine producing cells (Figure 3.17).

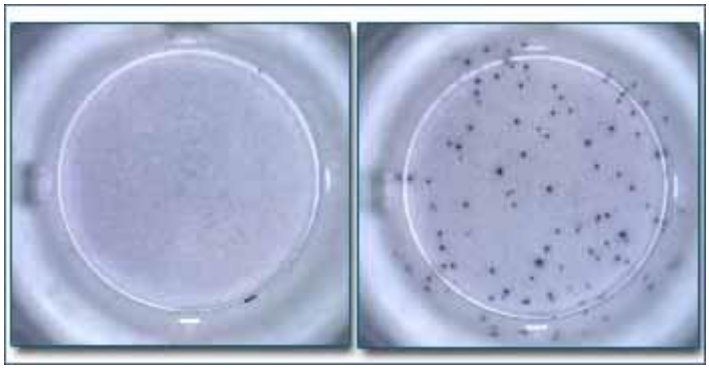


Figure 3.17 IFN-γ production in Elispot.

The left picture shows negative control for the assay and the right picture shows response of $1x10^5$ PBMC/well to the antigenic stimulation with CMV-derived antigens after overnight incubation as described in the method section. The number of dots was counted using AID Elispot reader.

Longitudinal analysis of 56 blood samples from 6 patients (05/36, 08/37, 03/43, 08/46, 05/40 and 05/47) demonstrated IFN-γ production in response to CMV derived epitopes

which fluctuated in accordance with the number of CMV-specific $CD8^+$ T-cells over time. Figure 3.18 shows the reconstitution of CMV-specific $CD8^+$ T-cells identified by Streptamers® and IFN- γ response (number of dots in Elispot plates).

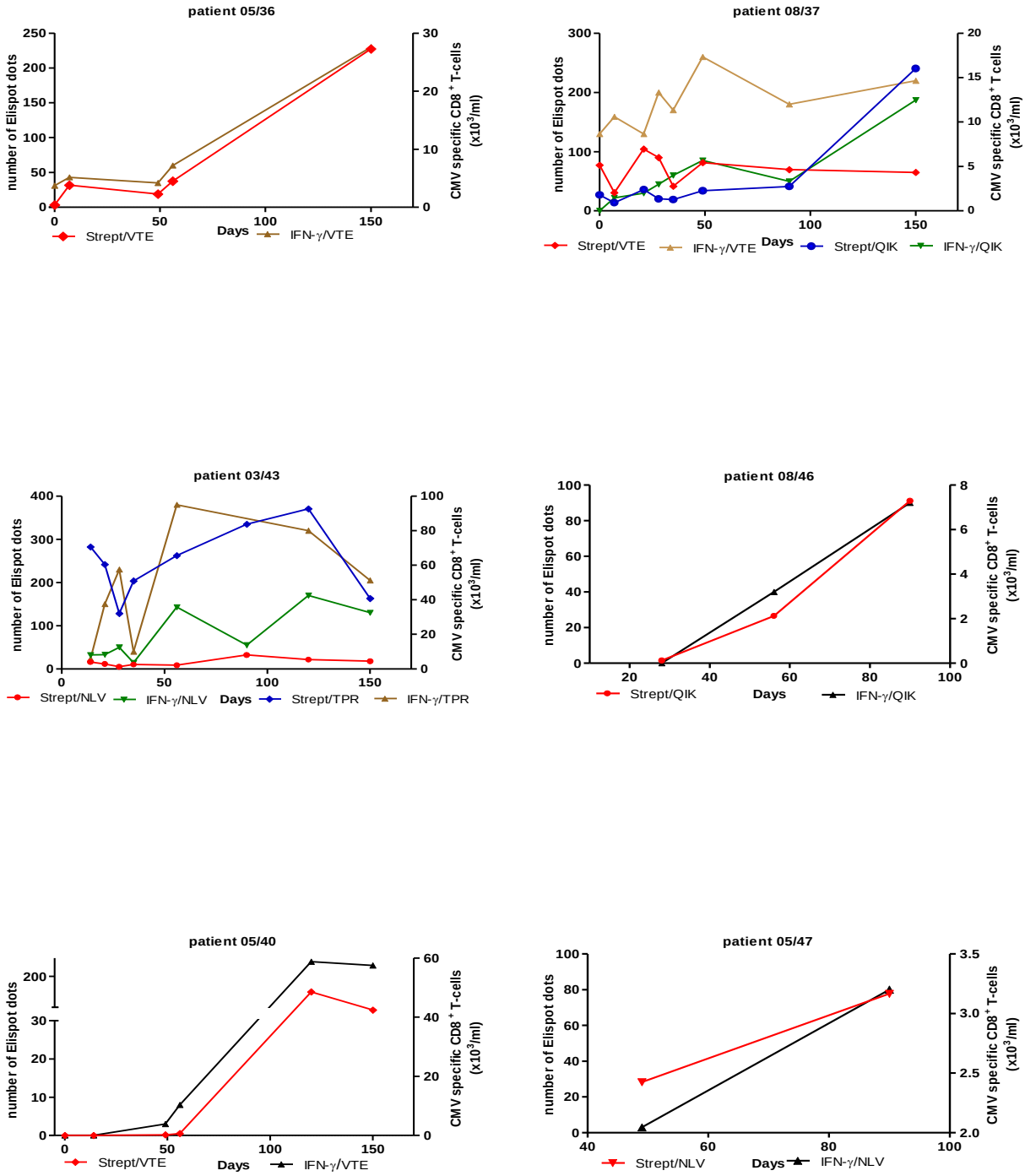


Figure 3.18 IFN-γ response to the CMV-derived peptides as measured by Elispot.

The figure shows Elispot responses (number of Elispot dots) and number of CMV-specific $CD8^+$ cells as determined by Streptamers® in 6 patients. Strept/peptide and IFN-γ/peptide represent the number of specific $CD8^+$ T-cells and Elispot dots in response to a particular peptide, respectively.

In the next step, the number of Elispot dots was converted to the number of CMV-specific IFN- γ producing cells/ml. To perform this, the number of dots were divided by the number of $CD8^+$ T-cells per 100µl of cell culture and then multiplied by the number of $CD8^+$ T-cells/ml. In all 6 patients, only a fraction of Streptamer$^{®+}$ cells made IFN-γ as the number of IFN- γ^+ cells were at range of 10 to 65% of Streptamer$^{®+}$ cells. Figure 3.18 represents the number of cells measured by Streptamer® staining and IFN-γ production in 2 patients. The ratio of IFN-γ^+/ Streptamer$^{®+}$ cells was in the range of 10-17% and 20-65% in these 2 patients (Figure 3.19 A and B, respectively). The red filled area in this figure was used for the better demonstration of these ratios.

Overall, there was a strong correlation between the number of CMV-specific $CD8^+$IFN-γ^+ cells/ml with the number of CMV-specific T-cells identified by Streptamer® staining (Figure 3.20, r=0.72, p<0.0001). These observations indicated functional activity of CMV–specific T-cells in the trial patients. However, it is very likely that due to the age of blood samples at the time of processing, the functional activity of T-cells was underestimated.

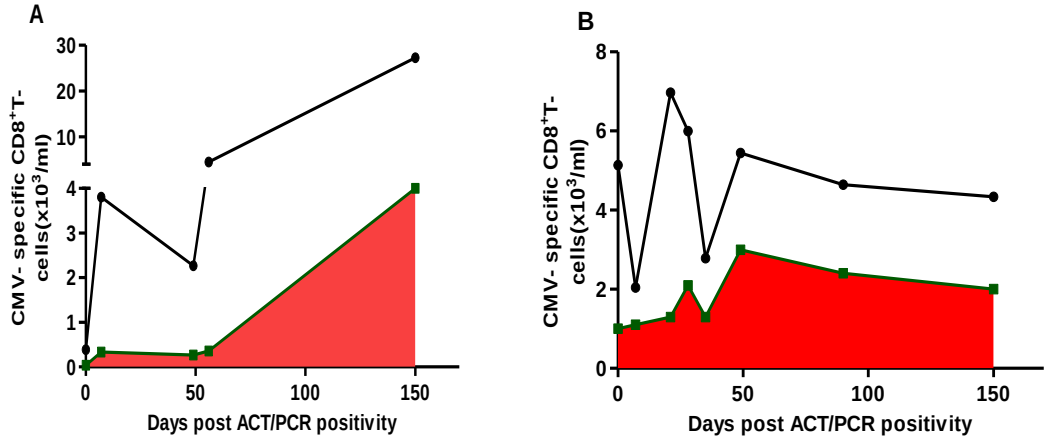


Figure 3.19 Longitudinal analysis of CMV-specific T-cells with Streptamer® staining and Elispot assay.

Figure 3.19 (continued). The frequency of CMV-specific $CD8^+$ T-cells by Streptamer® staining (black line) and IFN-γ production (red filled line). (A) and (B) represent 2 patients with low or high ratios of IFN-γ producing cells among CMV-specific $CD8^+$ T-cells.

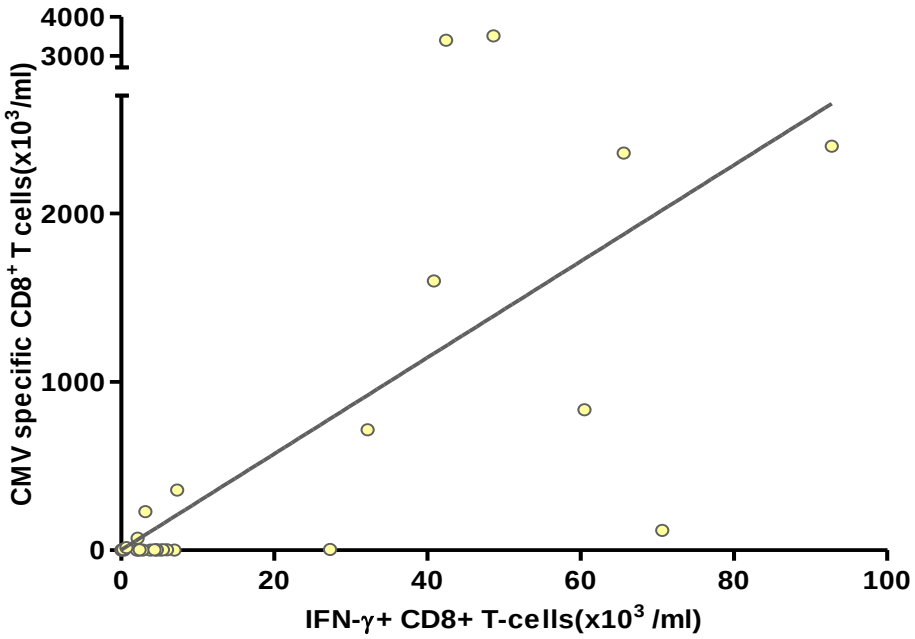


Figure 3.20 Correlation between the number of CMV-specific $CD8^+$ T-cells identified by Streptamer® staining and CMV-specific IFN-γ producing cells(r=0.72, p<0.0001).

IFN-γ production in response to the pooled peptides: pp65 and IE-1 was also analysed by Elispot. Longitudinal analysis showed changes in response to these peptides pools during the period of study which had some similarities with fluctuation of CMV-specific $CD8^+$ T-cell responses, as illustrated in Figure 3.21. It should be mentioned that pp65 and IE-1 contain antigenic epitopes for both $CD4^+$ and $CD8^+$ T-cells. In 3 patients, the CMV-specific $CD8^+$ T-cells, measured by Streptamer® staining, were specific for VTE peptide which is not present in pp65 or IE-1 (Figure 3.21).

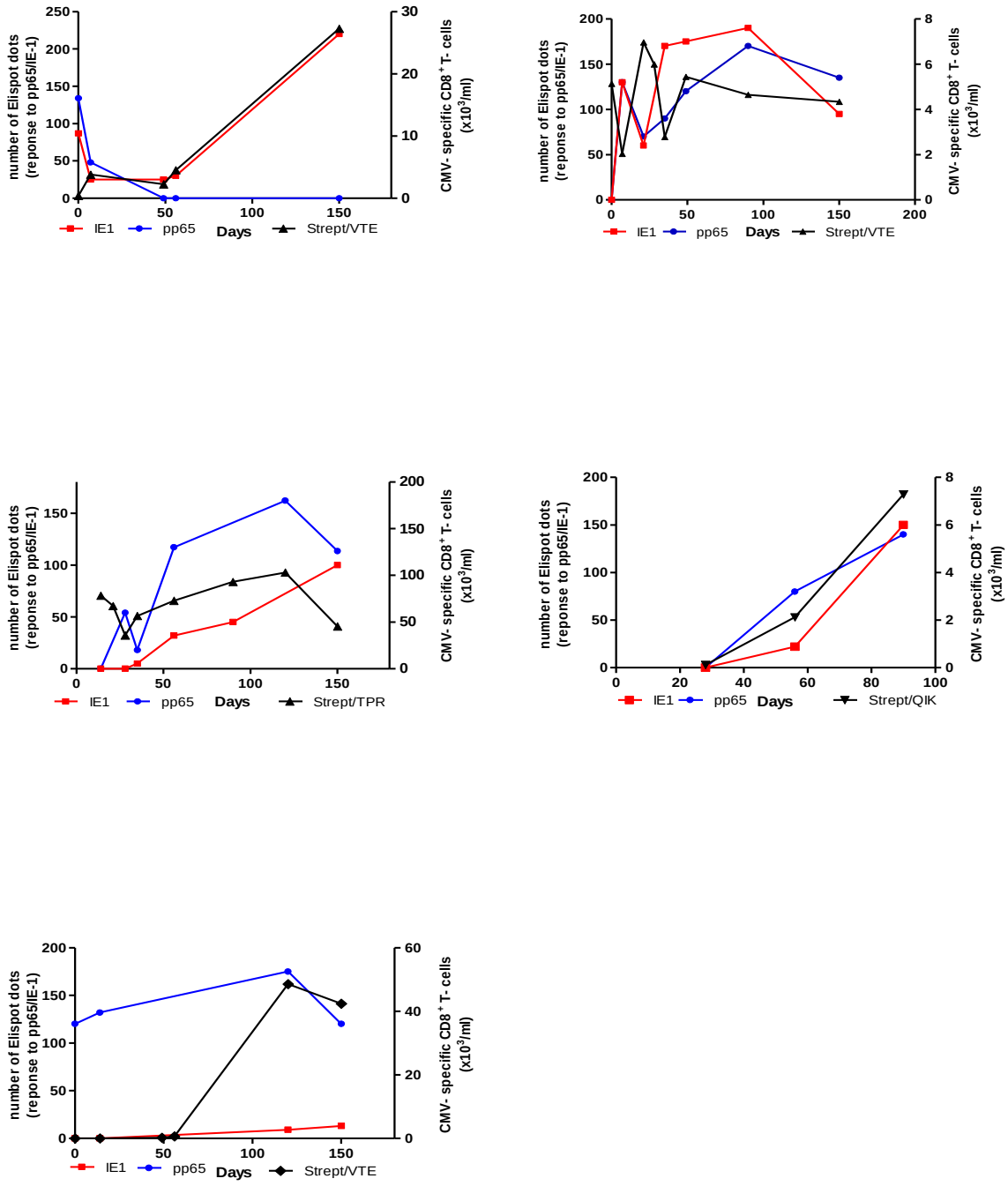


Figure 3.21 IFN-γ responses to pp65 and IE-1 peptide pools as detected by Elispot.

The figure shows the number of Elispot dots (IFN- γ response to pp65&IE-1**)** and number of CMV-specific $CD8^+$ T-cells as determined by Streptamers® in 5 patients. Strept/peptide represents the number of specific $CD8^+$ T-cells. Peptides: TPR and QIK belong to pp65 and IE-1 peptide pools, respectively (full list of CMV-derived peptide pools are in Table 2.2)

3.2.11b Multi-functional activity of CMV-specific T-cells as detected by intracellular cytokine production

Multiple cytokine production in response to antigens is an important indicator of functional properties of T-cells (Nebbia et al., 2008; Lichterfeld et al., 2004). In viral immunity, functional T-cells producing different cytokines have vital roles in control of viruses. Betts (2006) showed a strong association between the presence of multifunctional HIV-specific $CD8^+$ T-cells and control of HIV. In order to investigate the quality of CMV-specific T-cells, a flow cytometry-based intracellular staining of IFN-γ, IL-2 and TNF-α was conducted for 4 patients. PBMCs were stimulated in the presence of Brefeldin A in an overnight incubation with CMV-derived nanomeric peptides and CMV-lysate for stimulation of $CD8^+$ and $CD4^+$ T-cells, respectively. After cell surface and intracellular staining, flow cytometry based Boolean gating strategy was used to identify cytokine expression profiles of T-cells (Seder et al., 2008).This included analysis of cells producing all three cytokines (triple producers, TP) ($IL\text{-}2^+$ TNF^+ IFN- γ^+) or different combinations of two cytokines (double producers, DP) (TNF-α^+ $IL\text{-}2^+$, TNF-α^+ IFN- γ^+ , $IL\text{-}2^+$ IFN- γ^+) or only one cytokine (single producers, SP). In total, seven different conditions are possible: one TP, three DP and 3 SP cells.

As mentioned, the ACE trial blood samples were 24 hrs old at the time of cell processing and our preliminary experiments showed a negative effect of blood age on ability of cells to produce cytokines. Therefore, the quality of T-cells was underestimated in this study and in this regard, the assay did not continue beyond the 4 patients.Following subtracting the background values (from un-stimulated cells), the proportion of each of the seven subsets was expressed as a percentage of total cytokine producing cells. In overall, IFN- γ was the main produced cytokine during longitudinal

analysis of samples. Figure 3.22 represents cytokine production profile of CMV-specific T-cells in one patient during the period of study. In all four patients, IFN- γ was the main produced cytokine by both $CD4^+$ and $CD8^+$ T-cells and the frequency of IFN-γ^+ was much higher than the other two cytokines. This phenomenon was stable during the period of study.

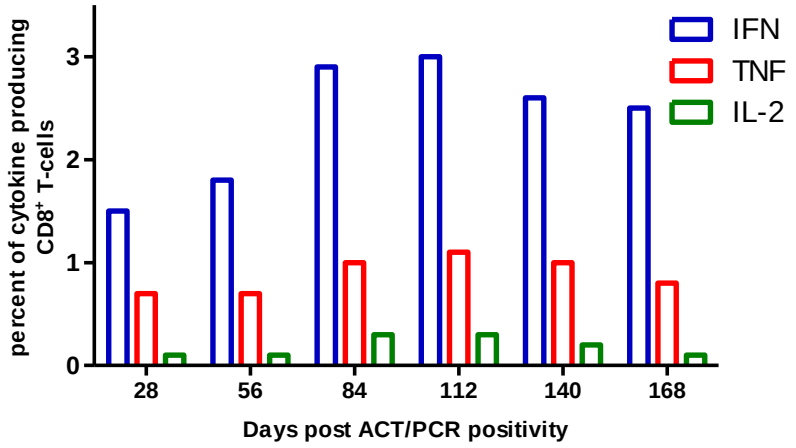
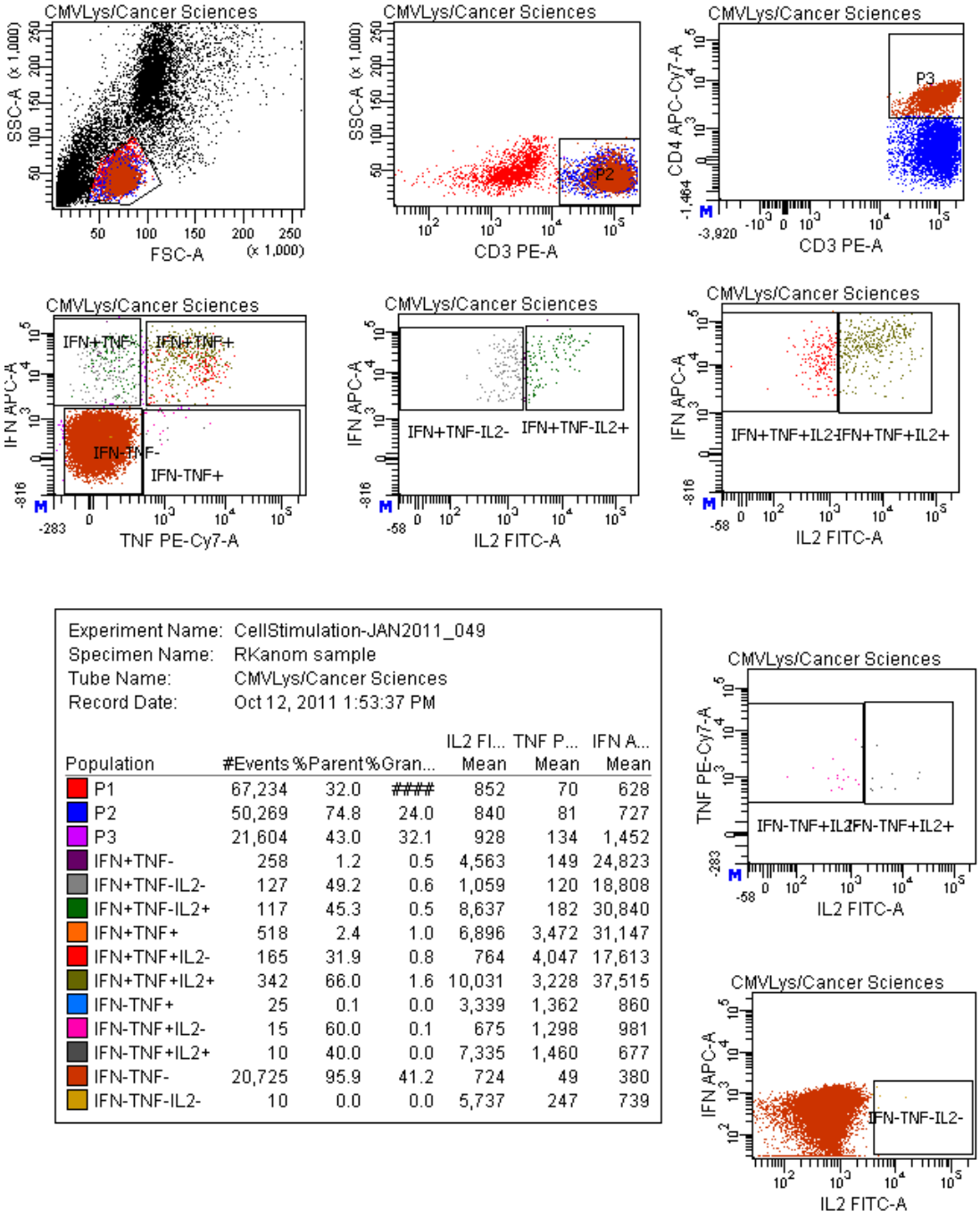


Figure 3.22 Cytokine production by $CD8^+$ T-cells following stimulation with CMV-derived peptides.

In the next step, the frequencies of SP, DP and TP cells were analysed. In $CD8^+$ population, the frequency of SP cells producing only IFN-γ (76%) was more than the other cells which was followed by TP (14%) and TNF-α $^+$ IFN-γ^+ (10%). There were no detection of cells making only TNF-α or IL-2 or both and IL-2^+ IFN-γ^+ cells.

In $CD4^+$ T-cells, SP cells making IFN-γ had higher frequency (72%). The other cells were: TP (15%), TNF-α $^+$ IFN- γ^+ (7%) and only IL-2^+ cells (6%).

Figure 3.23A is an example of flow cytometry panels used for the analysis of cytokine producing cells in response to CMV lysate in order to detect CMV-specific $CD4^+$ T-cells producing different cytokines. Figure 3.23B is an example of negative control for the cell stimulation with a cell lysate without CMV antigens.

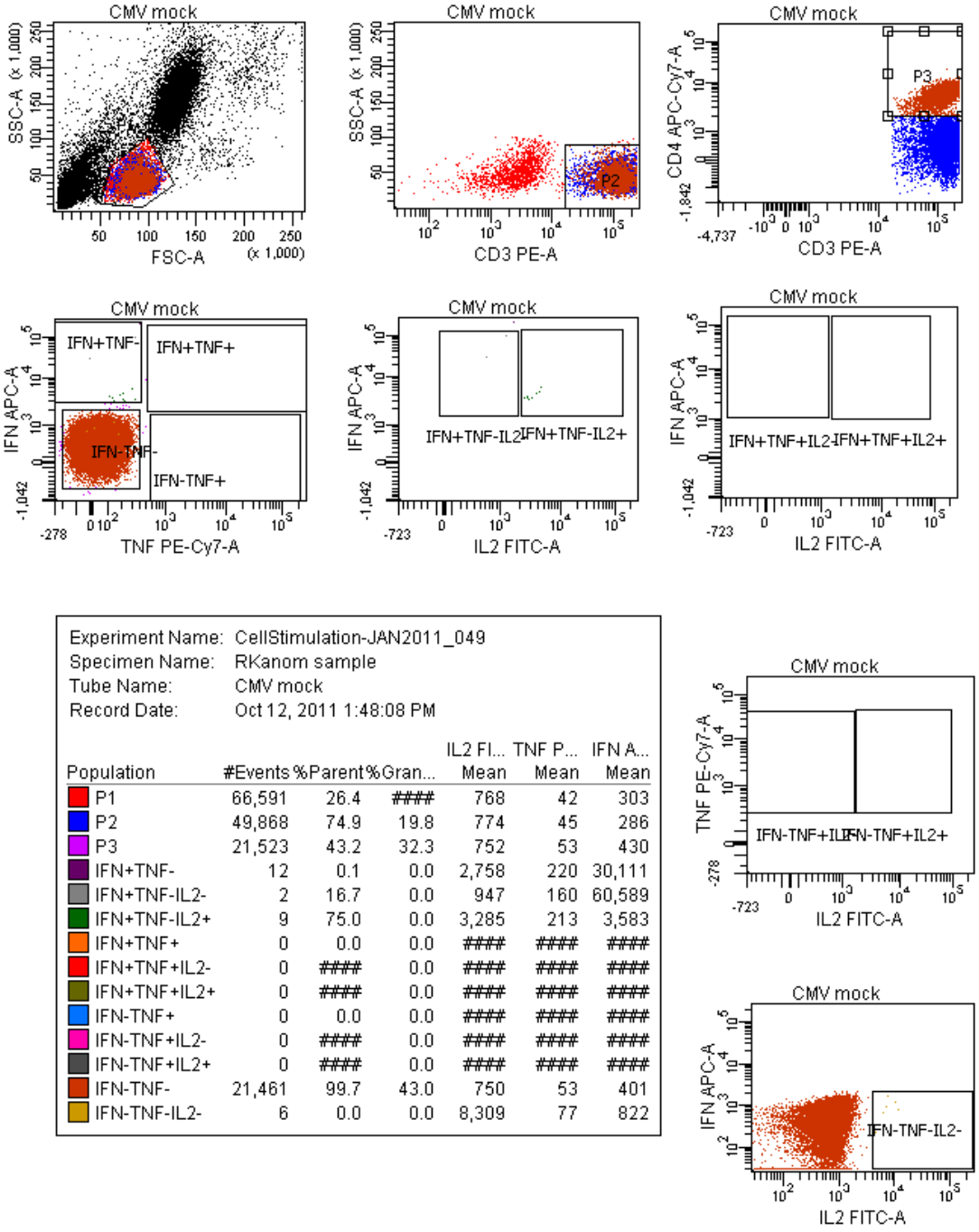


Experiment Name: CellStimulation-JAN2011_049
Specimen Name: RKanom sample
Tube Name: CMVLys/Cancer Sciences
Record Date: Oct 12, 2011 1:53:37 PM

Population	#Events	%Parent	%Gran...	IL2 FI... Mean	TNF P... Mean	IFN A... Mean
P1	67,234	32.0	####	852	70	628
P2	50,269	74.8	24.0	840	81	727
P3	21,604	43.0	32.1	928	134	1,452
IFN+TNF-	258	1.2	0.5	4,563	149	24,823
IFN+TNF-IL2-	127	49.2	0.6	1,059	120	18,808
IFN+TNF-IL2+	117	45.3	0.5	8,637	182	30,840
IFN+TNF+	518	2.4	1.0	6,896	3,472	31,147
IFN+TNF+IL2-	165	31.9	0.8	764	4,047	17,613
IFN+TNF+IL2+	342	66.0	1.6	10,031	3,228	37,515
IFN-TNF+	25	0.1	0.0	3,339	1,362	860
IFN-TNF+IL2-	15	60.0	0.1	675	1,298	981
IFN-TNF+IL2+	10	40.0	0.0	7,335	1,460	677
IFN-TNF-	20,725	95.9	41.2	724	49	380
IFN-TNF-IL2-	10	0.0	0.0	5,737	247	739

Figure 3.23A Intracellular cytokine production.

Production of IFN-γ, TNF-α and IL-2 in response to CMV antigens. The gating strategy was first to identify CD4+ CD3+ cells (third panel in the first row). Then, IFN-γ/TNF- α producing cells were identified (the forth panel) (double positive cells). This was followed by identification of IL-2 producing cells among IFN-γ and TNF-α positive cells (triple positive cells making all the three cytokines) (6th panel). Other possible double producing cells were also identified (5th, 7th and 8th panels).



Experiment Name: CellStimulation-JAN2011_049
Specimen Name: RKanom sample
Tube Name: CMV mock
Record Date: Oct 12, 2011 1:48:08 PM

Population	#Events	%Parent	%Gran...	IL2 Fl... Mean	TNF P... Mean	IFN A... Mean
P1	66,591	26.4	####	768	42	303
P2	49,868	74.9	19.8	774	45	286
P3	21,523	43.2	32.3	752	53	430
IFN+TNF-	12	0.1	0.0	2,758	220	30,111
IFN+TNF-IL2-	2	16.7	0.0	947	160	60,589
IFN+TNF-IL2+	9	75.0	0.0	3,285	213	3,583
IFN+TNF+	0	0.0	0.0	####	####	####
IFN+TNF+IL2-	0	####	0.0	####	####	####
IFN+TNF+IL2+	0	####	0.0	####	####	####
IFN-TNF+	0	0.0	0.0	####	####	####
IFN-TNF+IL2-	0	####	0.0	####	####	####
IFN-TNF+IL2+	0	####	0.0	####	####	####
IFN-TNF-	21,461	99.7	43.0	750	53	401
IFN-TNF-IL2-	6	0.0	0.0	8,309	77	822

Figure 3.23B Intracellular cytokine production.

FACS panels show lack of cytokine response in the negative control for the cell stimulation assay.

3.2.12 Other results for ACE-ASPECT trial

At the time of writing this thesis, there was no access to full CMV virology data, circulating levels of Alemtuzumab, frequency of CMV-specific $CD8^+$ T-cells in the donors, dose of infused cells and anti-viral treatment duration. Some data on these parameters were orally presented at BMT Tandem meeting, Texas in 2014 and published as an abstract (Chen et al., 2014, Appendix 2A). The following results have been released so far:

a) Alemtuzumab concentration; higher concentration of Alemtuzumab at the time of cell infusion led to a statistically significant lower number of reconstituting CMV-specific $CD8^+$ T-cells (Figure 3.24). This figure shows that patients in arm A who did not reach to the trial end point (10^4 cells/ml) had higher levels of circulating Alemtuzumab in comparison to the patients who reached to this point ($p=0.0076$). Therefore, Alemtuzumab level has direct effect on CMV-specific T-cell reconstitution.

b) Cell dose: number of infused cells had positive effect on reaching to the trial end point (Figure 3.24). Arm A patients who reached to the trial end point, on average, received $18x10^3$/kg CTLs whilst patients who did not, received around four times less specific cells ($5x10^3$/kg) ($p=0.0215$).

c) Donor CTL frequency: the frequency of CMV-specific CTLs in the donors had no effect on achieving the trial end point.

By considering a cut off level of Alemtuzumab at 0.15 mg/ml and cell dose of 1000/kg, patients who had less than this level of Alemtuzumab and received more than 1000 cells/kg, experienced bigger cell expansion following cell therapy ($p=0.0413$) (Figure 3.25).

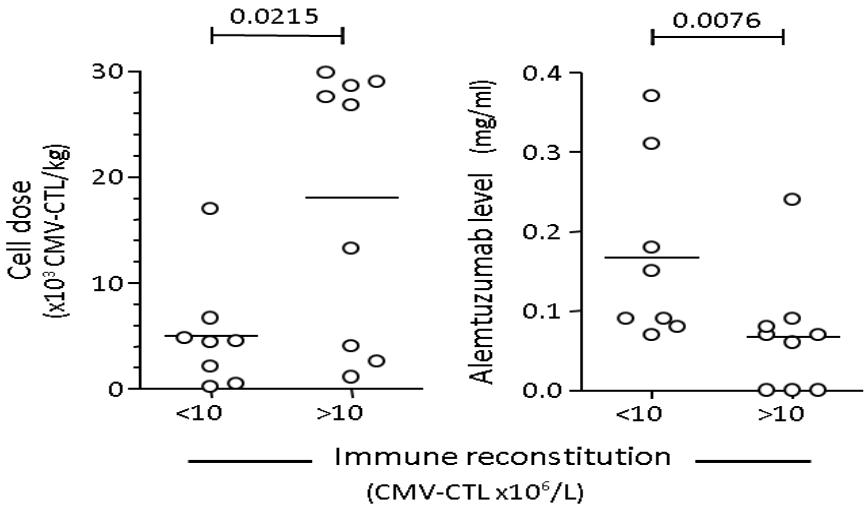


Figure 3.24 Effects of CTL dose and Alemtuzumab level on achieving the trial end point. (from Dr K.Peggs presentation, BMT Tandem meeting, 2014)

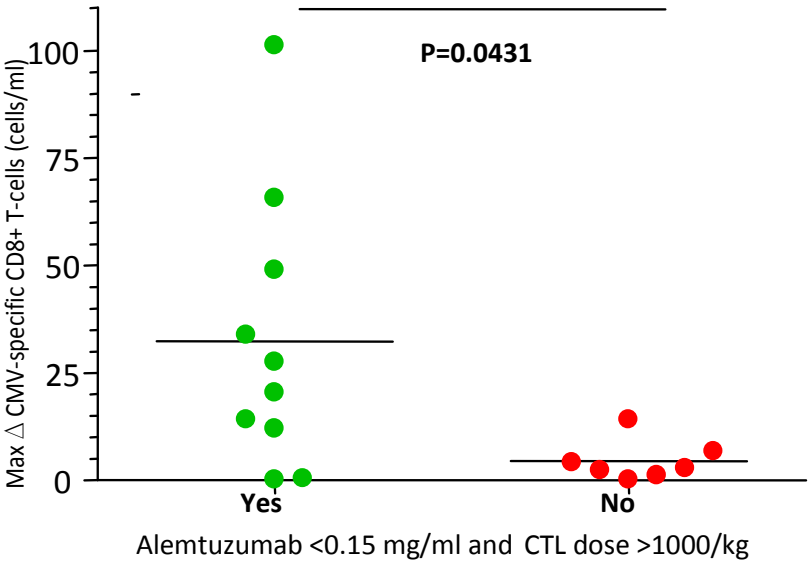


Figure 3.25 Combinational effects of Alemtuzumab level and CTL dose on reconstitution of CMV-specific CD8$^+$ T-cells. (Dr K.Peggs, BMT Tandem meeting, 2014)

As it is demonstrated in the above figure, the trial end point (10^4 cells/ml) was achieved in 8 out of 10 patients with baseline Alemtuzumab <0.15 mg/ml and CTL dose

>1000/kg and in 1/7 of those with either baseline Alemtuzumab >0.15 mg/ml or CTL dose <1000/kg (p = 0.0134).

It was also reported that incidence of new onset grade II-IV GVHD post ACT did not differ significantly between the cellular therapy and control arm (17.6% vs 27.3% respectively).The frequencies of new GVHD in the cellular therapy arm were: 1 Grade II and 2 Grade III and in arm B were: 2 Grade II and 1 Grade III (BMT Tandem meeting, 2014).

3.3 Discussion

This chapter presented the results of CMV-specific T-cell reconstitution in ACE-ASPECT trial patients. In this first randomized trial of adoptive immunotherapy of CMV-specific $CD8^+$ T-cells in T-cell depleted unrelated donor allogeneic HSCT patients, the primary end point was the reconstitution of CMV-specific T-cells above $1x10^4$/ml within two months post-ACT (or first positive CMV-PCR result in the control group).

In both therapeutic and control groups, the trial end point at 2 months was achieved in considerable numbers of patients and consequently, no statistically significant difference between these two groups was observed. However, a number of patients in the ACT group had low recovery of adoptively transferred T-cells, and this was found to be associated with the serum level of Alemtuzumab and the dose of infused cells. Patients who received on average $18x10^3$/kg CMV-specific $CD8^+$ T-cells and had lower level of Alemtuzumab at the time of cell infusion (below 0.15 mg/ml) were able to reconstitute more CMV-specific T-cells and reach to the trial end point.

It has been shown that Alemtuzumab remains in the circulation of HSCT patients in up to 56 days post-HSCT at the concentration above lympholytic level (> 0.1 µg/ml) (Morris et al., 2003). ACE-ASPECT trial patients received therapeutic cells at 28 days post-HSCT when residual Alentuzumab was still in circulation, which may explain the poor reconstitution of of the infused cells. The reported concentration of Alemtuzumab in ACE-ASPECT trial (Figure 3.23) at around day 28 post-HSCT is in the range of 100-400 µg/ml which is much higher than the level reported by Morris (2003). At the time of writing this thesis, the method used for measuring Alemtuzumab was still been reviewed. The mean number of cells infused cells into patients who did not reach to the

trial end point was less than $3x10^3$/kg. In the phase I CMV specific T-cell therapy trial, the lowest number of infused cells was around $1.5x10^3$/kg and interestingly, these patients had the lowest number of reconstituting CTLs at their peak level (Cobbold et al., 2005). However, these patients were able to produce CTLs above $10x10^3$ cells/ml. It is therefore important that both cell dose and Alemtuzumab should be considered as contributing factors to the efficacy of the ACT. As revealed by the data, 80% of patients with Alemtuzumab level <0.15 mg/ml and CTL dose $>10^3$/kg, reached the trial end point whilst only 14% of patients with Alemtuzumab level >0.15 mg/ml and CTL dose $<10^3$/kg did.

The mean peak numbers of CTLs in ACT patients and the control group within 2 months post-ACT/viraemia were around 3 times higher than the trial end point (35 and $25x10^3$ /ml, respectively). This indicates that some of HSCT patients were able to produce appropriate number of CMV-specific $CD8^+$ T-cells, regardless of immunotherapy. As discussed in the next chapter, half of HSCT patients receiving Alemtuzumab-containing conditioning chemotherapy experience only one episode of CMV viraemia and these patients were able to mount sufficient number of CTLs to control the viraemia. The minimum protective level of CTLs reported by this thesis (next chapter) and other studies range from 7 to $20x10^3$/ml (Gratama et al., 2010; Cwynarski et al., 2001; Aubert et al., 2001). In this regard, CMV specific T-cell therapy may not be necessary for boosting CMV immunity in patients who rapidly expand sufficient CMV-specific T-cells in response to the first CMV reactivation. In the next chapter we will discuss how patients could be stratified based on CMV-specific T-cell levels to help identify patients who could benefit most from immunotherapy.

Despite the presence of similar results in the patients of both arms in regard to the trial end point, the fold increase in CTL numbers from baseline to the peak within 2 months post-ACT was higher in ACT arm. This indicates that cellular therapy resulted in a greater CMV-specific $CD8^+$ T-cell expansion in response to CMV viraemia. Detailed analysis of the kinetics of response showed that this greater cell expansion in the cell therapy arm occurs only during the first month post-ACT. In this time period, there was a clear statistically significant difference between the 2 arms, with CTLs expanding exponentially in the ACT arm. However, over the whole 6 month of immune monitoring, both groups experienced similar overall degree of cell expansion. In other words, cell expansion in ACT patients was more rapid only at the beginning of cell therapy. In accordance with the bigger CTL expansion in a short time period post-ACT, patients also experienced bigger increase in their total $CD3^+$ and $CD8^+$ counts. Interestingly, CMV-specific T-cells that had antigen specificities other than the infused cells, also co-expanded rapidly in parallel with the infused CMV-specific T-cells. Expansion of $CD4^+$ T-cells was also positively influenced by ACT. This means that during the first month post-ACT of CMV-specific $CD8^+$ T-cells, both $CD8^+$ and $CD4^+$ populations expanded in parallel and that their fold increases from baseline to the peak were higher in comparison to the control group. It should be noted that the co-expansion of $CD4^+$ and $CD8^+$ T-cells is a feature of T-cell reconstitution which was also observed in the control group and non-trial HSCT patients (next chapter).

These findings suggest that adoptively transferred $CD8^+$ T-cells have a non-specific boosting effect on concomitant reconstitution of T-cell immunity shortly post-ACT. This impact of ACT might become less effective as time passes. This thesis did not have opportunity to explore some of physiological mechanisms behind the boosting

effect of T-cell therapy. Based up on the well documented data on T-cell reconstitution post-HSCT, it is possible to speculate involvement of different factors especially cytokines and co-stimulatory molecules in the rapid cell expansion in ACT patients. T-cell reconstitution after HSCT relies on thymopoiesis and expansion of mature T-cells. Thymopoiesis is a delayed process which occurs 1 up to 2 years post-HSCT (Krenger et al., 2011) and initial T-cell reconstitution depends on proliferation of mature T-cells derived from the donor infusion and driven by high homeostatic cytokine levels and antigen exposure, in a process called homeostatic peripheral expansion (HPE).

IL-7 and IL-15 are two important HPE cytokines that induce naïve and memory T-cell proliferation post-HSCT, respectively. However, HPE can also be constrained by regulatory T-cells and cytokines such as TGF-β. It has been shown that following lymphocyte depletion, there is an increase in the level of IL-7 possibly due to the less consumption of this cytokine. This high level of IL-7 leads to the considerable increase in the number of naïve T-cells (Fry and Mackall, 2001). As shown in chapter 5, naïve T-cells express IL-7Rα and following cell differentiation, frequency of IL-7Rα positive cells decreases.

In opposing pattern, expression of IL-15Rα increases following antigenic exposure (Chapter 5) (Geginat etal., 2003). IL-15 is produced constitutively by tissues and antigen-presenting cells and its level increases after severe lymphocyte depletion. Different studies have shown the important role of IL-15 in expanding of memory/effector memory T-cells. Proliferation of effector memory $CD8^+$ $CD28^-$ T-cells depends on IL-15 and its induced cytokines such as TNF-α (Chiu et al., 2006). Concomitant TCR engagement and IL-15 stimulation induce $CD4^+$ T-cell proliferation (Van Belle, et al., 2012). Interestingly, IL-15 is also a potent inducer of $CD4^+$ $FOXP3^+$

T-cells that are involved in the control of homeostatic lymphocyte expansion post-HSCT (Imamichi et al., 2008). Human and animal studies have shown that initial period post-HSCT with high levels of IL-7 and IL-15 provides an optimal milieu for T-cell immunotherapy (William and Gress, 2008). In this period, transferred cells consume cytokines and out-compete T-reg cells. Rapid increase in the number of CTLs within 1 month post-ACT may be a result of CTL response to antigenic stimulation and presence of cytokines such as IL-15. In this short time period, the number of transferred cells is initially greater than other cells including T-reg. This phase of immune response is followed by gradual proliferation of T-reg that can compete with CTLs and control their proliferation. The initial cytokine milieu and antigenic stimulation are similar in both arms of the trial and the only difference is the transfer of CTLs in ACT group. It is possible that the initial expansion of infused cells lead to greater consumption and competition for the cytokines by the infused CMV-specific $CD8^+$ T cells, thus outcompeting the T-regs. We can further speculate that these activated CTLs influence $CD4^+$ T-cell reconstitution through release of cytokines and expression of co-stimulatory molecules (such as CD40L). It has been shown that co-culture of effector memory $CD4^+$ and $CD8^+$ T-cells induces expression of CD40 and CD40L in both of these cells. Consequently, CD40/CD40L interaction causes a bi-directional stimulation of effector memory $CD40^+$ $CD4^+$ T-cells by effector memory $CD40L^+$ $CD8^+$ T-cells and of $CD40^+$ $CD8^+$ T (EM) by $CD40L^+$ $CD4^+$ T (EM) (Xydia et al., 2011). As demonstrated, CTLs were able to produce cytokines such as IFN-γ and IL-2. IFN-γ is a potent inducer of HLA-class II expression on APC. This facilitates more antigenic exposure for $CD4^+$ T-cells and leads to their augmented activation and proliferation. Murine $CD8^+$ T-cells do not transcribe MHC class II (MHC-II) genes, however it has

been reported that MHC-II molecules present on activated murine $CD8^+$ T-cells in vitro as well as in vivo. These MHC-II molecules are acquired via trogocytosis by $CD8^+$ T-cells from their activating APCs, particularly CD11c positive dendritic cells (DCs). These transferred MHC-II molecules on activated murine $CD8^+$ T-cells were functionally competent in stimulating specific $CD4^+$ T-cells to proliferate and produce high levels of Th1 cytokines. These $CD4^+$ T-cells in return help $CD8^+$ T-cells (Romagnoli et al., 2013). One of these cytokines is IFN-γ that activates IL-15 production by monocytes and DCs which in return induce T-cell proliferation (Perera et al., 2012). Therefore, activation of higher number of CTLs in ACT patients, in comparison to the control group, set the stage for augmented stimulation and activity of other cells such APCs and $CD4^+$ T-cells. One month after rapid and enhanced proliferation of CMV-specific T-cells in ACT patients, a decline in this process occurred and cell expansion became similar to the control group. These results may also provide evidence for a best time frame for immunotherapy. As it was shown, in one month post-ACT, cell expansion was greater and the biggest fold increase in T-cell counts occurred.

Functional behaviour of CMV-specific T-cells is also an important factor in control of viraemia (Seder et al., 2008). By using 2 different methods of Elispot and intracellular staining, the ability of reconstituting CMV-specific T-cells in producing IFN-γ was shown. The main challenge was the age of blood samples which were at least 24 hrs old, and our preliminary experiments showed the detrimental effect of age on cytokine production. Therefore, cytokine production was likely underestimated in our experiments. IFN- γ was the main cytokine produced and depending on the patient, 10 to 65% of CTLs (measured by Streptamer® staining) were able to make this cytokine.

Despite the considerable frequency of IFN-γ producing cells, production of TNF-α and IL-2 were low which may be related to the age of blood at the time of functional analysis.

In conclusion, the current data from the first randomised adoptive immunotherapy of CMV-specific $CD8^+$ T-cells indicated rapid and greater reconstitution of CMV-specific T-cells in patients who received ACT. Although there was no difference in reaching to the trial end point between ACT group and the control, immunotherapy not only led to early expansion of infused cells, but also induced bigger expansion of non-infused $CD4^+$ T-cells. The presented data also indicated that the infusion of antigen-specific therapeutic cells led to a general boost of T-cell immune reconstitution. Immunotherapy might not be necessary for patients who are able to produce enough CMV-specific T-cells and in this regard, CMV-specific T-cells quantification prior to the cell therapy could potentially identify high risk patients at need of T-cell immunity. This issue will be discussed in more detail in the next chapter.

Chapter 4

CHARACTERIZATION OF CMV- SPECIFIC $CD4^+$ T-CELL RECONSTITUTION POST-HSCT THROUGH THE USE OF HLA-CLASS II-TETRAMERS

4.1 Background

CMV infection is generally well controlled in the immunocompetent host, but viral reactivation may occur during immunosuppressive treatment following HSCT (Ljungman et al., 2011). CMV reactivation remains very common after T-cell depleted or allogenic transplants in which either the patient or donor are CMV sero-positive and up to half of such patients suffer from recurrent or late episodes of reactivation with impaired clinical outcome (Chakrabarti et al.,2002:, Boeckh et al., 2003).

Several studies have focussed on the role of $CD8^+$ T-cells in controlling CMV reactivation (Quinnan et al., 1982; Reusser et al., 1991, Li et al., 1994; Walter et al. 1995). Using HLA-multimers to enumerate CMV-specific $CD8^+$ T-cells permitted a rapid assessment of the magnitude and function of these cells. Different research groups reported protective level of CMV–specific $CD8^+$ T-cells which ranged from $7x10^3$/ml (Gratama et al., 2010) to $20x10^3$/ml (Aubert et al., 2001). Additionally, HLA-multimers have helped scientists to obtain valuable information on immunology of viral specific $CD8^+$ T-cells (Appay et al., 2002). Their utility in clinical practice remains to be established, but they have facilitated the development of adoptive cellular immunotherapy through the direct selection of CMV-specific T- cells from the transplant donor (Chapter 3) (Cobbold et al., 2005).

Despite these studies, little is known regarding the nature of the CMV-specific $CD4^+$ T-cell immune response, partly due to the lack of experimental tools for direct study of them. They appear important in sustaining a functional virus-specific $CD8^+$ immune response (Walter et all, 1995) and in renal transplantation they have been shown to protect against primary CMV infection (Gamadia et al., 2004). $CD4^+$ T-cells have also

been implicated in the success of adoptive immunotherapy for EBV-associated PTLD (Haque et al., 2007). However, the kinetics and role of CMV-specific $CD4^+$ T-cells following HSCT is not well defined (Widmann et al., 2008; Eid et al., 2009; Pourgheysari et al., 2009; Tormo et al., 2010). Some authors have reported a fall in the virus-specific $CD4^+$ T-cell response following viral reactivation whilst others have indicated the need for a minimum threshold to control viraemia (Eid et al., 2009; Tormo et al., 2010). However, most of this information regarding CMV-specific $CD4^+$ T-cells was acquired using indirect functional interferon-γ secretion assays which only detect cells that are capable of producing cytokine in response to antigen. These assays require antigenic stimulation of the virus-specific population, routinely overnight incubation, and cannot provide an accurate phenotype of the cells when taken directly from blood or lymphoid tissue. In an attempt to overcome this issue and establish a rapid assay, Sester's group introduced an indirect phenotyping method. They used effector memory $CD4^+$ T-cells with the phenotype of $CD27^-$ $CD28^-$ as a bio-marker of CMV-specific $CD4^+$ T-cells and showed association of $CD4^+$ $CD27^-$ $CD28^-$ $PD\text{-}1^+$ with viraemia in transplant recipients (Dirks et al., 2013). The identification of antigen-specific T-cells with HLA-tetramers is a direct method for visualising antigen specific T-cells and is based on HLA-peptide specificity and independent of the functional viability of the cells.

The observation of $CD4^+$ T-cell co-evolvement with $CD8^+$ T-cells in the clinical trial samples, persuaded another study with more focus on CMV-specific $CD4^+$ T-cell response following viraemia in HSCT patients. In addition, the presence of quality controlled HLA- class I multimer staining which had been developed for the trial, helped to design and conduct a HLA-class II multimer staining assay. The only

available HLA-class II multimer specific for CMV was found as HLA-DRB1*0701 restricted tetramer with CMV derived peptide: DYSNTHSTRYV. It has been shown that this peptide provokes a strong CD4 response (Crompton et al., 2008).Therefore, HSCT patients with HLA-DRB1*0701 were chosen for this study. The frequency of this HLA-class II is around 10% in the UK population and it became possible to study frozen PBMCs from 20 non-trial CMV sero-positive HSCT patients. These samples had been collected from 2003 to 2013.

The objective of the presented study in this chapter, was to use HLA-class II peptide-tetramers for the first time to interrogate the reconstitution of CMV-specific $CD4^+$ T-cells following transplantation. This information was used to examine the relative kinetics and phenotype of the CMV-specific $CD4^+$ alongside of $CD8^+$ T-cell immune response and to relate these findings to the clinical control of viraemia in order to inform clinical practice.

4.2 Results

4.2.1 CMV reactivation was common and over 40% of patients suffered from multiple episodes of viraemia

This study recruited 20 HSCT patients with 18 of them received a reduced-intensity conditioned transplant which incorporated TCD using either intravenous Alemtuzumab (CD52-specific monoclonal antibody) or ATG (Table 4.1). Two patients (04, 13) received myeloablative transplants without TCD. Patient 04 did not suffer from viral reactivation and patient 13 reactivated whilst receiving steroids for gut GVHD.

In 15 out of 19 patients with the CMV reactivation, viraemia occurred early and became apparent within 6 weeks. Of these 19, ten patients (53%) displayed only a single

episode of viral reactivation whereas the other nine (47%) suffered from multiple viremic relapses (Table 4.2). Examples of the viral reactivation patterns observed in the patient cohort are shown in Figure 4.1. Within the group which suffered from multiple viral reactivations, patients 05 and 06 had prolonged episodes of viraemia refractory to anti-viral treatment lasting 210 days and 77 days respectively. Patient 11 and 16 had three episodes of viral reactivation whereas patient 12 had four. Importantly, 8 out of 9 of this group had late recurrent reactivations beyond 100 days post-transplant (Table 4.2).

Table 4.1 Characteristics of HSCT patients.

UPN	Age	Gender	Diagnosis	Conditioning/ Donor	T-Cell Depletion	D/R (CMV serology)	HLA-class I restriction of HLA-multimers
01	56	M	NHL	RIC-Sib	C	+/+	B*0702
02	43	M	NHL	RIC-MUD	C	+/+	A*0202
03	49	M	NHL	RIC-Sib	C	+/+	B*0801
04	46	M	Myeloma	MA-Sib	N	+/+	A*1101
05	44	F	NHL	MA-MUD	C	+/+	A*0201
06	55	M	NHL	RIC-MUD	C	-/+	A*0201
07	64	F	MDS	RIC-Sib	C	+/+	A*0201
08	63	M	AML	RIC-MUD	C	+/+	A*0201
09	48	F	NHL	RIC-MUD	C	+/+	A*0201
10	54	M	AML	RIC-Sib	C	+/+	B*0702
11	68	M	AML	RIC-MUD	C	+/+	B*0801
12	74	M	AML	RIC-MUD	C	+/+	A*0101
13	27	M	AML	MA-Sib	N	-/+	A*0101
14	55	M	CLL	RIC-MUD	C	+/+	B*0801
15	44	F	MDS	RIC-MUD	C	+/+	A*0201
16	56	M	CLL	RIC-MUD	ATG	-/+	A*0201
17	57	M	MDS	RIC-Sib	C	+/+	A*0201
18	66	M	AML	RIC-MUD	C	-/+	A*0201
19	71	M	AML	RIC-MUD	C	+/+	A*0201
20	61	M	AML/MDS	RIC-MUD	ATG	+/+	A*0201

UPN: Unique patient Number, NHL:Non Hodgkin Lymphoma, AML: Acute Myeloid Lymphoma, MDS: Myelodysplastic Syndrome, CLL: Chronic Lymphocytic Leukaemia, RIC-Sib: reduced intensity conditioning, Sib: Sibling, MA: Myeloablative conditioning, MUD: Matched unrelated donor transplant, T-Cell Depletion: N: none, C: Alemtuzumab (Campath), ATG: anti-thymocyte globulins

Table 4.2 Summary of CMV reactivation and magnitude of CMV specific T-cell responses in HSCT patients.

UPN	No. CMV episodes	Duration of viraemia (days post-HSCT)	Maximum viral load (copies/ml	Maximum CMV-specific CD4+ T-cells* (% CD4)	Maximum CMV specific CD8+ T-cells* (% CD8)	Log expansion of CMV-specific CD4+ T-cells	Log expansion of CMV-specific CD8+ T-cells**
01	1	63-78	900	50(15)	370(9.7)	3.07	1.36
02	2	21-28 100-120	2600	1.5(5.2)	7(10)	1.2	2.40
03	1	28-70	700	17(7.5)	448(25)	2.03	3.04
04	0	0	-	28.7(3.5)	ND	0.17	ND
05	2	28-238 308-322	191633	ND	ND	ND	ND
06	2	27-54 97-174	7100	ND	1.9(0.5)	ND	3.5
07	1	24-50	11650	15.5(10)	82(9.4)	1.49	1.79
08	2	34-41 126-147	1400	1.2(1.4)	76(8)	3.07	1.94
09	1	35-56	1269	6.4(4)	146(12)	1.34	1.61
10	1	41-62	3500	0.5(0.23)	54(6.1)	0.17	0.77
11	3	63-91 144-164 203-224	16800	ND	297(36)	ND	3.14
12	4	25-46 83-114 127-141 195-226	15400	0.46 (0.35)	390(17)	1.60	2.57
13	2	17-39 77-84	15760	2.9(4.1)	15(2)	1.89	1.04
14	1	26-47	31000	ND	25(1.2)	ND	2.77
15	1	47-68	600	ND	37(13)	ND	ND
16	3	43-78 100-120 280-295	23000	30(25)	ND	3	ND
17	1	22-50	2155	ND	8.7(1.3)	ND	0.53
18	1	22-43	5100	ND	ND	ND	ND
19	1	28-56	1100	ND	20(5)	ND	4.75
20	2	27-62 99-118	600	ND	ND	ND	ND

Table 4.2 (continued)* Figures are cell numbers x10^3/ml peripheral blood, Figures in the brackets are percent of specific cells in whole $CD4^+$ or $CD8^+$ T-cells, **There was no statistically significant difference in expansion from baseline between CMV-specific $CD4^+$ and $CD8^+$ T-cells (median:1.7 log and 2.2log, respectively. p=0.54). ND; not detected

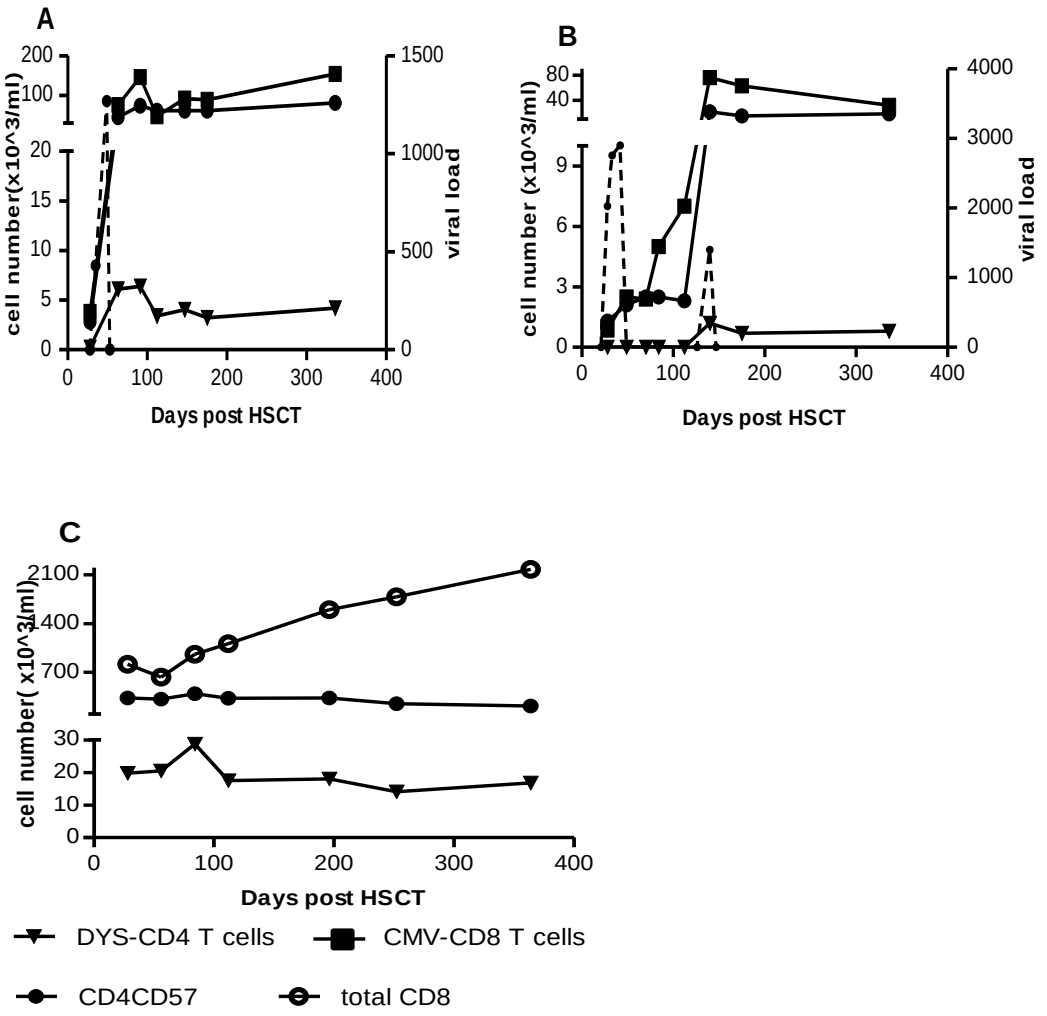


Figure 4.1 Pattern of reconstitution of CMV-specific $CD4^+$ and $CD8^+$ T-cells after transplantation in relation to episodes of CMV reactivation.

Graphs showing reconstitution of CMV-specific T-cells in (A) a patient with single episode of CMV viraemia, (B) a patient with multiple episodes of viraemia and (C) a patient with no CMV reactivation (due to lack of HLA-class I multimer-stainable cells for this patient, only total $CD8^+$T-cell levels are available). The frequency of DYS-specific $CD4^+$ T-cells in this patient was high (15 x 10^3/ml) and stable post-HSCT. Dashed line shows CMV copy number/ml.

4.2.2 Monitoring of CMV-specific $CD4^+$ T-cells using HLA-peptide tetramers showed that $CD4^+$ T- cells reconstituted with similar kinetics to virus-specific $CD8^+$ T-cells

The HLA-DRB1*0701-DYS tetramer was used in combination with a range of CMV-specific HLA-class I multimers (Table 2.2) to characterise CMV-specific $CD4^+$ and $CD8^+$ T-cell reconstitution (Figure 4.2A). DYS-specific $CD4^+$ T-cells were detected in 11 out of 20 HLA-DRB1*0701 HSCT patients and the frequency of tetramer-staining cells ranged at baseline from 0% to 4% of the $CD4^+$ T-cell pool (median: 0.11%) with absolute numbers ranging from 0 to $0.5x10^3$ /ml (median: $0.15x10^3$/ml) before first viral reactivation. The DYS-specific $CD4^+$ T-cell population expanded markedly after viral reactivation and reached a median peak of 4.8% of the $CD4^+$ T-cell pool (range 0.23% to 25%) corresponding to an absolute median count of 5.20 $x10^3$ /ml (range 0.46 to $50x10^3$ /ml). In comparison, the peak aggregate levels of tetramer-staining CMV-specific $CD8^+$ T-cells were markedly higher, with a median value over 14 fold higher at 76 $x10^3$ /ml (range 8.70 $x10^3$ /ml - $448x10^3$ /ml). However, the kinetics of reconstitution of both CMV-specific $CD4^+$ and $CD8^+$ T-cells were comparable expanding by approximately 2 logs (Table 4.2 and Figure 4.2 B-C). Monitoring was continued until 36 weeks post-transplant in nine patients with detectable levels of both CMV-specific $CD4^+$ and $CD8^+$ T-cells. A very strong correlation was found between the magnitude of the $CD4^+$ and $CD8^+$ CMV-specific immune response following CMV reactivation ($r=0.75$, $p<0.0001$) (Figure 4.2D).

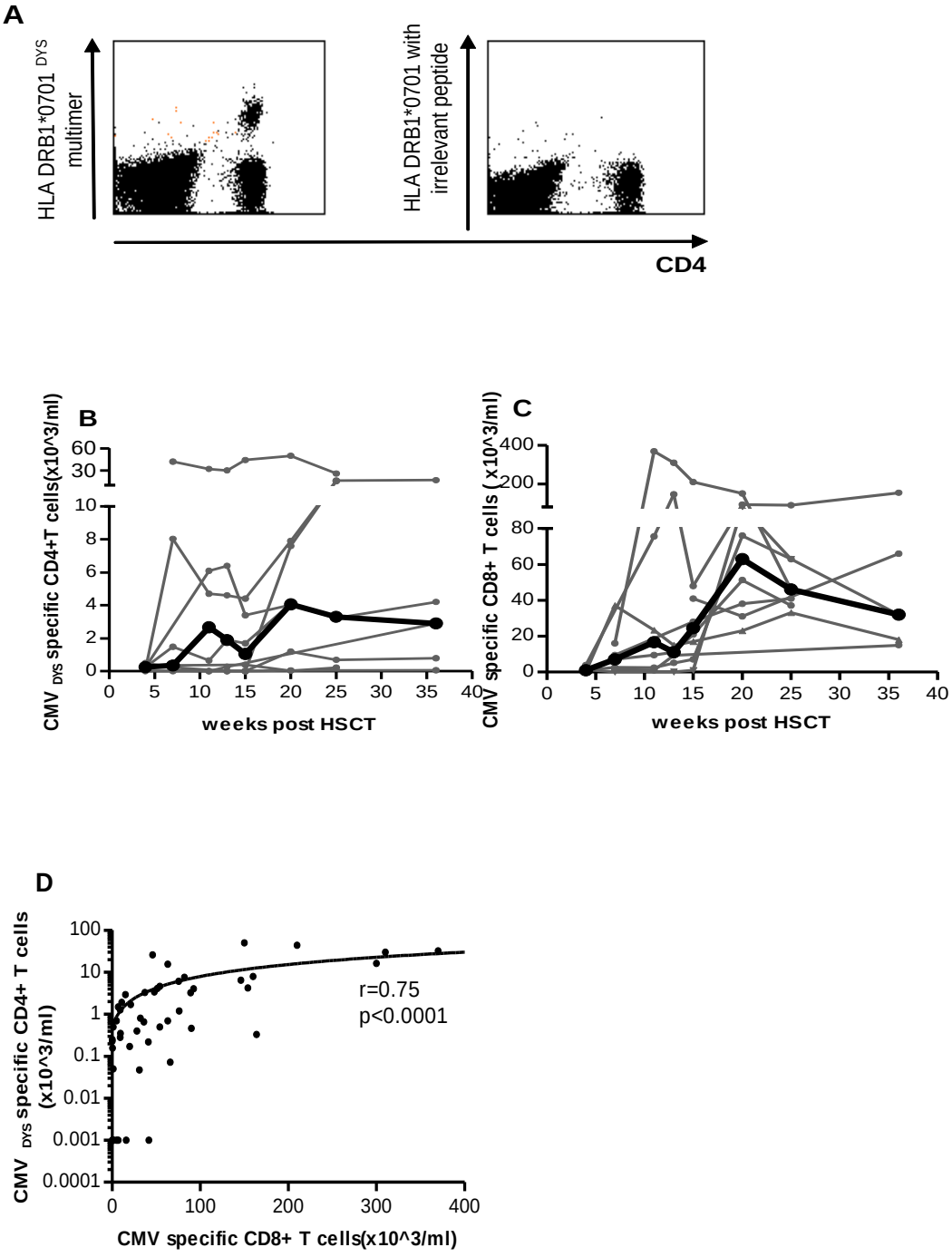


Figure 4.2 Longitudinal analysis of reconstitution of CMV-specific T-cells following HSCT.

(A) Flow cytometry dot plots of CMV-specific $CD4^+$ T-cells visualised using HLA-DRB1*0701 tetramers with CMV-derived DYS peptide. HLA-DRB1*0701 tetramers loaded with irrelevant peptides were used as negative control. x-axis, CD4 staining; y-axis, staining with tetramer.(B) Longitudinal monitoring of DYS-specific $CD4^+$ T- cells and (C) CMV-specific $CD8^+$ T-cells after HSCT. Thick black lines represent median cell numbers of CMV-specific T-cells. (D) Correlation between the number of CMV-specific $CD4^+$ and $CD8^+$ T-cells during immune reconstitution post-HSCT (r=0.75, p<0.0001).

4.2.3 T-cell responses before and after clearance of viraemia indicate a minimum threshold of CMV-specific T-cell immune response is required for viral control

Prior to reactivation, CMV-specific T-cells were detectable only at very low levels within peripheral blood. Although a difference in baseline CMV-specific $CD4^+$ T-cell levels between patients with single (median $0.25x10^3$ /ml) and multiple viremic episodes (median$0.05x10^3$ /ml) was observable, it was not statistically significant (p=0.12). This was also true for CMV-specific $CD8^+$ T-cells (Figure 4.3A, B). The expansion of CMV-specific immune response is usually triggered by CMV reactivation. HLA-class II tetramers were used to assess if the magnitude of the CMV-specific $CD4^+$ T-cell response after the first reactivation was predictive of sustained viral control. Indeed, patients who experienced only a single viraemic episode showed a median peak CMV-specific $CD4^+$ level of 6.2 $x10^3$ /ml and cleared the virus without later relapses (Figure 4.3D). In contrast, patients who went on to experience multiple episodes of viraemia had a median CMV-specific $CD4^+$ T-cell level of only $0.04x10^3$/ml after the first reactivation (p=0.02). A similar pattern was seen with CMV-specific $CD8^+$ T-cells, where the count after resolution of viraemia was 41.10 $x10^3$/ml in patients with single reactivation compared to $4.30x10^3$ /ml in patients with multiple reactivations (p=0.049) (Figure 4.3E). The peak CMV-specific T-cell count after the initial viraemic episode is therefore predictive of the ability to maintain control of further reactivations. We estimated the minimum thresholds of CMV-specific T-cell counts predictive of long-term viral control based on the median peak T-cell level following the last episode of viraemia. A median level of $>0.7x10^3$ /ml CMV-specific $CD4^+$ and $>20x10^3$/ml specific $CD8^+$ T-cells after clearance of CMV was associated with absence of further reactivations. This magnitude of CMV specific $CD8^+$T-cells is consistent with previously reported protective levels (Cwynarski et al., 2001; Aubert et al., 2001).

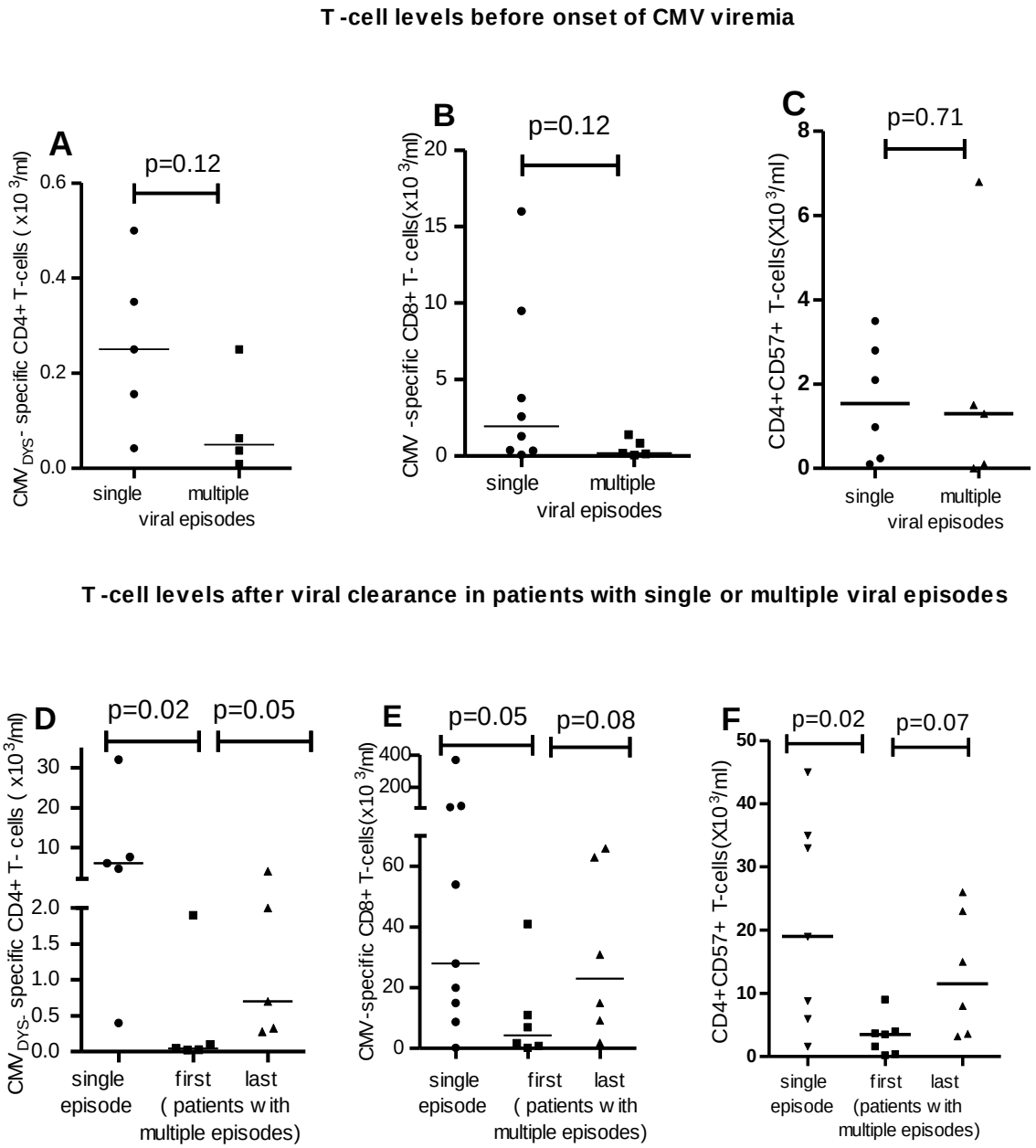


Figure 4.3 Frequencies of CMV specific T-cells before and after viraemia in relation to occurrence of single or recurrent episodes of reactivations.

(A)Number of CMV-specific $CD4^+$ T-cells, (B) CMV-specific $CD8^+$ T-cells and (C) $CD4^+CD57^+$ T-cells before the onset of initial CMV viraemia in patients who underwent single or multiple episodes of viral reactivation.(D) Number of CMV-specific $CD4^+$ T- cells , (E) CMV-specific $CD8^+$ T-cells and (F) $CD4^+CD57^+$ T-cells after clearance of viraemia in patients with single of reactivation, or after first and final episode of reactivation in patients who suffered from multiple episodes of CMV reactivation. Patients with a single episode of CMV reactivation had higher numbers of all cell subsets after the first episode of viraemia compared to patients with multiple reactivations (p=0.02).

4.2.4 Expansion of $CD4^+CD57^+$ T-cells during viral reactivation indicates a broader CMV-specific $CD4^+$ T-cell response

As the level of DYS-specific $CD4^+$ T-cells was predictive of control for viral reactivation following the first episode of viraemia, it became important to ascertain how this correlated with the total CMV-specific $CD4^+$ T-cell immune response. $CD4^+$ $CD57^+$ T-cells are present almost exclusively in CMV-seropositive patients (next chapter) and CD57 is present in CMV-specific $CD4^+$ T-cells (van Leeuwen et al., 2005; Crompton et al., 2008; Strioga et al., 2011). Indeed, the number of tetramer-binding CMV-specific $CD4^+$ T-cells correlated strongly with the $CD4^+$ $CD57^+$ T-cell count ($r=0.93$, $p<0.0001$) (Figure 4.4). Similar to DYS-specific $CD4^+$ T-cells, there was a statistically significant difference in $CD4^+$ $CD57^+$ T-cell peak levels after first CMV reactivation between patients with single reactivation (median 19 $x10^3$/ml) and those who went on to develop multiple reactivations (median 4 $x10^3$/ml) ($p=0.02$) (Figure 4.3F). The median level of protective number of $CD4^+$ $CD57^+$ T-cells after final viral clearance in multiply reactivated patients was 11.5 x 10^3/ml (Figure 4.3F).

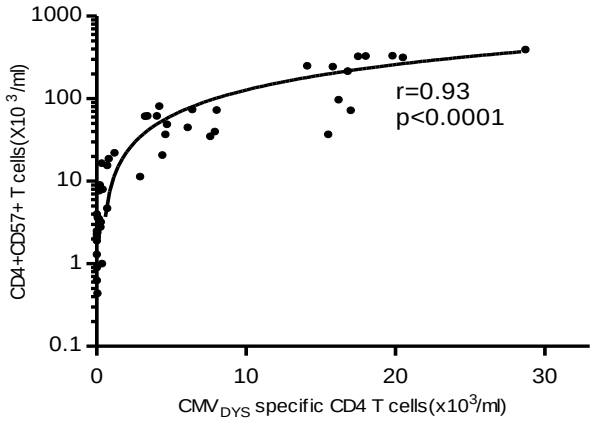


Figure 4.4 Correlation between the number of CMV-specific $CD4^+$ T-cells and number of $CD4^+$ $CD57^+$ T-cells during T-cell reconstitution post-HSCT.

Reconstitution of $CD4^+$ $CD57^+$ T-cell correlates strongly with appearance of CMV-specific $CD4^+$ T-cells and therefore could serve as a surrogate marker for detection of CMV-specific $CD4^+$ T-cells.

4.2.5 Diagnostic value of CMV-specific T-cell quantification

To address whether measuring the frequency of CMV-specific T-cells or $CD4^+$ $CD57^+$ T-cells could be used as a diagnostic test in predicting recurrent CMV reactivations after the first episode of viraemia, ROC (Receiver Operating Characteristic) analysis was performed. Due to the low number of samples with the measurable frequencies of CMV-specific $CD4^+$ T-cells, this analysis was only performed for CMV-specific $CD8^+$ and $CD4^+$ $CD57^+$ T-cells. Furthermore, in order to increase the number of samples with detectable CMV-specific $CD8^+$ T-cells, additional data from 14 HSCT patients were kindly provided by Dr David Lewis (School of Cancer Sciences, University of Birmingham, submitted paper). These data and data from 15 patients (presented in Figure 4.3) (in total 29 patients) were analysed by ROC test. This analysis showed that the presence of lower than $7.22x10^3$/ml CMV-specific $CD8^+$ T-cells after the clearance of first episode of viraemia, could predict the recurrent CMV reactivation with a sensitivity of 84.56% and specificity of 75.00%. The area under the curve (AUC) was 82% and the test was statistically significant at p=0.003 (Figure 4.5A). These values for specificity and sensitivity were also found by X square analysis which is presented in the next pages. Figure 4.5B shows the comparison between CMV-specific $CD8^+$ T-cells after the clearance of first viral reactivation in 29 patients with single or multiple episode(s) of viraemia. Similar to Figure 4.3E which illustrated the data from 15 patients, Figure 4.5B confirms a median frequency of CMV-specific $CD8^+$ T-cells after first episode of viraemia in patients with single episode of $20x10^3$/ml. However, according to the ROC analysis, this level of CMV-specific $CD8^+$ T-cell frequency gives a sensitivity of 92.34% and a specificity of 50.10% specificity for detecting patients with multiple episodes.

ROC analysis of $CD4^+$ $CD57^+$ T-cell frequencies in 14 HSCT patients (7 with single episode and 7 with multiple episodes of CMV viraemia), also showed that $CD4^+CD57^+$ T-cell levels can be used as a reliable surrogate marker for predicting recurrent reactivation. This analysis showed that after the resolution of the first episode of viraemia, a level of $CD4^+$ $CD57^+$ T-cells below $5x10^3$/ml is associated with patients suffering from further reactivations (85.70% sensitivity and 85.70% specificity , AUC=86% , p-0.022) (Figure 4.5C). Additionally, X square analysis confirmed these sensitivity and specificity values.

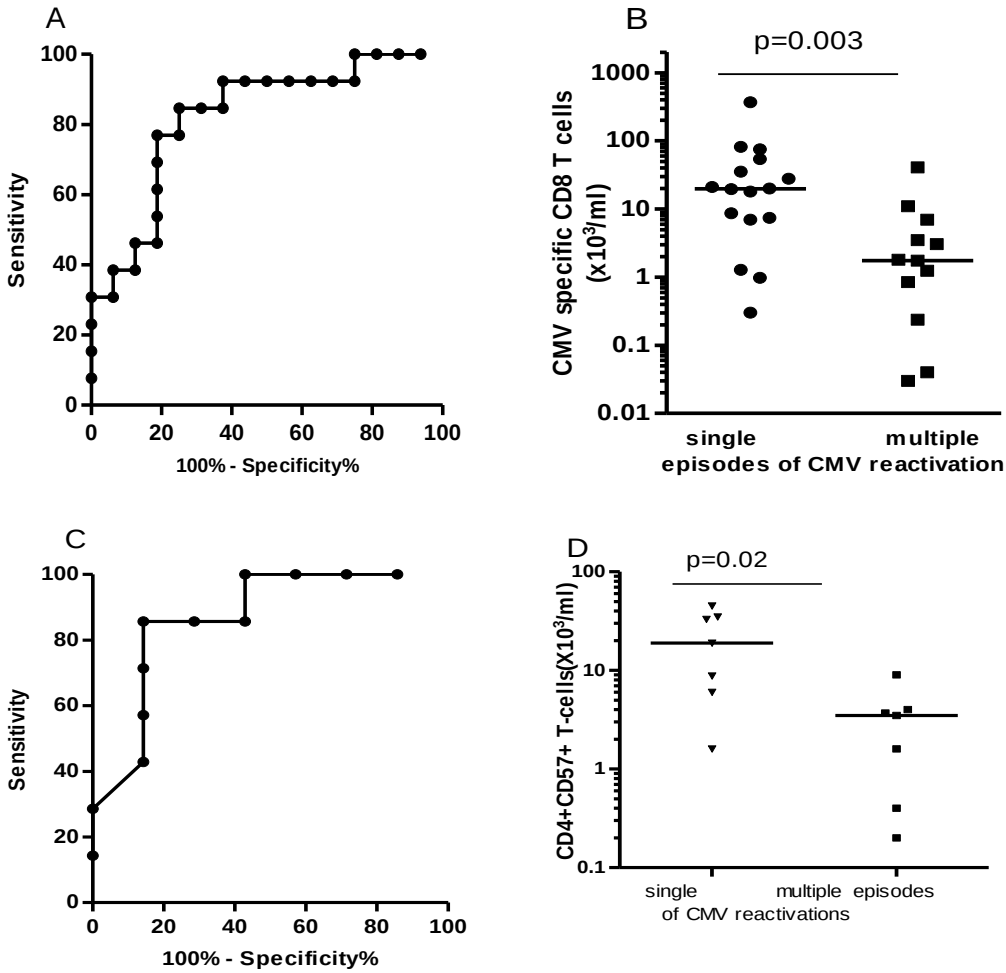


Figure 4.5 ROC analysis of CMV-specific T-cell numbers in HSCT patients with single or multiple episode(s) of CMV reactivation.

(A) CMV-specific $CD8^+$ T-cell levels were analysed in 29 HSCT patients with single or multiple CMV viraemia by ROC analysis test or (B) compared by Mann Whitney test. (C) ROC analysis of $CD4^+$ $CD57^+$ T-cells in 14 HSCT patients with single or multiple episodes of CMV reactivation. (D) Comparison of $CD4^+$ $CD57^+$ T-cell levels. This figure is part of Figure 4.3F.

Despite the small number of patients analysed by ROC test, the results clearly show the potential advantage of quantifying the frequency of CMV-specific T-cells, especially after a first episode of viraemia, for identifying patients at risk of recurrent viraemia. The CMV-specific T-cells can thus be used to risk-stratify HSCT patients into those at high risk of recurrent reactivation and those at low risk. This can help to guide clinical

decision making on how frequently the individual patient should be monitored for CMV reactivation, and possibly on when to use salvage CMV-specific therapy. The data support a role for CMV immunotherapy as salvage therapy in this setting, rather than as first line prophylactic or pre-emptive therapy.

HLA-class I tetramer (CMV/CD8$^+$ T-cells)

	multiple react.	single react
<7000/ml	11	4
>7000/ml	2	12

Specificity=12/16=75%
Sensitivity=11/13=84.6%

CD4$^+$ CD57$^+$ T-cells

	multiple react.	single react
<5,000/ml	6	1
>5000/ml	1	6

Specificity=6/7=85.7%
Sensitivity=6/7=85.7%

4.2.6 CMV-specific CD4$^+$ T-cells exhibit an effector phenotype

To assess the functionality of CMV-specific CD4$^+$ T-cells in the post-transplant setting, HLA-class II tetramers were co-stained with a range of different antibodies against cell surface or intracellular molecules (Figure 4.6). Longitudinal monitoring demonstrated largely stable CMV-specific CD4$^+$ and CD8$^+$ T-cell phenotypes throughout the period of viraemia (Figure 4.7A, B). The majority of CMV-specific CD4$^+$ T-cells maintained a CCR7$^-$ CD45RA$^-$ effector memory phenotype (EM) (median 82%), whereas a mean of 77% CMV-specific CD8$^+$ T-cells skewed towards a CCR7$^-$ CD45RA$^+$ (EMRA phenotype) and a mean of 21% towards EM phenotype. CMV-specific CD8$^+$ T-cells exhibited an increase in EMRA phenotype during viraemia. No naïve or central memory subsets were detected in this population. This indicates that following CMV-reactivation in HSCT patients, CMV-specific CD4$^+$ and CD8$^+$ T-cells do not express

lymph node chemokine receptor CCR7. Despite this similar phenotype, most of CMV-specific $CD4^+$ T-cells do not re-express CD45RA whilst $CD8^+$ T-cell undergo further cell differentiation and regain CD45RA molecule.

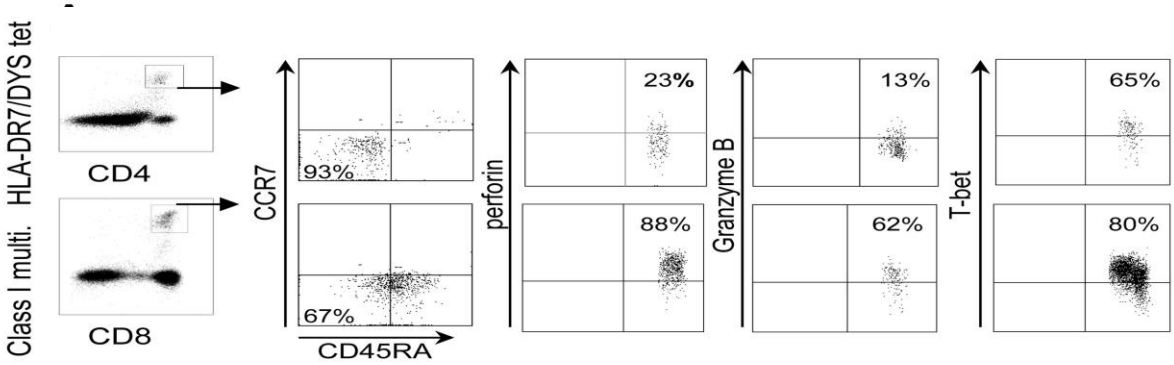


Figure 4.6 Immunophenotyping of CMV specific T-cells by flow cytometry.

CMV-specific T-cells were co-stained with antibodies against cell surface molecules of CD45RA, CCR7 and intracellular perforin, granzyme B and T-bet. The percentages show the frequency of cells expressing a particular phenotype among CMV-specific $CD4^+$ (HLA-DR7/DYS tetramer) and $CD8^+$ (HLA-class I multimers) cells.

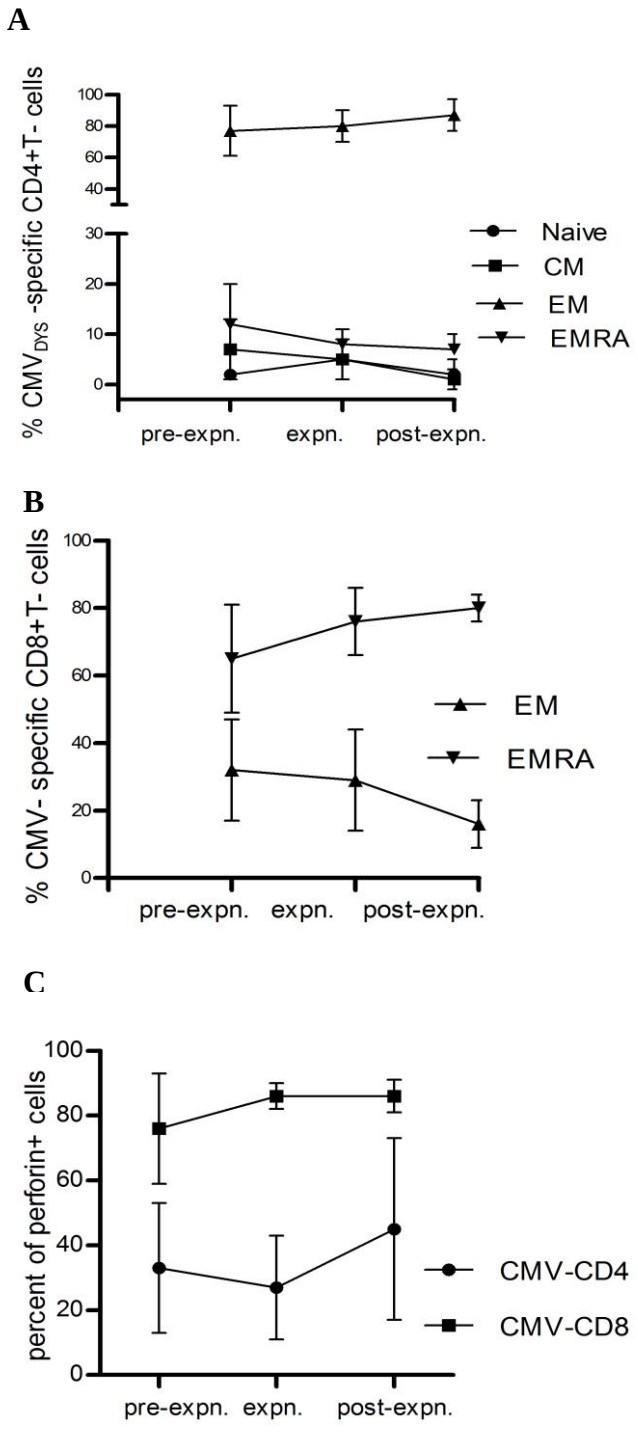


Figure 4.7 Immune phenotype of reconstituting CMV-specific T-cells post-HST.

(A, B) Frequencies of T-cell subsets; naïve, central memory, effector memory (EM) and revertant EM (EMRA) during T-cell reconstitution (pre, during and post cell expansion). (C) Frequencies of perforin expressing CMV-specific CD4 (CMV-CD4) and CD8 (CMV-CD8) T-cells post-HSCT. These figures are median frequencies of positive cells from 4 HSCT patients.

4.2.7 Helper and Cytotoxic potential of CMV specific $CD4^+$ T-cells

Several studies have shown that a considerable percentage of CMV-specific $CD4^+$ T-cells from healthy individuals display cytotoxic activity, a property that is less commonly observed in other antigen-specific $CD4^+$ T-cells (Casazza et al., 2006). Indeed, perforin and granzyme B expression was observed in 32% and 22% of patients' CMV-specific $CD4^+$ T-cells, respectively, clearly demonstrating the cytotoxic potential of some of these cells (Figures 4.6-8). However, the expression level of T-bet within CMV-specific T-cells was similar in specific $CD4^+$ and $CD8^+$ T-cells, present at a median of 62% of the antigen-specific subset (Figures 4.6-8). T-bet is a T-box transcription factor with an important role in development of Th1 cells (Edwards et al., 2014) and the proportion of T-bet positive cells within the CMV-specific $CD4^+$ T-cell population was significantly higher compared to the global $CD4^+$ T-cell repertoire ($p<0.03$), supporting the predominant Th1 profile of CMV-specific $CD4^+$ T-cell pool.

On the other hand, the T-bet expression by CMV-specific $CD8^+$ T-cells supports anti-viral activity. T-bet expression by CMV-specific $CD8^+$ T-cells after primary infection has been reported to predict control of recurrent pulmonary CMV viraemia in lung transplant patients (Pipeling et al., 2011). As anticipated of CMV-specific $CD8^+$ T-cells, being professional cytotoxic cells, the expression of perforin and granzyme B was much higher within 85% and 61% of the population, respectively. This remained largely stable during and after cell expansion in response to CMV (Figure 4.7C).

Analysis of CD57 expression by CMV-specific $CD4^+$ T-cells revealed that up to 65% of these cells expressed CD57. Interestingly, co-staining of CMV-specific T-cells with anti-CD57 and anti-perforin Abs showed that in both total and CMV-specific $CD4^+$ T-cells, perforin expression is much higher in the cells expressing CD57 molecule (Figure

4.9).This is in accordance with a previous finding on $CD8^+$ population showing that CD57 expression represents perforin positive cytotoxic T-cells (Chattopadhyay et al., 2009).

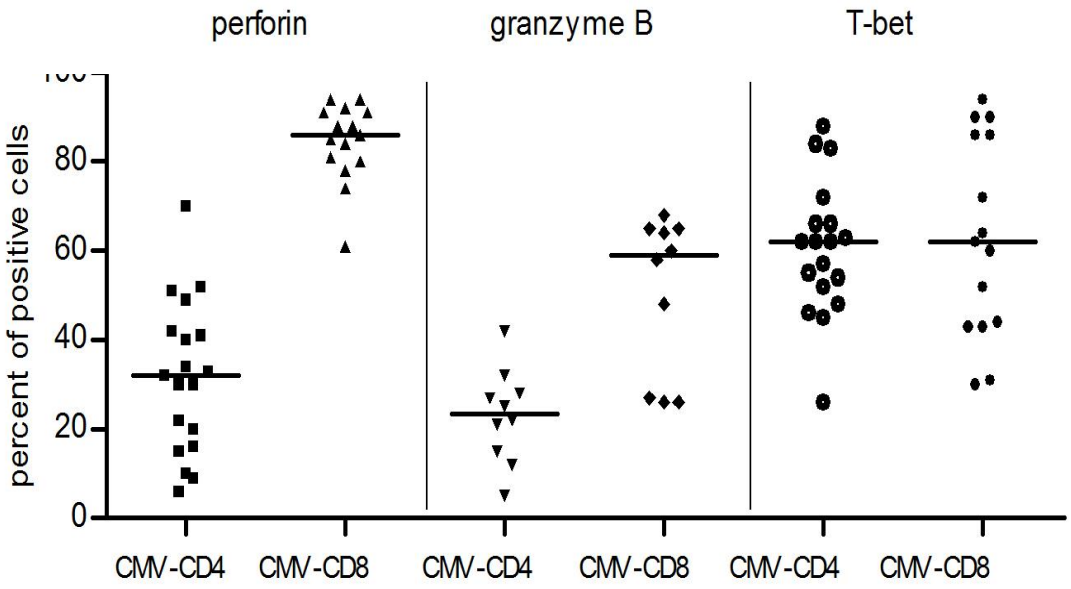


Figure 4.8 ***Expression levels of perforin, granzyme B and T-bet in CMV-specific T- cells.***

Percentages of CMV-specific T-cells with intracellular expression of perforin, granzyme B and T-bet during immune reconstitution in 4 HSCT patients. Expression of perforin and granzyme B were higher in CMV-specific $CD8^+$ compared to CMV-specific $CD4^+$ T-cells ($p<0.0001$, $p<0.002$, respectively).

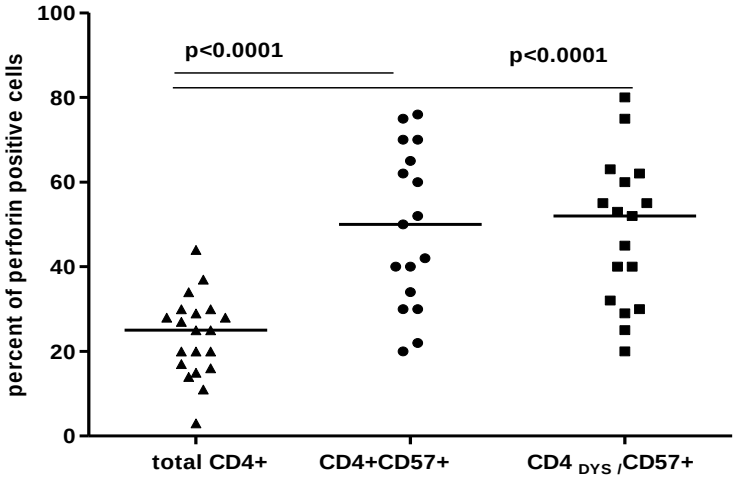


Figure 4.9 Perforin expression in total CD4$^+$ population, CD4$^+$CD57$^+$ and CD57$^+$ DYS -specific T-cells.

Higher perforin expression in CD4$^+$CD57$^+$ and CD57$^+$ DYS-specific CD4$^+$ T-cells in comparison to total CD4$^+$ population. The perforin expression profile of CD4$^+$ CD57$^+$ cells was very similar to the expression profile of CMV-specific CD4$^+$ CD57$^+$ T-cells.

4.2.8 Cytokine receptor expression in the reconstituting CMV-specific T-cells

Cytokines play a major role in post-transplant immune reconstitution. We therefore co-stained tetramer-positive cells with anti-CD25 (IL-2Rα) and -CD127 (IL-7Rα), receptors for two key homeostatic cytokines. Neither was detected in CMV-specific CD8$^+$ T-cells but a mean 46% of CMV-specific CD4$^+$ T-cells retained stable expression of IL-7Rα but not IL-2Rα (Figure 4.10), suggesting that CD4$^+$ cells have the potential to respond to local IL-7 production, and to provide help to CD8$^+$ T-cells. The absence of CD25 expression indicated that CD25$^+$ T-regulatory cells were absent amongst CMV-specific CD4$^+$ T-cells.

TNF-α is produced rapidly following HSCT and as it is discussed in the next chapter, there are two receptors for this cytokine which only one of them (TNFR2) is detectable on cell surface by using flow cytometry based cell staining. Co-staining with anti-

TNFR2 (CD120b) antibody showed that all CMV-specific T-cells expressed CD120b after CMV viraemia. The expression level of CD120b per cell (as determined by mean fluorescent intensity (MFI) of stained cells) increased rapidly during viraemia (MFI from 3000 to 10500) and decreased following viral clearance (Figure 4.11).This finding may indicate an important role for TNF-α in the process of T-cell response to antigens (next chapter).

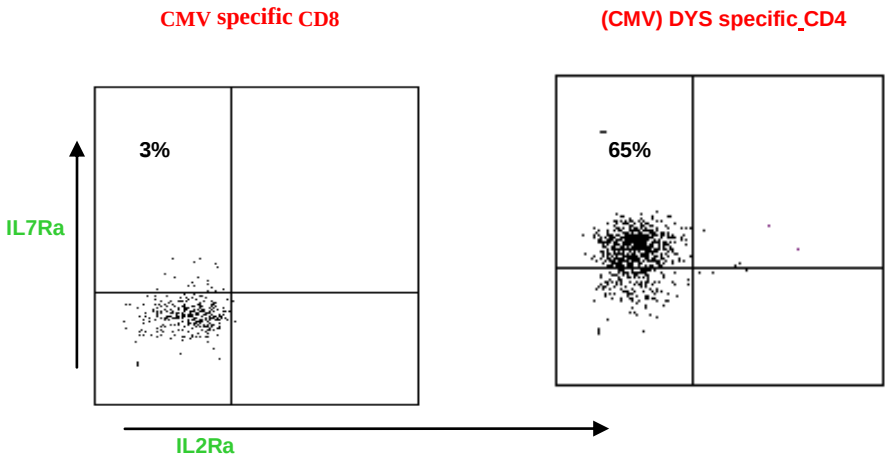


Figure 4.10 Flow cytometry dot plots of cytokine receptor expression in CMV-specific T-cells.

Co-staining of CMV-specific T-cells with IL-7Rα and IL-2Rα showed that these receptors were almost absent in CMV-specific $CD8^+$ T-cells whilst the majority of CMV-specific $CD4^+$ T-cells expressed IL-7Rα with no IL-2Rα expression.

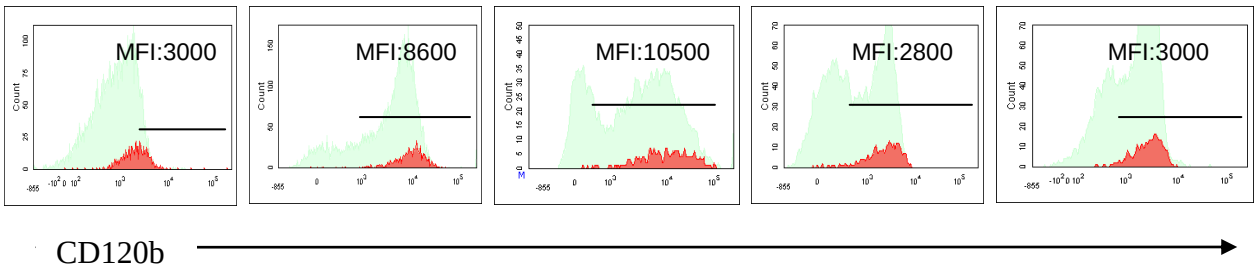


Figure 4.11 TNFR2 (CD120b) expression by CMV-specific T-cells in response to CMV reactivation.

CMV-specific T-cells expressed TNFR2 in different level per cell as measured by mean fluorescent intensity (MFI) (X axis). During cell response to CMV reactivation, TNFR2 expression per cell increased considerably and returned to its steady level following viral clearance. Light green and red graphs show TNFR2 expression in whole peripheral and CMV-specific $CD8^+$ T-cell populations, respectively.

4.2.9 The CMV-specific humoral immune response correlates with the reconstitution of CMV-specific $CD4^+$ T-cells

As $CD4^+$ T-cells are known to support antibody production through both Th1 and Th2 help (Swain et al., 2012), it became interesting to study whether CMV-specific $CD4^+$ T-cells were involved in supporting anti-CMV humoral response. Longitudinal analysis of CMV-specific antibody titres showed correlation with the reconstitution of CMV-specific $CD4^+$ T-cells. This correlation was found between the magnitude of the CMV-specific IgG and CMV-specific $CD4^+$ T-cells ($r=0.45$, $p=0.01$) (Figure 4.12).

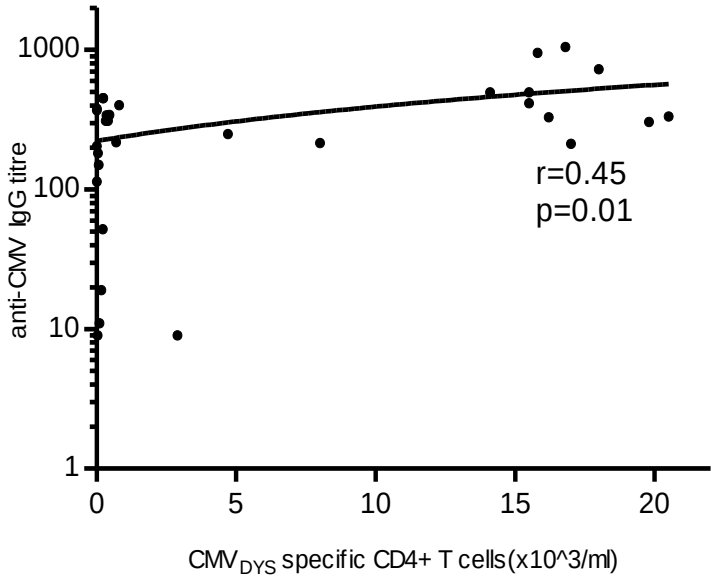


Figure 4.12 Coordinated humoral and cellular immune responses to CMV reactivation post-HSCT.

There was a correlation between the number of DYS-specific $CD4^+$ T- cells (x-axis) and anti CMV- IgG titre during immune reconstitution.

4.3 Discussion

Cytomegalovirus reactivation continues to be a considerable challenge in stem cell transplantation, and the risk is particularly high with non-myeloablative regimens that incorporate TCD (Chakrabarti et al., 2002). The management of viral reactivation has improved in recent years with use of QPCR for early detection of viral reactivation, guiding effective anti-viral medication and application of CMV-specific T-cell therapy. However, tests which can predict the onset of viraemia would be of considerable benefit in optimizing preventative therapies.

The CMV-specific T-cell immune response is almost certainly the most important effector arm in the control of viral reactivation and studies have shown that regeneration

of a robust T-cellular response can predict effective viral control. The introduction of HLA-class I-peptide tetramers permitted the rapid assessment of the magnitude and phenotype of the CMV-specific $CD8^+$ immune response. However, the lack of availability of CMV-specific HLA-class II tetramers has limited the incorporation of CMV-specific $CD4^+$ immune response into management algorithms.

This study used a CMV-specific HLA-class II tetramer for the first time to analyze the magnitude, kinetics and functional properties of regenerating CMV-specific $CD4^+$ T-cells in the post-transplant period. Three major findings were observed within the study: firstly, following the onset of CMV viraemia, CMV-specific $CD4^+$ and $CD8^+$ T-cells were found to expand in parallel and with similar kinetics. Both populations showed an average increase of around 2 logs over a period of 8 weeks. Rapid expansion of CMV-specific T-cells following viral detection has been reported in many HSCT studies, and subclinical reactivation is thought to provide a basis for the high prevalence of CMV-specific cells in healthy donors. These studies show that CMV-specific $CD4^+$ T-cells have comparable proliferative capacity to their $CD8^+$ counterparts, and demonstrate the importance of robust T-cellular expansion in the setting of viral replication. However, it was notable that the magnitude of and variation in the CMV-specific $CD4^+$ T-cell response was much smaller than that of CMV-specific $CD8^+$ T-cells.

A second important finding was that we were able to use the peak CMV-specific T-cell response following initial viraemia to define a minimum number of CMV-specific $CD4^+$ T-cells that was associated with protection from recurrent viral reactivations. A level of $0.7x10^{3}$/ml CMV-specific $CD4^+$ T-cells was required to provide long term control of viral reactivation. This level is comparable to protective value of $1x10^3$/ml previously reported using functional assays and confirms that reconstitution of CMV-

specific CD4 T-cells is protective (Widmann et al., 2008; Pourgheysari et al., 2009; Tormo et al. 2010, Lilleri et al., 2008). This estimate of minimum protective CMV-specific CD8 T-cell level of $20x10^3$/ml is also comparable with previous reports of levels between 10 and 20 $x10^3$/ml (Cwynarski et al., 2001; Aubert et al., 2001).

Finally, the use of HLA-peptide tetramers allowed us to make a detailed phenotypic analysis of the CMV-specific $CD4^+$ T-cell immune response in the post-transplant setting. This property is one of the most valuable aspects in the use of HLA-peptide multimers, as functional assays based on response to peptide stimulation, alter the membrane phenotype following cellular activation.

Although, this study focused on a single glycoprotein B-derived epitope DYS with the available tetramer, the immune response to glycoprotein-B constitutes a large component of the overall CD4 and humoral response to CMV infection (Crompton et al., 2008).

Nevertheless, the total CMV-specific CD4 response is likely to be considerably larger as CD4 responses generally have a broad range of specificities, as seen in EBV-specific CD4 responses (Long et al., 2013). Using $CD4^+$ $CD57^+$ T-cells as surrogate markers for CMV-specific $CD4^+$ T-cells, it was estimated that the numbers of $CD4^+$ $CD57^+$ T-cells required for viral control were between 11.5 and 19 $x10^3$/ml. These were the levels measured after resolution of viraemia in patients with single reactivation and after resolution of the last viraemic episode in patients with multiple reactivations, respectively (Figure 4.3F). Given the statistically significant difference in levels between single and multiply relapsed patients, it may be feasible to use $CD4^+$ $CD57^+$ T-cell levels to predict recurrent viraemia.

$CD4^+$ T-cells contribute diverse functions to antiviral protection (Swain et al., 2012). The best known role of them is providing help to B-lymphocytes for antibody production, but it also mediates direct cytotoxicity as a number of studies of CMV-specific $CD4^+$ T-cells from healthy donors have shown (Casazza et al., 2006). In our cohort, cytotoxic molecules was observed in only up to 30% of CMV-specific $CD4^+$ T-cells in contrast to CD8 counterparts that have much higher content . It is possible that exhaustion or selective migration from peripheral blood to the site of infection had taken place but the high expression of intracellular T-bet suggests that the predominant function of CMV-specific $CD4^+$ T-cells is to provide Th1 support for $CD8^+$ T-cells with only a minority component mediating cytotoxicity.

As nearly half of HSCT patients with high-risk CMV serology have been shown to go on to develop recurrent reactivations in this and previous studies (Chakrabarti et al., 2002), using HLA-peptide tetramers to measure the CMV-specific T-cell response after resolution of the first CMV reactivation may allow the stratification of patients into high and low risk groups to guide the appropriate introduction of prophylactic CMV-specific adoptive T-cell immunotherapy or second line anti-viral drug therapy.

Chapter 5

CYTOKINE RECEPTOR EXPRESSION PATTERN IN T-CELL SUBSETS AND FUNCTIONAL ACTIVITY OF TNFR2

5.1 Background

Cytokines are vital mediators of a broad range of physiological processes including cell growth and differentiation, tissue formation and repair, inflammatory and anti-inflammatory mechanisms and immune response. Cytokines' activities are dependent on their interaction with specific receptors expressed by target cells. Cytokine receptors are grouped into six structurally related super-families. Among them are type I cytokine receptors and Tumour Necrosis Factor Receptor (TNFR) family (Wang et al. 2009; Hehlgans and Pfeffer, 2005). The type I cytokine receptor family includes 3 subgroups: glycoprotein 130 (gp130), common beta and common gamma chain molecules. Cytokine receptors in this family mainly interact with cytokines that regulate a hematopoietic system and immune responses (Figure 5.1). Gp130 is a common signal transducing molecule in receptors for cytokines such as Interleukin 6 (IL-6) and IL-11 (Taga, 1997**).** Common gamma chain is a signal transducing chain in receptors for cytokines such as IL-2, IL-7 and IL-15. Each of these cytokines has its own private receptor (IL-2Rα, IL-7Rα and IL-15Rα, respectively) which in combination with IL-2R-γ and beta receptor (in case of IL-2 and IL-15) make functional receptor complexes (Wang et al., 2009). Cytokines of the common gamma chain family are vital for the development of T cell memory. Antigenic stimulation of T- cells in the presence of IL-2 leads to strong T-cell proliferation which is followed by IL-2 induced cell death during the contraction phase of the immune response. Activation-induced cell death is mediated through FAS/FASL and TNF/TNFR interactions (Grell et al., 1999; Green and Ware, 1997). In contrast, IL-7 and IL-15 are involved in the rescue of T-cells from apoptosis and induce cell growth which finally leads to the generation of memory T-cell pool (Schluns and Lefrancois, 2003).

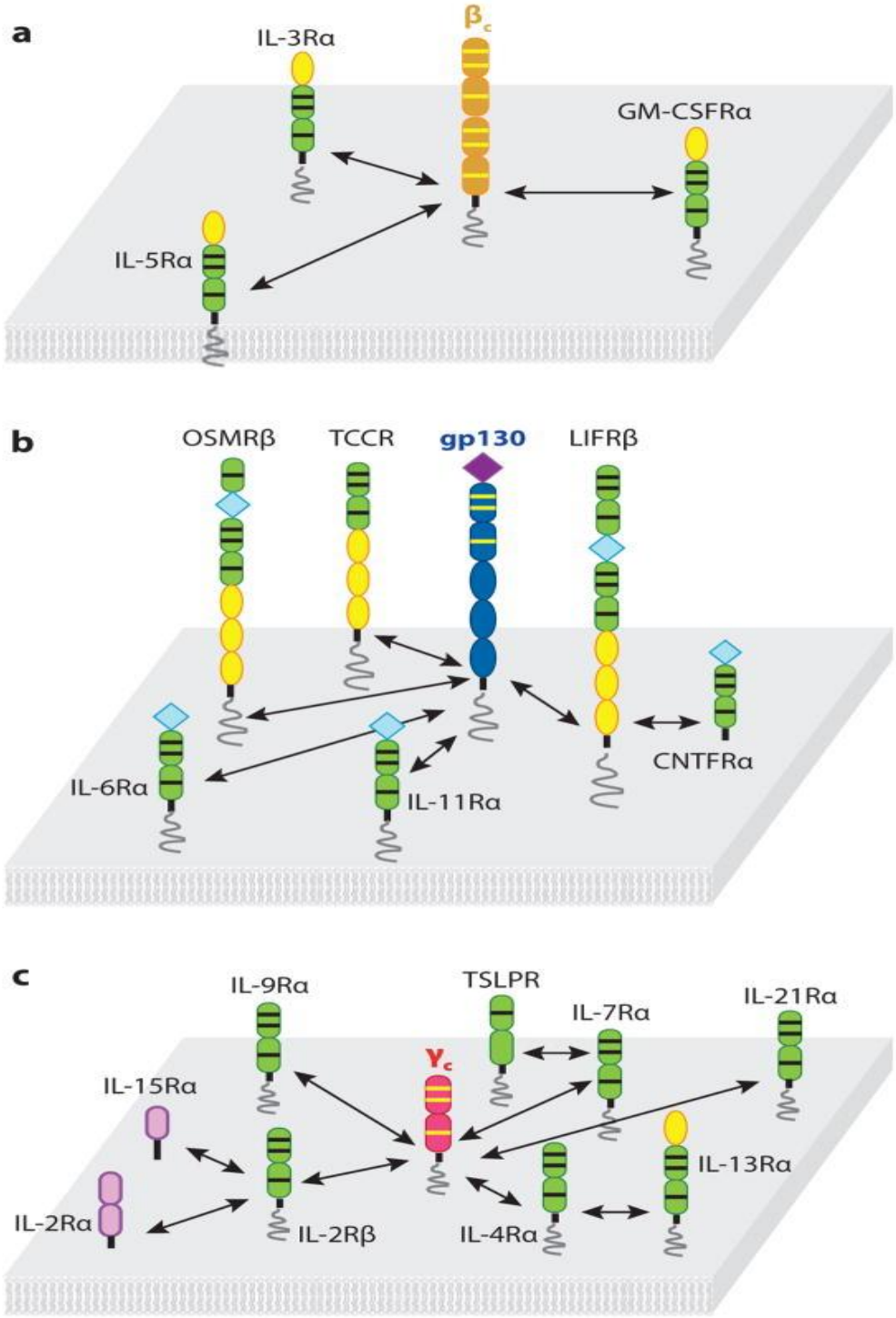


Figure 5.1 Cytokine-receptor interactions of type I family cytokine receptors.

Type I family cytokine receptors include 3 subgroups; shared cytokine receptors β_c (*a*) gp130 (*b*) and γ_c (*c*) are represented schematically on a cell membrane. The respective interacting cytokines with known three-dimensional structures are shown with cylinder representations of the four-helix bundles. (CNTF: ciliary neurotrophic factor, LIF: leukemia inhibitory factor, OSM: oncostatin M, TCCR: T cell cytokine receptor, TSLPR: thymic stromal-derived lymphopoietin receptor) (Wang et al., 2009)

IL-15 interacts with the responding cells through a unique pathway of trans-presentation (Figure 5.2) which leads to the activation of Bcl-2 and Bcl- xL genes and therefore suppresses cell apoptosis (Malamut et al., 2010). IL-7 is also able to induce expression of anti-apoptotic Bcl-2 and Bcl-xl molecules.

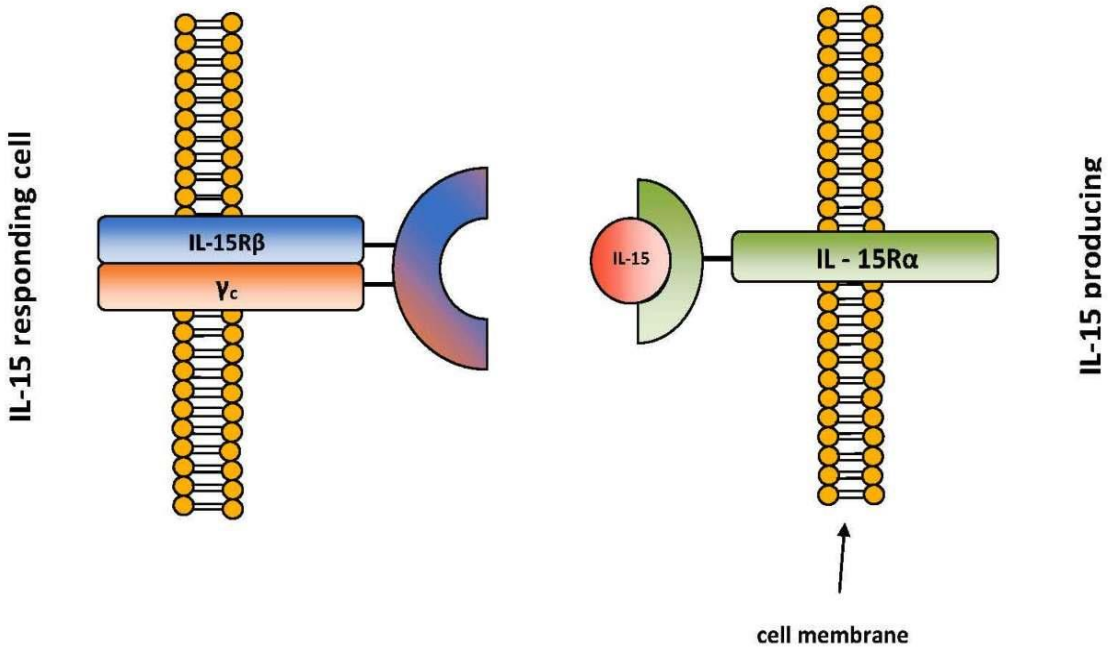


Figure 5.2 Trans-presentation of IL-15.

The main pathway of IL-15 interaction with its receptor is trans-presentation which starts with binding to IL-15Rα in IL-15 producing cells and subsequently presentation to the responding cells bearing IL-15Rβγc complex on their cell surface.

The TNF superfamily includes 19 soluble and membrane-bound ligands and 32 receptors. TNF superfamily ligands and receptors interact to regulate cell responses such as activation, proliferation, differentiation, and apoptosis (Aggarwal , 2003). Cell signalling through TNF superfamily members is a critical process in the regulation of immune responses and haematopoiesis, and is also implicated in tumorigenesis, transplant rejection, virus replication, and diabetes. Discovery of TNF/TNFR superfamily functions has led to successful development of multiple therapeutic interventions such as Infliximab (anti-TNF Ab) and Etanercept (consist of soluble TNFR2).

Some TNFR family members such as 4-1BB (CD137, TNFRSF9), OX40 (CD134, TNFRSF4), CD27 (TNFRSF7), CD30 (TNFRSF8), CD40 (TNFRSF5), GITR (CD357, TNFRSF18), HVEM (TNFRSF14), and TACI (CD267, TNFRSF13B), have been studied widely for their roles as co-stimulatory molecules in T-cell activation (Figure 5.3) (Croft, 2003 and 2009). Most of these molecules are not present in naïve T-cells and appear at high level following T-cell activation and differentiation toward effector T- cells. Generation of memory T-cells is accompanied by decrease in cell surface expression of TNF/TNFR members (Table 5.1) (Watts, 2005).

Table 5.1 Expression properties of TNF/TNFR molecules by T-cells and APCs.

Molecule	**T-cells**			**APCs**	
	Naïve	Effector	Memory	Resting	Activated
CD27	++	+++	++/-	-	B
CD70(CD27L)	-	+++	-	-	B, DC, MØ
HVEM	+++	+	+++	B, DC	B, MØ
OX40	-	+++	+/-	DC	B, DC
0X40L	-	+++	-	-	B, DC, MØ
4-1BB	-	+++	+/-	-	B, DC
4-1BBL	-	+++	-	-	B, DC, MØ
CD30	-	+++	+/-	-	-
CD30L	-	+++	-	-	B, MØ

Expression levels: -: absent, +: low level, ++: moderate level, +++: high level

Figure 5.3 TNF/TNFR family members with co-stimulatory roles.

TNF ligands are shown as homotrimeric trans-membrane proteins. The TNFR family molecules are presented as monomers that are thought to associate in trimers when interacting with their ligands. The commonly used names are in bold, with alternate names and TNF/TNFR superfamily (SF) designation in parentheses. The total amino-acid length and number of intracellular amino acids (parentheses) are also shown. Web access to TNF/TNFR nomenclature and sequence can be found at: *http://www.gene.ucl.ac.uk/nomenclature/genefamily/tnftop.html*.(Croft, 2003)

TNF has two receptors: TNFR1 (TNFRSF1A/CD120a) and TNFR2 (TNFRSF1B/CD120b). TNFR1 is expressed by a wide array of cell types but TNFR2 has restricted expression found in selective lymphoid, neuronal, mesenchymal and endothelial cells (Sedger et al., 2014). TNF as a pleiotropic cytokine induces both apoptotic and cell survival/proliferation signals (Brenner et al., 2015). Despite using different intracellular signalling pathways, both TNFR1 and TNFR2 promote cell survival through activation of NF-κB. The intracellular part of TNFR1 also contains a 'death domain' which facilitates interaction with caspases molecules and consequently induces cell apoptosis. TNFR2 does not have a 'death domain', but can relay apoptotic signals through engagement of caspases (Depuydt et al., 2005). It is still not clear how TNFR1 and TNFR2 signalling are regulated. Most of immune cells express both TNFR2 and TNFR1 and it has been shown that the relative levels of TNFR1 and TNFR2 on the surface of immune cells and their activation status are vital factors in determining cell fate and in the regulation of survival versus cell death (Pimentel-Muinos et al., 1999).

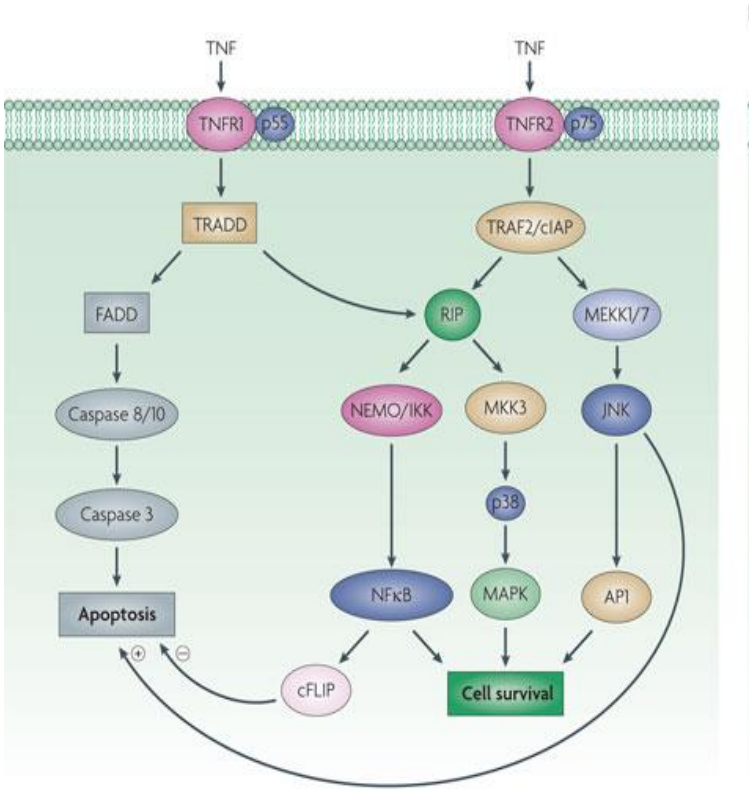


Figure 5.4 TNF interaction with TNFR1 and TNFR2 induces two opposing signalling events.

TNFR signalling can result either in cell survival or apoptosis. This is dependent upon the cell type, the state of activation of the cell and the cell cycle. Engagement of both TNFR1 and TNFR2 can induce NF-KB activation which leads to cell survival through BCL-2 activation. In addition, binding of TNF of to its receptors could rely apoptotic signals through activation of caspases. AP1, activator protein 1; cFLIP, cellular FLICE-like inhibitory protein (also known as CFLAR); cIAP, cellular inhibitor of apoptosis protein; FADD, Fas-associated death domain; IKK, inhibitor of κB kinase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEKK, mitogen-activated protein kinase kinase kinase; MKK3, dual specificity mitogen-activated protein kinase kinase 3; NEMO, nuclear factor-κB (NF-κ B) essential modulator; RIP, receptor interacting protein; TRADD, TNFR1-associated death domain; TRAF, TNF receptor-associated factor. (Faustman and Davis, 2010)

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$CD8^+$ and $CD4^+$ T-cells are currently categorised into 4 main subsets based on the expression of lymph- node homing receptor CCR7 and membrane CD45RA molecule. These subsets represent T cells in their differentiation pathway following antigenic response: $CD45RA^+$ $CCR7^+$ (Naïve), $CD45RA^-$ $CCR7^+$ (central memory, CM), $CD45RA^-$ $CCR7^-$ (effector memory, EM) and $CD45RA^+$ $CCR7^-$ (effector memory $CD45RA^+$, EMRA). Phenotypic analysis of T-cell subsets has shown distinct expression of functional molecules by naïve and memory T-cells (Mahnke et al., 2013). IL-7Rα is mainly expressed by naïve, CM and EM subsets and this serves IL-7 induced homeostatic expansion and survival of naïve and memory T-cells. IL-6R (gp130) is expressed by naïve and CM T-cells and its signalling leads to IL-7 production and consequently T- cell proliferation. In contrast, T-cells with late differentiation EMRA phenotype have less proliferation and survival potential, particularly in response to IL-7. In humans, infection with CMV has a profound effect on the frequency of T cell subsets in the peripheral blood (Wertheimer et al., 2014). The majority of CMV-specific T-cells have a late differentiation EM/EMRA phenotype and express CD57, discussed in chapter 4. It is well documented that the number of memory/effector memory cells is increased in whole T-cell population of CMV sero-positive elderly individuals. CMV infection is accompanied by production of Th1 derived cytokines such as IFN- γ and TNF-α which are made by differentiated T- cells (van de Berg et al., 2010).

In this study, cytokine receptor expression was studied in T-cell subsets of CMV-sero-positive and sero-negative healthy individuals, and found that the expression of TNFR2 was uniquely skewed towards more differentiated cells. This observation was

investigated in more functional detail using a cognate ligand to stimulate peripheral blood $CD8^+$ and CMV specific T-cells from healthy blood donors and HSCT patients. The results showed that CMV specific T-cells, which were predominantly differentiated effector memory T-cells, had high TNFR2 expression and that *in vitro* ligation of TNFR with a specific ligand led to the apoptosis of these cells.

5.2 Results

5.2.1 T-cell subset distribution and CMV infection

First, the distribution of differentiation subsets was studied amongst $CD4^+$ and $CD8^+$ T-cells derived from healthy blood donors. These were young and middle age individuals, with a mean age of 42 years. The blood donors were laboratory workers and platelet donors of the NHS Blood and Transplant Donor Centre, Birmingham, UK.

$CD4^+$ and $CD8^+$ T-cells have different distribution patterns. Amongst $CD4^+$ T-cells, the size of the differentiation subsets followed a decreasing trend from naïve to effector cells with the frequency of naïve cells ($CCR7^+$ $CD45RA^+$) highest (median; 47.50%), followed by CM ($CCR7^+$ $CD45RA^-$) (30.20%), EM ($CCR7^-$ $CD45RA^-$) (15.30%) and EMRA cells ($CCR7^-$ $CD45RA^+$) (4.15%) (Figure 5.5A). The pattern was different for $CD8^+$ T-cells. Naïve and EMRA cells occupied similar proportions with lower percent of EM and CM cells and the lowest frequency represented by CM cells (5.20%) (Figure 5.5A). However, this is likely a reflection of the influence of exposure to CMV in a proportion of the donors. Previous reports have shown that CMV infection changes the T-cell subset frequencies in favour of more differentiated T-cells which become more prominent with ageing (Khan et al., 2002). By segregating the data according to their

CMV sero-status we confirmed that the decreased frequency of naïve cells and corresponding increase in effector cells amongst both $CD4^+$ and $CD8^+$ T-cells were accentuated in the CMV sero-positive group (Figure 5.5B,C). The 2 groups of donors were CMV serostatus and age-matched (median age: 47.2 and 43.1 years). However, these differences did not reach statistically significant levels. CD57 is another marker of late T –cell differentiation and $CD4^+$ and $CD8^+$ $CD57^+$ T-cells have been linked to CMV infection (discussed in chapter 4). When we analysed our healthy donors for CD57 expression, we found that the frequency of $CD8^+$ $CD57^+$ T-cells were higher in CMV sero-positive compared to CMV sero-negative individuals, and this was statistically significant ($p=0.01$, Figure 5.5D). Importantly, $CD4^+$ $CD57^+$ T-cells were barely detectable in CMV sero-negative donors, with median frequency of 0.4 percent, whilst the median frequency in CMV sero-positive blood donors was 12 percent ($p<0.0001$, Figure 5.5D). As presented in the chapter 4, this molecule could be used as a biomarker for clinical diagnostic purposes in the management of CMV reactivation.

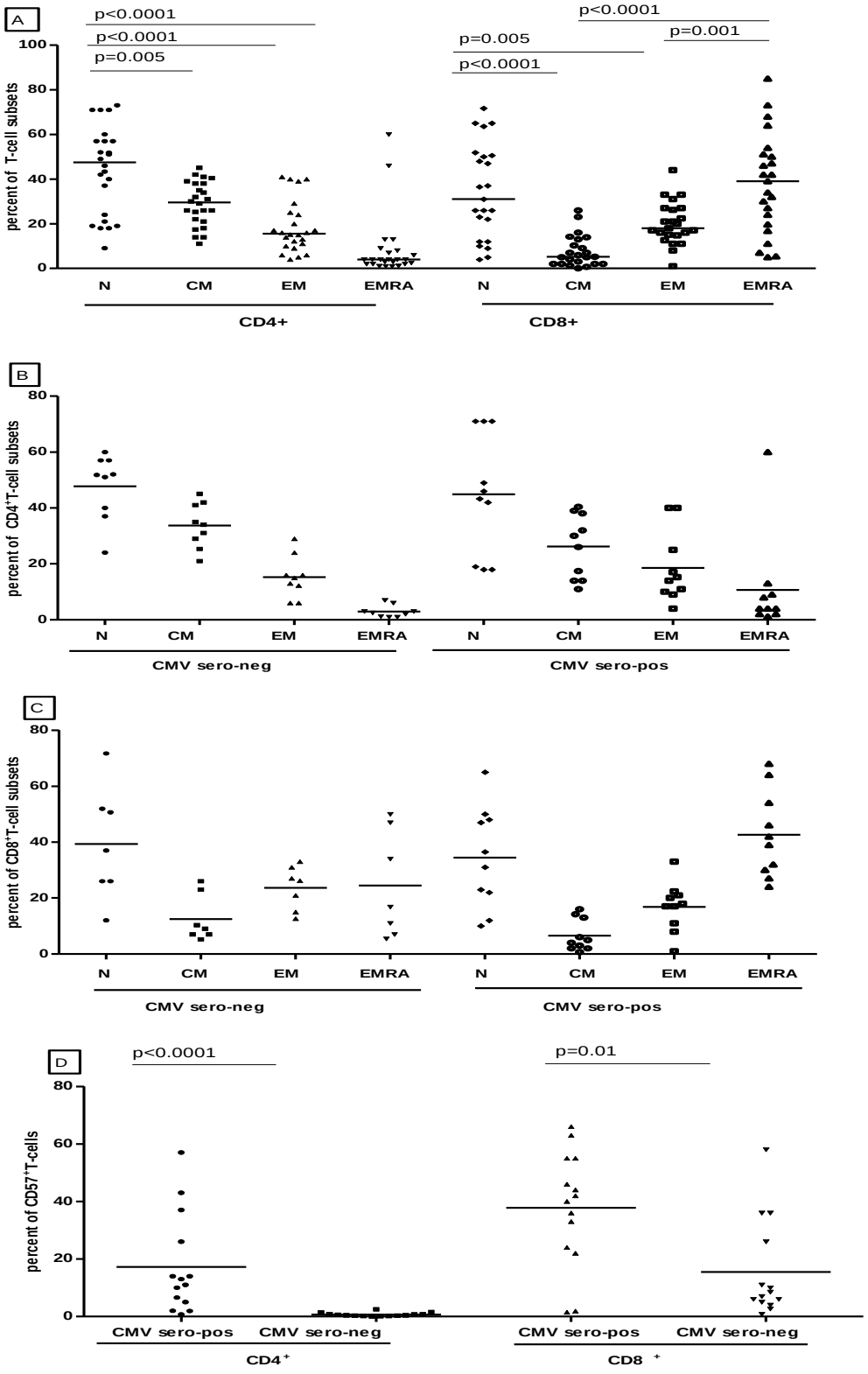


Figure 5.5 Distribution of T-cell subsets in healthy individuals and CMV infection.

Figure 5.5 (continued). (A) Distribution of T-cell subsets in $CD4^+$ and $CD8^+$ populations of healthy individuals (n=27) which shows statistically significant differences in their frequencies. Frequencies of $CD4^+$ T-cell subsets in (B) and $CD8^+$ (C) cells with respect to CMV infection which showed no difference in T-cell subset distribution in CMV^+ versus CMV^- healthy donors. D) Frequency of $CD57^+$ T-cells in CMV^+ versus CMV^- individuals. CMV infection is associated with increase in the frequency of $CD57^+$ T-cells. This was especially prominent in the $CD4^+$ population with a mean of less than 1% frequency of $CD4^+$ $CD57^+$ T-cells in CMV^- donors.

5.2.2 Expression of IL-2Rα (CD25) in T cell subsets

IL2 is a cytokine that promotes survival and homeostatic proliferation, so the presence of CD25 (IL2R) on T cell surface should be an indicator of the survival and proliferative capacity of the cells. Therefore, the overall frequency of CD25 positive cells was determined within the $CD4^+$ and CD8+ populations and found this receptor was more abundant amongst $CD4^+$ (median: 12.43%) than $CD8^+$ T-cells (median: 2.59%) (p=0.001) (Figure 5.6A). However, when segregated according to differentiation subsets, CD25 expression was found to increase from a low base in naïve cells to a median of 21.50% amongst CM cells, and reaching a peak in the EM subset (median: 37.41%). Further differentiation toward EMRA, however, led to a decrease in $CD25^+$ expressing T-cells. Amongst $CD8^+$ T cells, CD25 expression increased following cell differentiation and had its highest level in the CM subset (5.55%) and its frequency decreased with increasing differentiation (Figure 5.6B).The frequency of $CD25^+$ cells was higher amongst CMV^+ individuals (Figure 5.6C), despite the higher proportion of highly differentiated cells. Nevertheless, the frequency of CD25 expression was lower in $CD57^+$ when compared to $CD57^-$ T-cells (Figure 5.6D), in line with low CD25 expression amongst highly differentiated T-cells.

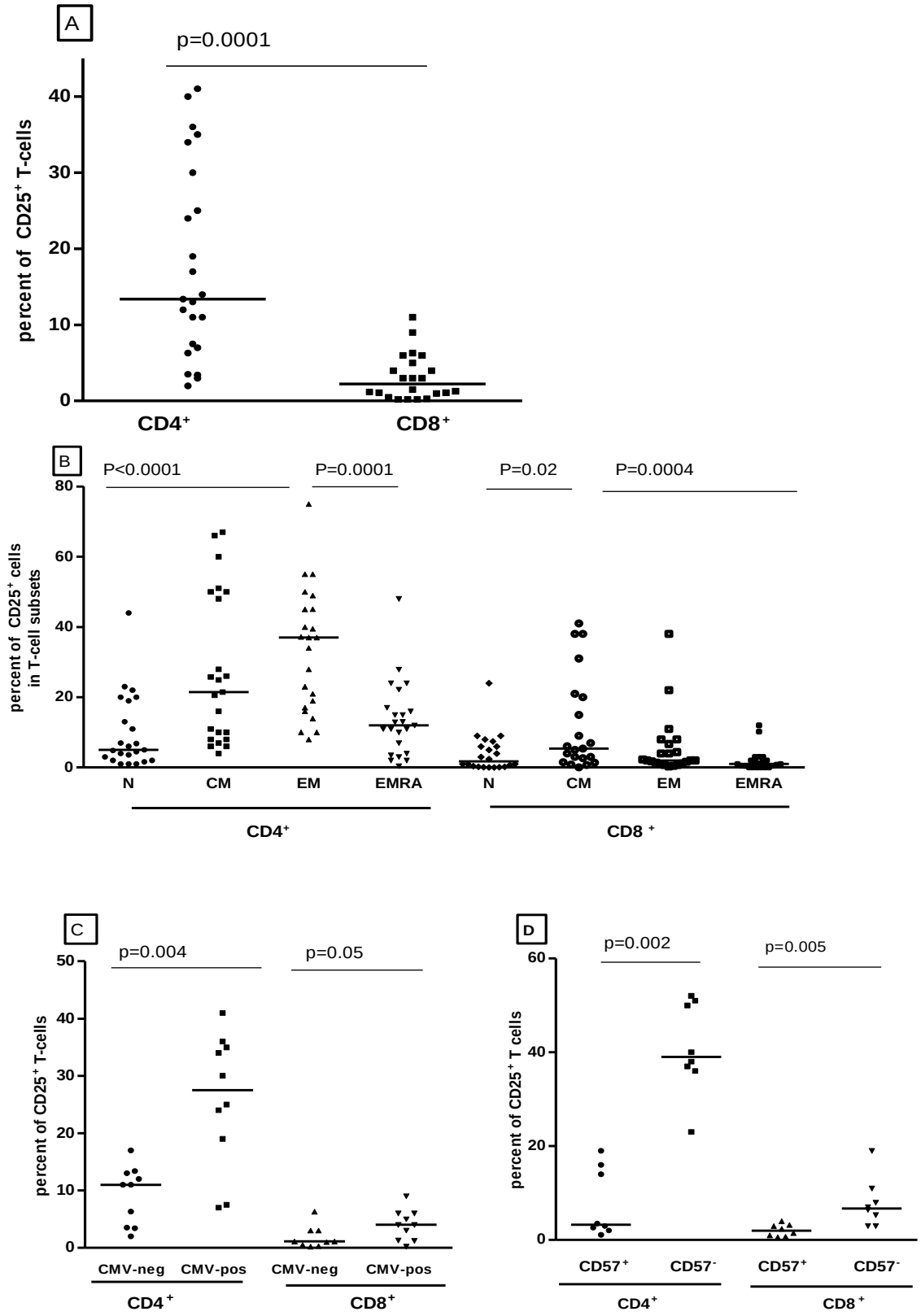


Figure 5.6 CD25 expression in T-cell subsets of healthy blood donors.

Figure 5.6 (continued). (A) Higher prevalence of CD25 expression in $CD4^+$ T-cells in comparison to $CD8^+$ population. (B) In both $CD4^+$ and $CD8^+$ T-cells, all the subsets expressed CD25 and this expression increased following cell differentiation with the highest level of $CD25^+$ cells were observed in EM and CM subsets of $CD4^+$ and $CD8^+$ T-cells, though less pronounced amongst CD8. (C) Increase in the frequency of $CD25^+$ T-cells was a global effect of CMV infection in healthy individuals. (D) A low number of $CD57^+$ T-cells expressed CD25.

5.2.3 Expression of IL-7Rα (CD127) in T-cell subsets

IL7 is a key cytokine responsible for the maintenance and survival of T cells and it was observed that how the cognate receptor is mainly expressed by early differentiated T cells.

Overall, fewer $CD8^+$ T-cells expressed IL-7Rα (38.23%) in comparison to $CD4^+$ T-cells (61.20%) (p=0.003) (Figure 5.7A). In the naïve subsets of $CD4^+$ and $CD8^+$ T-cells, around half of the cells expressed IL-7Rα and this frequency increases in CM cells to 62.55% and 68.00% for $CD4^+$ and $CD8^+$ T-cell, respectively (Figure 5.7B), but then diminishes following further differentiation with medians of 28% and 17% for EMRA subsets of $CD4^+$ and $CD8^+$ T-cells, respectively. Our results showed no statistically significant differences in the overall frequency of IL-7Rα positive T-cells between CMV^+ versus CMV^- individuals (Figure 5.7C), but highly significant when we compared IL7Rα expression between $CD57^+$ with $CD57^-$ T cells. Similar to the low expression of IL-2Rα by $CD57^+$ T-cells, IL-7Rα was also low in CD57+ cells. The median frequencies of $CD4^+$ $CD57^+$ $CD127^+$ and $CD8^+$ $CD57^+$ $CD127^+$ were 34.10% and 15.00%, respectively in CMV sero-positive individuals (Figure 5.7D).

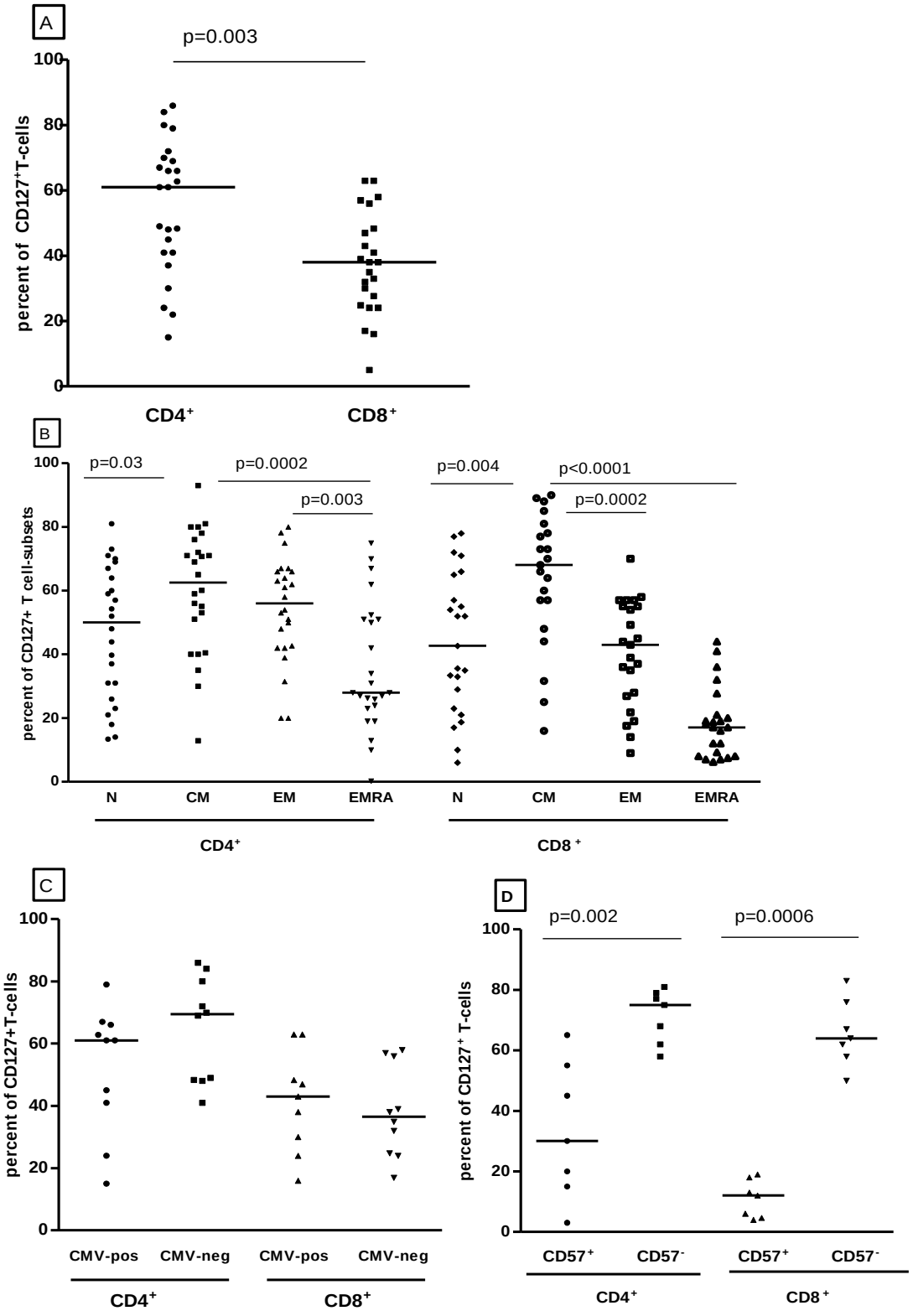


Figure 5.7 CD127 (IL7Rα) expression in T-cell subsets of healthy blood donors.

Figure 5.7 (continued). (A) Higher prevalence of CD127 expression in $CD4^+$ T-cells in comparison to $CD8^+$ population. (B) In both $CD4^+$ and $CD8^+$ T-cells, all the subsets expressed CD127 and this expression increased following cell differentiation with the highest level of $CD127^+$ cells was observed in CM subsets of $CD4^+$ and $CD8^+$ T-cells. Expression of this cytokine receptor decreased in fully differentiated $CD4^+$ and $CD8^+$ T-cells. (C) CMV infection did not have any effect on the frequency of $CD127^+$ $CD4^+$ and $CD8^+$ T-cells. (D) In comparison to $CD57^-$ T-cells, significantly lower number of $CD57^+$ T-cells expressed CD127.

5.2.4 Expression pattern of IL-15Rα

Detection of IL-15Rα positive T-cells was inefficient due to the trans-presentation of this receptor (Figure 5.2). The method used in this study detected low level of IL-15Rα in all T-cell subsets of $CD4^+$ and $CD8^+$ cells with no statistically significant difference between them (Figure 5.8A). In $CD4^+$ T-cells, naïve and EMRA cells had slightly higher frequencies of IL-15Rα positive cells (medians: 3.50% and 8.55%, respectively). In $CD8^+$ T-cells, detectable level of IL-15Rα was lower but the highest frequency was observed in CM cells with median of 2.50% (Figure 5.8B). Co-staining of cells with anti IL-15Rα and CD57 antibodies revealed higher expression of IL-15Rα in $CD4^+$ $CD57^+$ and $CD8^+$ $CD57^+$ T-cells in comparison to $CD57^-$ T cells (Figure 5.8C). This is in keeping with previous report showing important role of IL-15 in the proliferation of late differentiated T-cells (Broux et al., 2015; Picker et al., 2006).

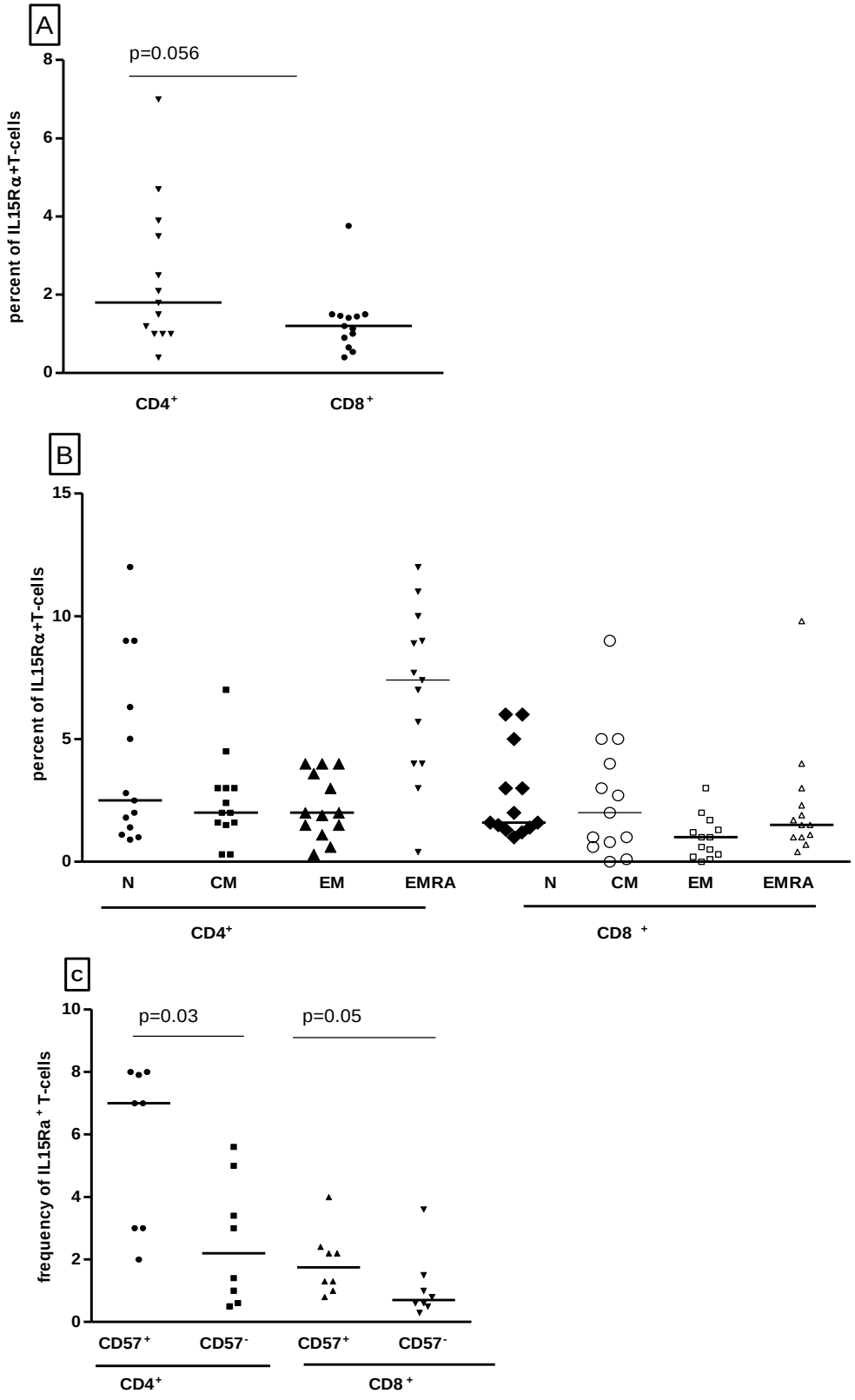


Figure 5.8 IL-15Rα expression in T-cell subsets of healthy blood donors.

Figure 5.8 (continued). (A) IL-15Rα expression was more frequent in $CD4^+$ T-cells in comparison to $CD8^+$ population. (B) In $CD4^+$ and $CD8^+$ T-cells, frequencies of detectable IL-15Rα^+ T-cells were low and in $CD4^+$ T-cells, EMRA cells had the highest frequency of T-cell subsets expressing IL-15Rα. (C) In comparison to $CD57^-$ T-cells, IL-15Rα expression was more frequent in $CD57^+$ T-cells.

5.2.5 Expression pattern of CD130 (IL-6Rα)

IL-6 is a potent inflammatory cytokine. The frequency of its cognate receptor CD130 expression was considerably higher in $CD4^+$ (median: 47.10%) than in $CD8^+$ T cells (median:7.22%) (p=0.0001, Figure 5.9A), although its distribution amongst the differentiation subsets is comparable (Figure 5.9B). Unlike IL-2, IL-7 and IL-15 receptors, CD130 expression was highest in the naïve T-cell population. In $CD4^+$ and $CD8^+$ T cells, the median CD130 frequency amongst naïve cells were 74.10% and 24.20% for $CD4^+$ and $CD8^+$ T-cells, respectively, and decreased rapidly with differentiation, reaching its lowest level in EM/EMRA cells (medians: 11.22% and 1.20% , in $CD4^+$ and $CD8^+$ T-cells, respectively) (Figure 5.9B).

The CD130 expression was independent of CMV status (Figure 5.9C). $CD57^+$ T-cells had much lower CD130 frequency (medians: 8.10% and 4.15%, respectively) than $CD57^-$ T cells (medians: 58.45% and 18.12%) (p=0.0009, p=0.006, respectively) (Figure 5.9D). This finding supports the previous observation of preferential CD130 expression amongst naïve and less differentiated cells.

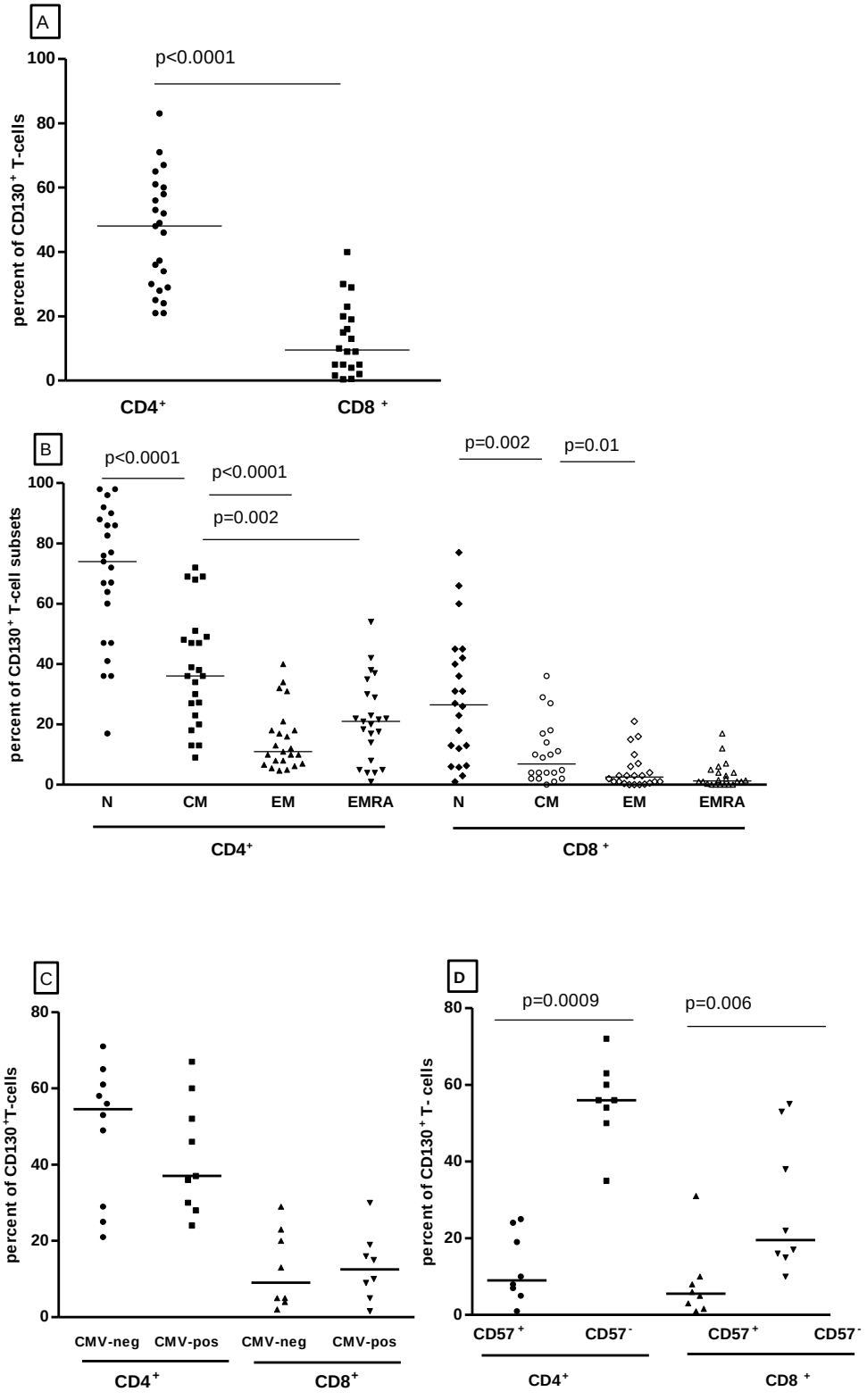


Figure 5.9 CD130 (IL-6Rα) expression in T-cell subsets of healthy blood donors.

Figure 5.9 (continued). (A) Higher prevalence of CD130 expression in $CD4^+$ T-cells in comparison to $CD8^+$ population. (B) In both $CD4^+$ and $CD8^+$ T-cells, the highest frequency of $CD130^+$ cells belonged to naïve T-cell subsets and cell differentiation led to the rapid decrease in the expression of this receptor. In this line, very low percent of EMRA cells expressed CD130. (C) CMV infection did not have any effect on the frequency of $CD130^+$ $CD4^+$ and $CD8^+$ T-cells. (D) Frequency of $CD57^+$ $CD130^+$ T-cells was very low whilst considerable percent of $CD57^-$ T-cells expressed CD130.

5.2.6 Expression pattern of CD120b (TNFR2)

Another commonly studied inflammatory cytokine is TNF which interacts with two receptors: TNFR1 and TNFR2. TNFR1 was not detectable in T-cell through flow cytometry method used in this study. TNFR2 was easily detectable in T-cell subsets.

Peripheral blood $CD4^+$ and $CD8^+$ T-cells had similar frequencies of TNFR2 (CD120b) expression (medians: 15.60% and 21.00%, respectively) (Figure 5.10A). However, opposite to CD130 and uniquely different from other cytokine receptors, TNFR2 expression increased with T-cell differentiation (Figure5.10B). The lowest CD120b expression was detected in naïve T cells (medians: 4.50% and 4.00% in $CD4^+$ and $CD8^+$ T-cells, respectively) and this expression gradually increased through CM, EM and reached its highest level amongst EMRA cells, with medians of 45.50% and 48.00% for $CD4^+$ and $CD8^+$ T-cells, respectively) (Figure 5.10B). CMV infected individuals showed even higher frequency of TNFR2 positive T-cells (Figure 5.10C). This is the first report of a possible effect of CMV infection on TNFR2 expression by peripheral blood T-cells. Accordingly, $CD4^+$ $CD57^+$ and $CD8^+$ $CD57^+$ T-cells also had significantly higher frequencies of TNFR2 expression in comparison with $CD57^-$ T-cells (Figure 5.10D). Nearly all $CD57^+$ T-cells expressed TNFR2 whilst only 25.15% $CD4^+$ $CD57^-$ T-cells expressed this receptor, suggesting a close relationship of TNFR2 expression with late T-cell differentiation.

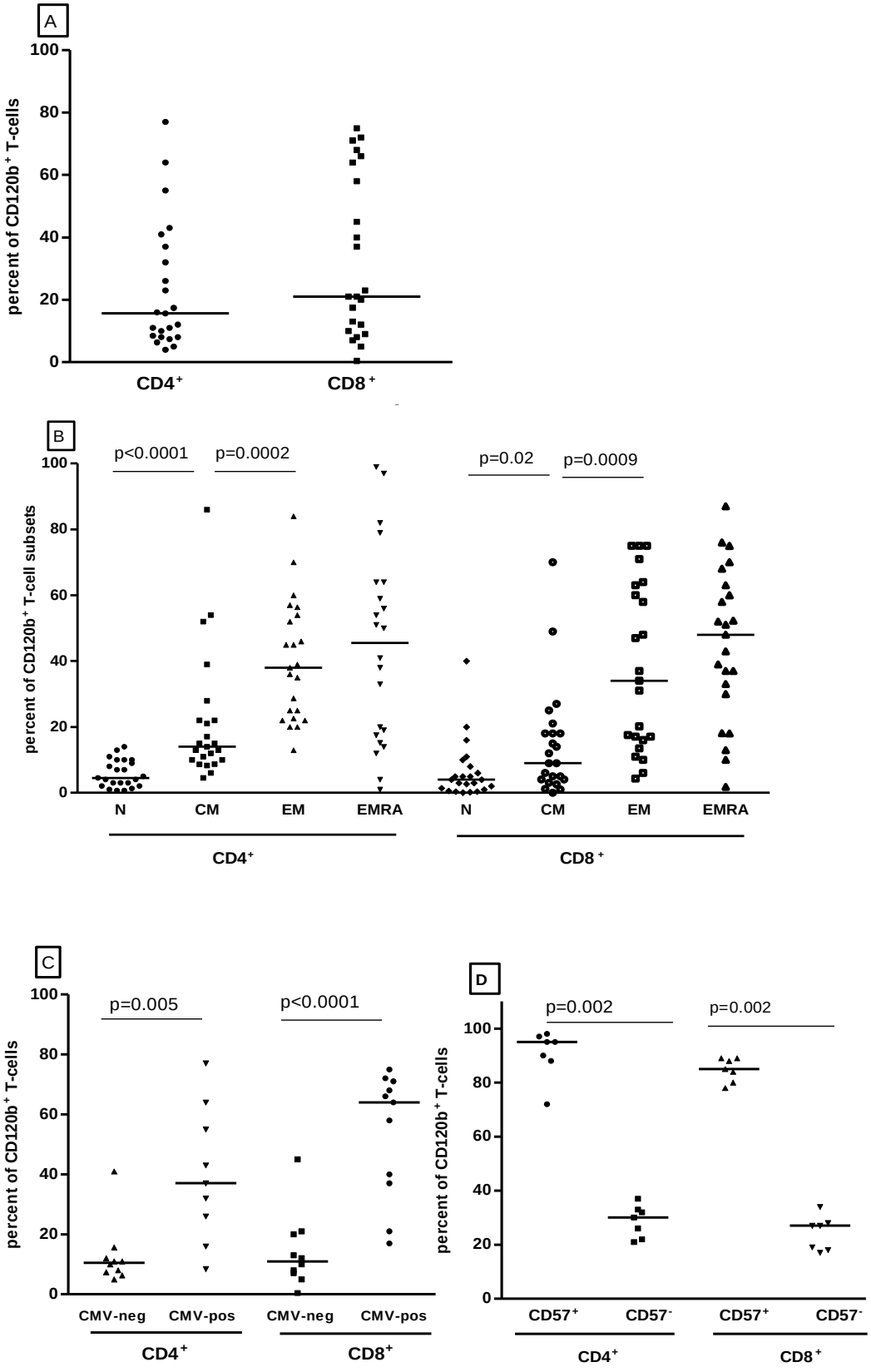


Figure 5.10 CD120b (TNFR2) expression in T-cell subsets of healthy blood donors.

Figure 5.10 (continued). (A) In overall, frequency of $CD120b^+$ cells was similar in $CD4^+$ and $CD8^+$ populations. (B) In $CD4^+$ and $CD8^+$ T-cells, the highest frequency of $CD120b0^+$ cells belonged to the fully differentiated cells whilst naïve T-cell subsets had the lowest frequency of cells expressing CD120b. (C) CMV infection had significant effect on CD120b expression. CMV sero-positive blood donors had statistically significant higher levels of circulating $CD4^+$ $CD120b^+$ and $CD8^+$ $CD120b^+$ cells. (D) Nearly all $CD57^+$ T-cells expressed CD120b whilst less than half of $CD57^-$ T-cells expressed this receptor.

5.2.7 TNFR2 expression in CMV-specific T-cells

Increased frequency of TNFR2 positive cells amongst highly differentiated $CD57^+$ T-cell populations persuaded the analysis of TNFR2 expression in CMV specific $CD8^+$ T-cells of healthy blood donors and stem cell transplant patients. Using CMV-specific HLA-tetramers, the peripheral blood lymphocytes from 6 CMV sero-positive healthy individuals were studied. This showed that a range of 28-100% of CMV-specific $CD8^+$ T-cells expressed TNFR2. Due to low number of samples, it was not possible to draw any conclusion on reason(s) behind this wide range of $TNFR2^+$ expression levels amongst CMV-specific T-cells. As described in the chapter 4, longitudinal analysis of PBMCs collected from 4 HSCT patients up to 21 months post-transplant, showed that TNFR2 was expressed by nearly all CMV-specific $CD4^+$ and $CD8^+$ T-cells at all studied time points with the exception of one sample (below). During CMV reactivation which induces CMV-specific T-cell expansion (inflation phase), the density of TNFR2 expression at the single cell level increased sharply as demonstrated by the increase in the median fluorescence intensity of TNFR2 staining (Figures 4.11 and 5.11). This is possibly due to increase in TNFR2 expression in activated or recently activated cells.

In patient 1, where 92% of CMV specific $CD8^+$ T-cells had an EMRA phenotype, CD120b was strongly expressed. At the single cell level, the MFI of CD120 expression increased during T-cell expansion, and then decreased to reach a new low steady state level following CMV viral clearance and contraction of T-cell response (monitored up to 6 months post transplant). A sample obtained 21 months post transplant, long after the resolution of CMV reactivation, revealed that around 30% of CMV specific $CD8^+$ T-cells still expressed CD120b, but with considerably diminished MFI (Figure 5.11A).

CMV-specific $CD4^+$ T-cells also expressed CD120b (90%) and alongside this, their MFI also changed during its response to CMV with the highest level of MFI observed at peak level of cell expansion, and reached its lowest level 21 months post transplant.

In patient 2, CMV-specific T-cells expressed CD120b and its expression level increased during T-cell expansion and decreased during the contraction phase of T-cell response to CMV (Figure 5.11B). CD120b expression in CMV-specific $CD8^+$ T-cells of patients 3 and 4 peaked during cell expansion in response to CMV and decreased following viral clearance and decrease in T-cell number (Figure 5.11 C-D). Comparison of CD120b levels during (median MFI: 7500) and after CMV viraemia (median MFI: 3200) in these patients showed statistically significant difference in the expression level of this cytokine receptor in CMV-specific T-cells ($p=0.03$) (Figure 5.12). These results are in line with the previous data on increased expression of TNFR family members following T-cell activation (Table 5.1) (Watts, 2005).

These results suggest that CD120b up-regulation is a response to T cell activation by antigens. We therefore activated PBMCs from healthy blood donors with IL-2 and anti-CD3 Abs for 3 days, and observed the increased CD120b expression in T-cells (Figure 5.13).

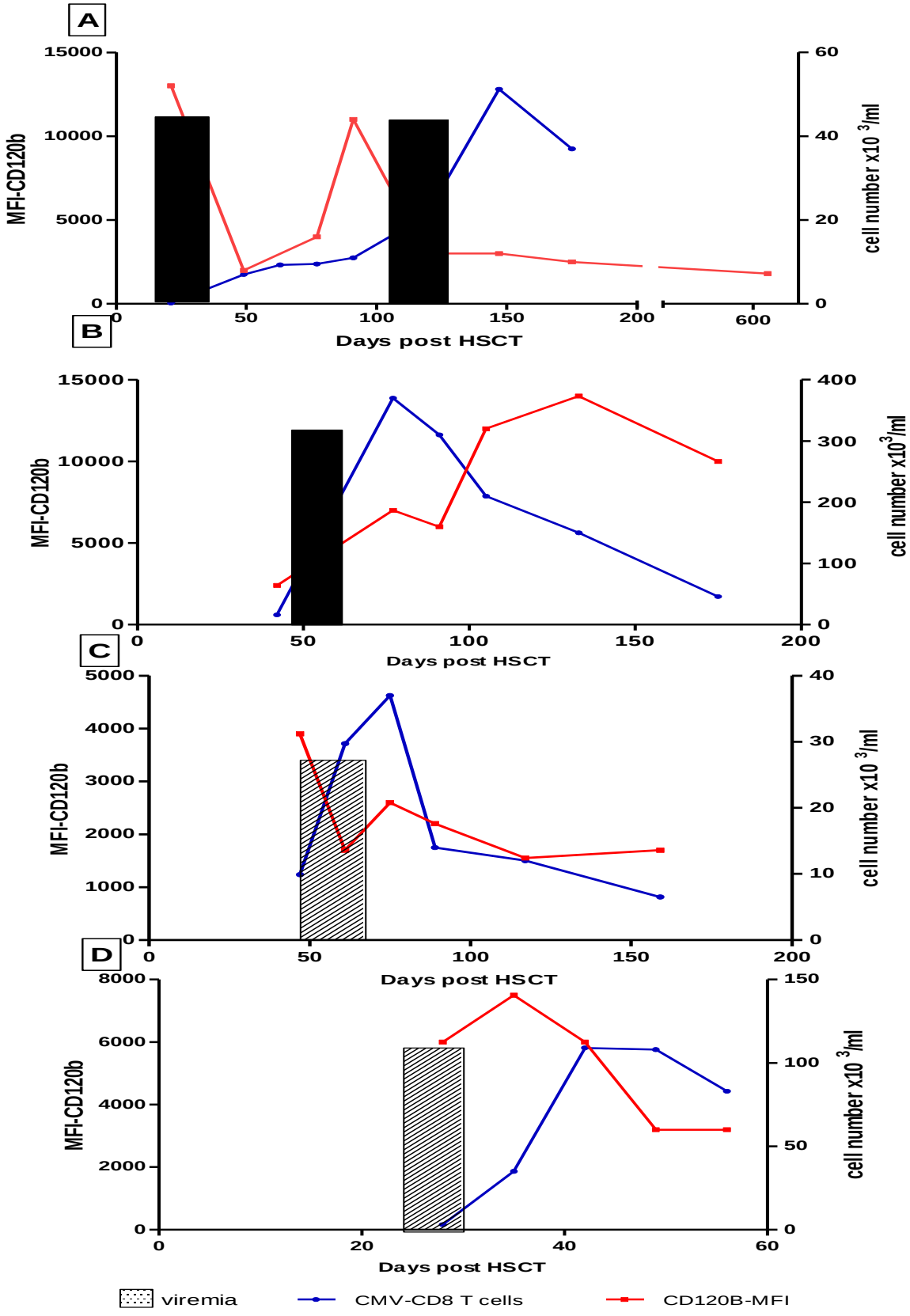


Figure 5.11 CD120b (TNFR2) expression level of CMV-specific T-cells in response to CMV viraemia.

Figure 5.11 (continued) CD120b expression in CMV-specific $CD8^+$ T-cells of 4 HSCT patients (A-D) was studied up to 21 months post transplantation. Following viraemia and during inflation phase of T-cell response, CD120b expression per cell increased rapidly. In 3 patients (A, C, D), CMV–specific T-cell numbers decreased (contraction phase) shortly after reduction in CD120b expression level. In overall, there were parallel changes in the level of CD120b expression and T-cell frequencies.

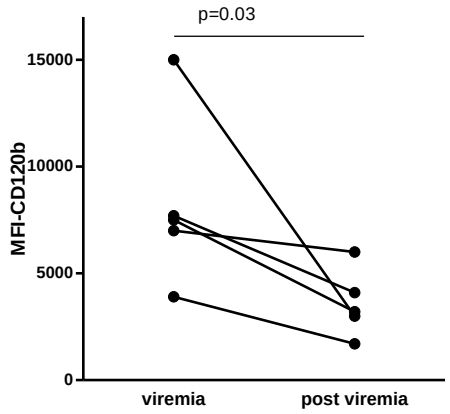


Figure 5.12 Comparison of CD120b expression level in CMV-specific T-cells of HSCT patients.

Significant increase in the density of CD120b expression at the single cell level (as detected by MFI) during T-cell response to CMV viraemia.

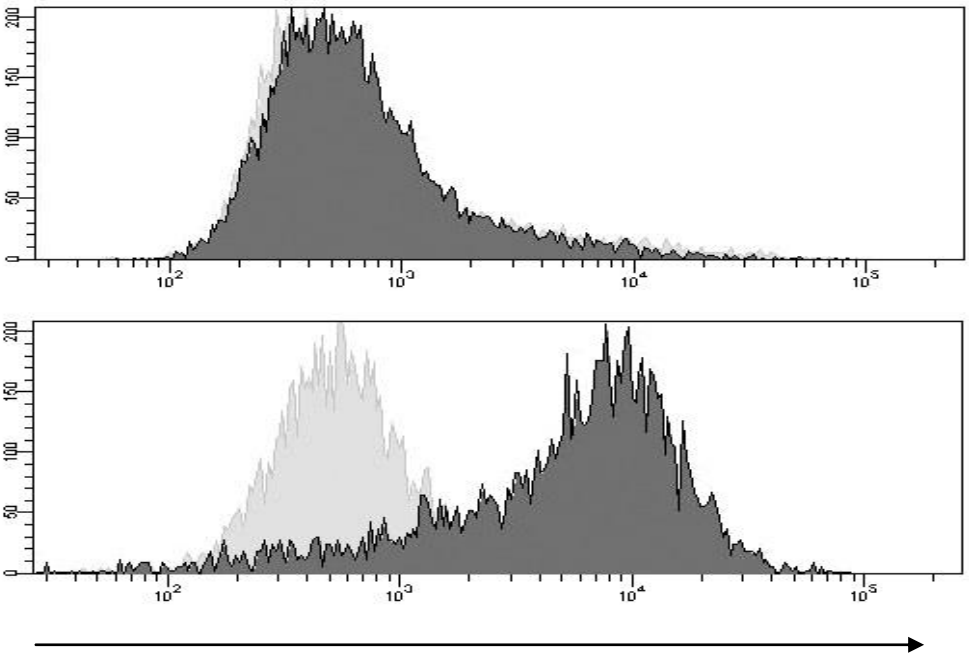


Figure 5.13 CD120b expression and in vitro T-cell activation.

T-cell activation with anti-CD3 and IL-2 Abs led to increase in CD120b (TNFR2) expression. Top and bottom histograms show CD120b expression (dark area) before and after T-cell activation, respectively.

5.2.8 Ligation of TNFR2 with a specific agonist induces cell apoptosis

In order to study what purpose the increased expression of TNFR2 in differentiated cells may serve, PBMCs isolated from healthy individuals and HSCT patients were incubated with a custom-made TNFR2 agonist, called Flag-TNC-scTNF (143N/145R) (Fischer et al., 2011) (Figure 5.14). TNFR2 preferentially binds to membrane TNF (Grell et al., 1995) and ligation of soluble Flag-TNC scTNF (143N/145R) mimics binding of TNFR2 to membrane TNF (m TNF).

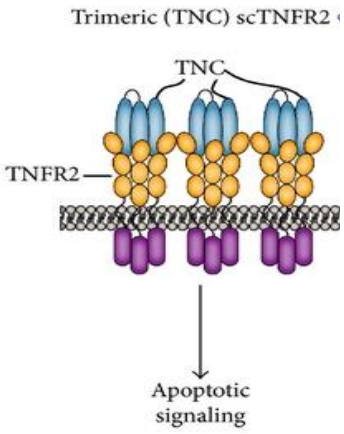


Figure 5.14 Interaction of TNFR2 and its soluble agonist (Flag-TNC-* scTNF (143N/145R) *which mimics TNFR2 binding to membrane TNF (main ligand for TNFR2). (Bremer et al., 2013)

Experiments were first conducted to determine the optimal concentration of the ligand. PBMC were incubated with the ligand at concentrations of 50, 100 and 200 ng/ml of Flag-TNC-scTNF (143N/145R). T-cell numbers, mainly $CD8^+$ $CD3^+$ population (Figure 5.15) decreased with increasing concentration. $CD8^+$ T-cell number dropped from 272 cells/µl (un-stimulated cells) to 194/µl, 142/µl and 142/µl following cell incubation with 50, 100 and 200 ng/ml of Flag-TNC-scTNF (143N/145R), respectively.

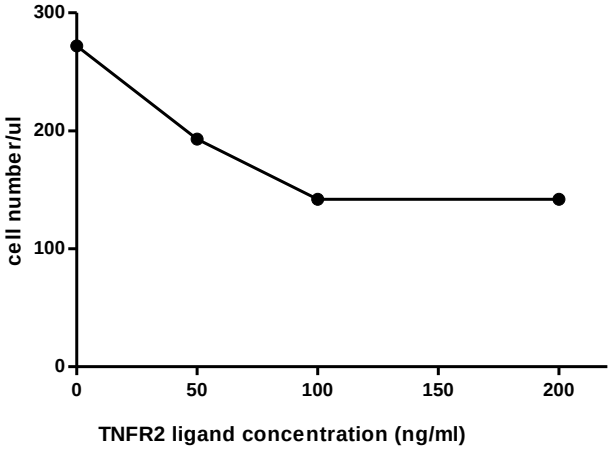


Figure 5.15 Titration of Flag-TNC-scTNF (143N/145R) for study of TNFR2 ligation.

In order to determine the working concentration of Flag-TNC-scTNF (143N/145R), PBMCs from healthy lab workers were stimulated with 50, 100 and 200 ng/ml of this reagent for 3 days. Following incubation and in comparison to the control (cells with no added ligand), a reduction in the CD8 T-cell number was observed. The biggest cell loss occurred in the presence of either 100 or 200ng/ml of Flag-TNC-scTNF (143N/145R).

Next, PBMCs from 3 healthy CMV sero-positive individuals were incubated with Flag-TNC-scTNF (143N/145R) in order to analyse cell loss in detail. This experiment was repeated 3 times for each individual. Phenotypic analysis showed that in all 3 samples, cell loss was restricted to EM/EMRA T-cell subsets (Figure 5.16). As the Figure 5.16 with data from one of the donors shows, the number of naïve (N) (beige bars) and CM subsets (red bars) did not change following cell incubation with different concentrations of Flag-TNC-scTNF (143N/145R). On the other hand, this incubation caused up to 60% and 34% cell loss in EM (green bars) and EMRA (blue bars) subsets, respectively. It has been mentioned in the section 5.2.6 that EM/EMRA T-cell populations were the main subsets of T-cells expressing TNFR2 and accordingly, engagement of TNFR2 with Flag-TNC-scTNF (143N/145R) resulted in the cell loss of only $TNFR2^+$ EM/EMRA cells. Analysis of data obtained from 3 blood donors showed that the percentages of cell loss in $TNFR2^+$ populations were significantly higher than in

TNFR2 negative cells (Figure 5.17). Following TNFR2 ligation, the average reduction in the number of $CD8^+$ $TNFR2^+$ T-cells was 50.33% in comparison to 2.00% in $CD8^+$ $TNFR2^-$ T-cells (p=0.001). Also, the median reduction in EM/EMRA $TNFR2^+$ T-cell subsets was 54.66% in comparison to 6.30% in EM/EMRA $TNFR2^-$ cells (p=0.004). Therefore, incubation with Flag-TNC-scTNF (143N/145R) specifically targeted $TNFR2^+$ cells and reduced their frequency.

The cell loss in $CD57^+$ T-cells was also observed following TNFR2 ligation in 3 healthy individuals. The percentages of reduction in $CD57^+$ $CD57^+$ T-cells were 74.41%, 33.12% and 34.00% in those individuals.

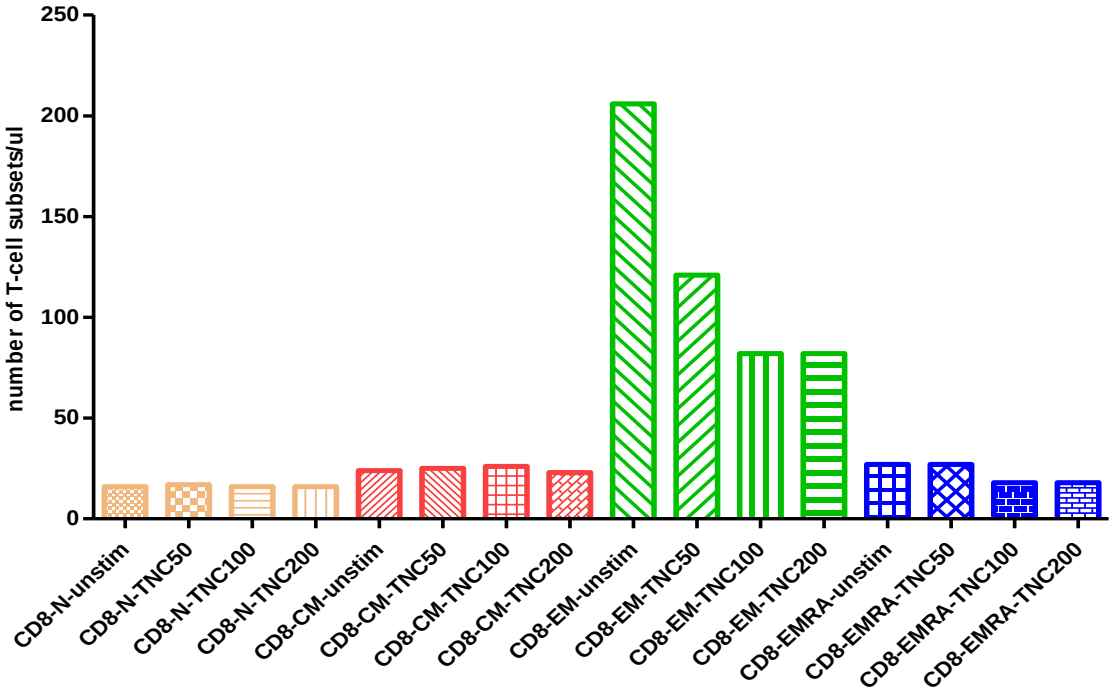


Figure 5.16 Effect of TNFR2 ligation on the frequency of CD8$^+$ T-cell subsets.

PBMCs from healthy blood donors were incubated with 50, 100 and 200 ng/ml of Flag-TNC-scTNF (143N/145R). The Figure represents data obtained from one of donors and shows reduction in the number of EM and EMRA CD8$^+$ T-cells following TNFR2 ligation whilst the number of naïve (N) and CM subsets did not change.

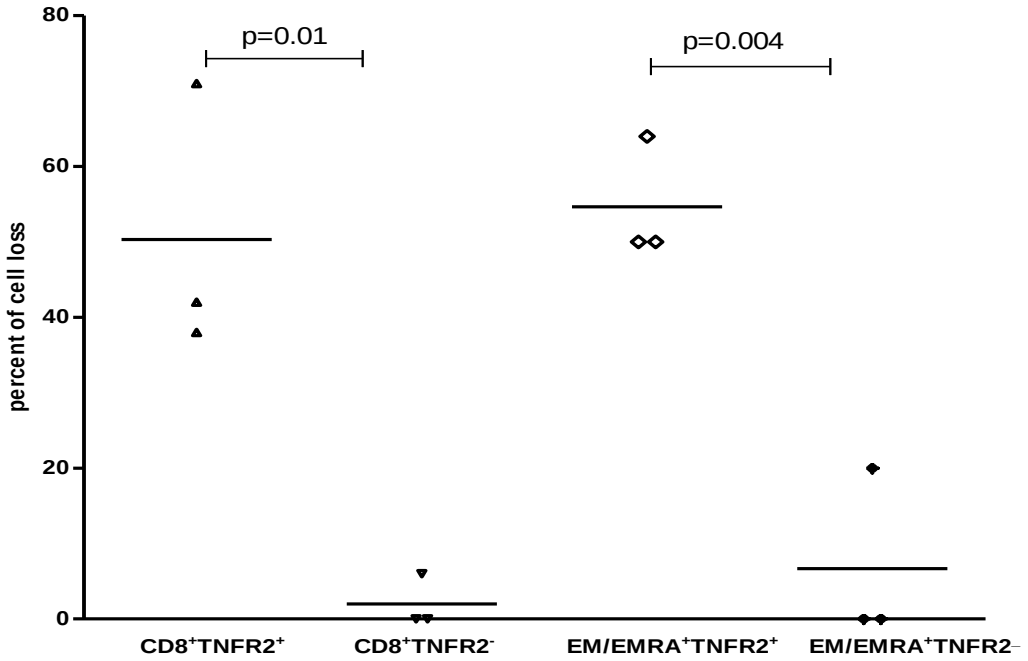


Figure 5.17 TNFR2 ligation and cell loss in TNFR2 positive population.

Analysis of data from 3 healthy blood donors showed that TNFR2 ligation caused statistically significant cell loss only in TNFR2 positive cells.

5.2.8A TNFR2 ligation associated with apoptosis

To investigate the mechanism of cell loss following TNFR2 ligation, TNFR2-PE labelled $CD8^+$ cells were isolated from a healthy blood donor by using anti-PE labelled magnetic beads. The immunomagnetically selected cells were 91% pure for $CD8^+$ TNFR2 pos cells. The negative fraction was used as a control for this experiment. The cells in positive and negative fractions were then incubated with Flag-TNC- scTNF (143N/145R) for 21 hrs. After the incubation, cells were stained with anti Annexin-V antibody and PI (Propium Iodyte) to demonstrate whether cell loss was due to apoptosis or necrosis. Indeed, the cells stained positively for Annexin V but with less frequency for PI (marker of necrotic death or late apoptosis). The presence of $CD8^+$ cells (Annexin

V positive and PI negative) confirmed the presence of apoptosis (Figure 5.18). Table 5.2 shows the frequencies of necrotic and apoptotic cells in positive (TNFR2 pos) and negative fractions (TNFR2 neg) following isolation of TNFR2 positive cells and incubation with different concentrations of Flag-TNC-scTNF (143N/145R). The highest frequency of stained cells belonged to Annexin V stained cells in the positive fraction which ranged form 13.70% to 16.20% depending on the concentration of Flag-TNC-scTNF (143N/145R). These frequencies for TNFR2 ligation of the negative fraction ranged from 1.90% to 3.90%. In TNFR2 positive fraction, frequencies of necrotic cells following TNFR2 ligation was two times less than the percent of apoptotic cells and ranged from 2.30% to 3.60%.

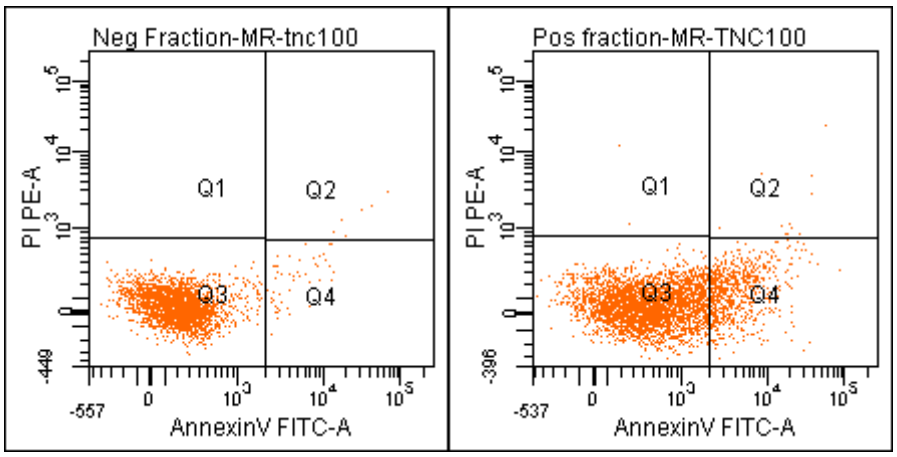


Figure 5.18 Induction of apoptosis following TNFR2 ligation.

Following separation of $TNFR2^+$ and $TNFR2^-$ CD8 T-cells and overnight incubation with Flag-TNC-scTNF (143N/145R), $CD8^+$ cells were stained with PI-PE and Annexin V-FITC in order to detect necrotic (Annexin V and PI positive) and apoptotic cells (Annexin V positive, PI negative). The left and right FACS panel show necrotic and apoptotic cells in the $CD8^+$ $TNFR2^-$ and $CD8^+$ $TNFR2^+$ T-cells, respectively.

Table 5.2 Frequencies of apoptotic and necrotic cells following TNFR2 ligation in TNFR2 positive and negative fractions of isolated CD8$^+$ cells

Condition	% necrotic cells		% apoptotic cells	
	positive fraction	negative faction	positive fraction	negative faction
Untreated	2.3*	2.1	4.1	1.3
Flag-TNC 50ng/ml	2.3	3.3	**16.2**	2.7
Flag-TNC 100ng/ml	2.4	2.2	**13.7**	3.9
Flag-TNC 200ng/ml	3.6	3.1	**15.1**	1.9

* These frequencies are mean frequencies of two experiments.

5.2.8B Ligation of TNFR2 in CMV-specific T-cells

The expression of TNFR2 by CMV-specific T-cells and the increase in its expression at single cell level during T-cell response to CMV reactivation have been discussed in this chapter and chapter 4). In this regard, it became important to study the possible effects of TNFR2 ligation on CMV-specific T-cells. For this study, blood samples from 3 HSCT patients with high frequency CMV-specific T-cells were used. These samples were collected longitudinally and cryopreserved until it was possible to assay them simultaneously. Ligation of TNFR2 with Flag-TNC-scTNF (143N/145R) caused a decrease in the number of CMV-specific CD8$^+$ T-cells in 2 out of 3 patients. In the first patient, cells were taken during and after CMV reactivation, which occurred between days 46 to 67 post HSCT. The cells were incubated with the TNFR2 ligand which led to a similar decrease in the number of CMV-specific T-cells in all samples. The

frequencies of cell loss were 66.66%, 58.00%, 58.60% and 70.20% for samples collected at: 56, 105, 148 and 252 days post HSCT. Although the TNFR2 expression was at its highest on day 56, there was no difference in ligand-induced cell loss at different time points.

In the second HSCT patient, CMV reactivated from days 21 to 100 and TNFR2 had its highest expression level at day 90. Incubation of samples collected during the time of active infection from days 56, 70, 98 and 112 with TNFR2 ligand did not cause cell loss in CMV-specific T-cells. However, 48.10% and 42.00% ligand-induced cell losses were seen in samples taken on days 168 and 420 after resolution of CMV viraemia. These experiments showed the possibility of cell death in CMV-specific T-cells following TNFR2 ligation. It should be noted that there was no any relation between the expression level of TNFR2 and apoptosis of CMV-specific $CD8^+$ T-cells following TNFR2 ligation. No cell death due to TNFR2 ligation in CMV-specific T-cells was observed in the third HSCT patient. These experiments need further investigations with more patient samples in order to precisely clarify the susceptibility of TNFR2 expressing CMV-specific T-cells to cell death from ligand induction.

5.3 Discussion

This chapter discussed cytokine receptor expression patterns in $CD4^+$ and $CD8^+$ T-cell subsets in order to explore the role of cytokines in T-cell development. Following HSCT, massive cytokine production (cytokine storm) governs the reconstitution and homeostasis of lymphocytes. Among highly produced cytokines post HSCT, are IL-6 and IL-15 (first wave of cytokine storm) and TNF-α, IL-7 and IL-2 (second wave of

cytokine storm) (Melenhorst et al., 2012). Therefore, receptors for these cytokines were chosen for the study of cytokine receptor expression in whole T-cell population and in antigen specific T-cells of healthy individuals and HSCT patients. In healthy individuals, the frequencies of IL-2Rα, IL-7Rα, IL-15Rα and IL-6Rα expressing $CD4^+$ T-cells were higher than the frequencies of $CD8^+$ T-cells bearing these receptors. However, when the analysis was segregated according to differentiation subsets, the pattern of cytokine receptor expression was similar for $CD4^+$ and $CD8^+$ T-cells. Importantly, each differentiation subset from healthy steady state lymphocytes had a unique pattern of cytokine receptor expression. Of the common gamma chain cytokine receptors, IL-7Rα is predominantly expressed by naïve and CM cells with peak expression amongst the latter population. IL2Rα was predominantly expressed by CM and EM peaking in the EM subset. Both IL-2Rα and IL-7Rα expression were steeply reduced in the EMRA subset. Detection of IL-15Rα in $CD4^+$ and $CD8^+$ T-cells was challenging, and only a small fraction of T-cells showed Il-15Rα expression and $CD4^+$ EMRA cells had highest frequencies of IL-15Rα positive cells. This may be in keeping with the previous reports of IL-15 being important for the maintenance of differentiated cells (Chiu et al., 2006), although the true expression may be masked by the trans-expression of IL-15R. IL-6Rα expression was heavily skewed towards naïve cells with minimal expression in the EMRA cells. In contrast, TNFR2 was skewed towards EMRA and nearly absent in the naïve population. Further cell differentiation and activation caused rapid expression of this cytokine receptor which reached to its highest frequency in EM, EMRA and $CD57^+$ T-cells. This suggests that the receptor is associated with highly differentiated cells. TNFR2 expression is more selective to lymphocytes than TNFR1. Indeed TNFR1, although considered to be ubiquitously

expressed, was not detectable on lymphocytes using surface staining with antibodies in our experiments, and confirms previous reports (Zola et al., 1995).

Considerable differences in the T-cell subset distribution were observed between $CD4^+$ and $CD8^+$ T-cells. Amongst $CD4^+$ T-cells, the most frequent subset was naïve cells followed by CM, EM and EMRA subsets, whereas amongst $CD8^+$ T-cells, the highest frequency belonged to the EMRA subset, followed by naïve, EM and CM cells. This is likely to have been skewed by previous CMV exposure. All the studied cytokine receptors demonstrated higher expression amongst $CD4^+$ than $CD8^+$ cells, except for TNFR2 which had highest frequency amongst $CD8^+$ EM and EMRA cells.

CMV infection is known to induce changes in T-cell subset distribution in the elderly in favour of late differentiated cells (EM/EMRA) (Di Benedetto et al, 2015). In our CMV sero-positive group, the frequency of EMRA subset of $CD4^+$ T-cells (median: 12.53%) was much higher than in CMV sero-negative individuals (median: 2.12%). However, in our data, this difference did not reach to a statistically significant level ($p=0.091$). This is in accordance with the recent finding that $CD4^+$ EMRA subset presents only in CMV sero-positive individuals and is independent of age (Di Benedetto et al, 2015). . This supports our data that $CD4^+$ $CD57^+$ T-cells were found almost exclusively in CMV sero-positive individuals. However, it should be mentioned that the healthy individuals in the current study were mostly in their middle age and the impact of CMV on T-cell subset distribution is more profound in the elderly.

CMV infected individuals had higher frequencies of TNFR2 and IL-2Rα positive cells. This may represent one of the early impacts of CMV infection on the T-cell compartment which leads to the increase in the percent of activated cells. The presented data demonstrated that cell activation increased TNFR2 expression. CD57 molecule is

expressed by highly differentiated cells, and by many CMV-specific T-cells alongside TNFR2, but not IL-2Rα, IL-7Rα-, IL-6Rα, that are primarily associated with less differentiated cells. Taken together, TNFR2 appears to be associated with highly differentiated cells.

To my knowledge, this study was the first to investigate the profile of cytokine receptor expression amongst steady state T-cell subsets in a group of healthy adult blood donors by flow cytometry. H.Zola and colleagues (1995) published the first paper on cytokine receptor expression in PBMCs of adult and cord blood. Their finding showed lower frequencies of IL-2Rα, IL-7Rα and TNFR2 positive T-cells in cord blood in comparison to adult cells. This is in accordance with the fact that the majority of cord blood T-cells are antigen inexperienced cells (Frumento et al., 2013) and cell activation increased the frequencies of cells bearing cytokine receptors (Zola et al., 1995). Interestingly, they reported that IL-6Rα was the only cytokine receptor which had similar frequency in adult and cord blood cells, indicating its utility as another marker for identifying naïve cells.

M.Callan's group investigated the molecular signatures of $CD8^+$ T-cell subsets and focused on cytokine receptor expression pattern at mRNA level (Willinger et al., 2005). This group found differential expression of cytokine receptors mRNA that supports the present findings. Naïve T-cells were shown to have higher mRNA expression of IL-6R and IL-7Rα. Cell activation and differentiation led to increase in the mRNA for receptors of Th1 related cytokines including: TNFR2, IL-12RB1, IL-18R and IFNγR1. Functional analysis of cytokine receptor signalling pathways showed naïve, CM and EM subsets responded to IL-7 through STAT-3 phosphorylation, and TNFR2 signalling

induced higher NF-κB expression in EM/EMRA subsets in comparison to the other subsets (Willinger et al., 2005).

Taken together, all these data support the role of common gamma chain receptors and their ligands in the differentiation and homeostasis of T-cells. IL-7 is important for cell survival and proliferation of naïve and CM cells and IL2 has fundamental role in T-cell proliferation. The increased frequency of IL-7Rα in CM subset may explain their greater expansion potential than effector memory cells.

It has been shown that IL-15 induces generation and growth of EMRA cells (Chiu et al., 2006) which is consistent with the limited observation of increased frequencies of IL-15Rα within the EMRA subset. In addition it has been documented that IL-15 increases expression of cytotoxic molecules, granzyme B and perforin in EMRA subset of $CD4^+$ T-cells (Alonso-Arias et al., 2011).

TNFR2 expression is limited to certain cell types including lymphocytes and believed, sometimes controversially, to mediate a broad range of activities. There are multiple reports on the role of TNFR2 in the induction of apoptosis in lymphocytes. Zheng and colleagues (1995) were the first to show that TNFR2 ligation was involved in cell apoptosis of $CD8^+$ T-cells following T-cell receptor activation**.** Then, it was shown that TNFR2 deficient $CD8^+$ (and not $CD4^+$ T-cells) were resistant to Fas/FasL induced apoptosis (Teh et al., 2000). This group also reported that TNFR2 lowered the T-cell activation threshold, and as one of the earliest co-stimulatory molecules of TNFR family, help T-cell proliferation alongside CD28 for optimal IL-2 production and T-cell survival (Kim et al., 2001, 2004 and 2006). Interestingly, in murine models, $TNFR2^{-/-}$ $CD8^+$ T-cells had superior anti-tumour activity due to their resistance to activation induced cell death (Kim et al., 2009). Therefore, TNFR2 has a role in providing initial

activation signals for T-cells and is also important for limiting the duration of T-cell responses by promoting activation induced cell death (AICD) (Twu et al., 2011). Based on the observation made by Teh's group, a model has been proposed to explain the opposing activities of TNFR2. The model proposes that TNFR2 activation leads to the consumption of TRAF2 (Figure 5.4) which connects TNFR2 to the apoptosis machinery. In conditions with high level of TRAF2 (such as in $TNFR2^{-/-}$ cell lines), TNFR1 signalling pathway interacts with this molecule and activates the canonical NF-κB pathway and induce cell survival (Twu et al., 2011). This model suggests a cross talk between TNFR1 and TNFR2 and in addition, TNFR1 can induce a pro-survival signal.

The current study had access to a special ligand for TNFR2 (Flag-TNC-scTNF (143N/145R) which mimicked membrane TNF. T-cell culture in the presence of this ligand caused cell death through induction of apoptosis. This research was the first that show a link between TNFR2 expression in EM/EMRA subsets of T-cells and induction of apoptosis in these highly differentiated subsets of $CD8^+$ T-cells. Preliminary experiments did not show $CD4^+$ T-cell death following TNFR2 ligation, which is likely explained by the low frequency of EM/EMRA subsets amongst $CD4^+$ and hence absence or low frequency of $CD4^+$ T-cells expressing TNFR2. The induction of cell death through TNFR2 primarily in $CD8^+$ and not $CD4^+$ T-cells has been previously reported (Zheng et al., 1995).

The possible function of TNFR2 molecule in CMV–specific T-cells is not clear but it has been reported that in HIV infection $CD8^+$ T-cell loss is due to cell apoptosis which in turn is mediated by the interaction between mTNF in macrophages and TNFR2

expressed by $CD8^+$ T-cells. Expression of these cell surface proteins is up-regulated by lymphocytes in HIV sero-positive individuals (Herbein et al., 1998).The findings of this chapter contribute towards the understanding of the possible role of TNFR2 in inducing apoptosis of antigen specific T-cells in humans. In an interesting observation, stimulation of TNFR2 specifically killed autoreactive insulin specific $CD8^+$ T-cells in type I diabetes, as well as autoreactive cells in multiple sclerosis, Graves, and Sjogren's disease (Ban et al., 2008). TNFR2 stimulation did not induce loss of non-autoreactive T-cells. Although the authors did not look for TNFR2 expression in the autoreative T-cells, we speculate whether these autoreactive cells, which are continuously activated in autoimmune disorders, express high levels of TNFR2 and go through apoptosis process following TNFR2 ligation. In another recent study, TNFR2 expression was shown to be the key molecule in the induction of apoptosis in inflammatory T-cells (Punit et al., 2015; Dube et al., 2015). In a mouse model of inflammatory bowel disease where $CD8^+$ T-cells played an essential role in the immunopathogenesis of IBD, the presence of TNFR2 was associated with the mouse's ability to inhibit the function and expansion of these inflammatory cells, whereas the absence of TNFR2 drove the aggressive inflammation by $CD8^+$ T-cells. Therefore, approaches that can increase the expression of TNFR2 or agents such as ligands or antibodies that can interact with TNFR2 expressing cells may provide new ways of treating inflammatory conditions caused by $CD8^+$ T-cells and their Th1 related cytokines.

In this work, a significant increase in the levels of TNFR2 in CMV-specific T-cells following CMV viraemia was observed. This is in line with well documented increase in the levels of other TNFR family molecules after cell activation (Table 5.1). Although this study did not define the physiological role of TNFR2 in T-cell activation, it was

demonstrated how TNFR2 engagement could induce apoptosis in EM/EMRA subsets of $CD8^+$ T-cells as well as CMV-specific $CD8^+$ T-cells. Pathophysiologically, cell death due to TNFR2 ligation could be a putative mechanism for reducing the number of lymphocytes following massive cell expansion in response to CMV reactivation, thus limiting CTL toxicity. Expansion of inflationary T-cells with late differentiated phenotype ($CCR7^-$ $CD45RA^-$ $CD27^{-/+}$ $CD28^{-/+}$ $IL\text{-}7R^-$) is one of the main features of the immune response to CMV reactivation. These cells undergo apoptosis as part of their rapid turnover (Klenerman and Dunbar, 2008). The results of this chapter suggest that TNFR2 may be important for the homeostasis of expanded T-cells and controls the inflation of antigen-specific T-cells through induction of apoptosis.

The number of inflammatory diseases that CMV infection might exacerbate their inflammatory conditions, grows continuously (chapter 1) (Halenius and Hengel, 2014). A common feature of several inflammatory diseases such as IBD, RA and CVID, is the accumulation of late differentiated T-cells (EM/EMRA subsets). Some of these T-cells are CMV-specific (Domènech et al., 2008; Rothe et al., 2015; Marashi at al., 2011). Deficiency in TNFR2 signalling pathway has been described in some of inflammatory diseases (Faustman and Davis, 2013). It is possible that the accumulation of terminally differentiated T-cells in these diseases may be due patly to dysfunctional TNFR2 signalling and consequently, disruption of cell apoptosis. Molecules such as Flag-TNC-scTNF (143N/145R) that targets TNFR2 or its signalling pathway, could potentially be used for therapeutic purposes in Th1 mediated inflammatory conditions to control these responses. In conclusion, study of cytokine receptor expression pattern may become useful in clarifying physiological properties of T-cell subsets in more detail and apply the relevant information in management of broad range of human diseases.

GENERAL DISCUSSION

CMV disease resulting from the progression of CMV reactivation is a major cause of infection-related morbidity and mortality in the HSCT setting (Ljungman et al., 2011). The probability of CMV reactivation is approximately 85% in the reduced intensity conditioning allogeneic HSCT patients with 'high risk CMV serology' (D/R CMV+/+ and -/+) (Chakrabarti et al., 2002). CMV viraemia is the consequence of the immunosuppression that these patients receive as part of transplant conditioning chemoimmunotherapy, and of the delayed immune reconstitution following transplantation. T-cell immunity is essential for controlling CMV reactivation and recovery of CMV-specific T-cells in the early post-transplant period abrogates the development of CMV-related disease. In the current study, most of patients underwent reduced intensity conditioning HSCT. With the use of reduced-intensity conditioning transplant regimens which incorporate Alemtuzumab, the probabilities of multiple reactivations before and after 100 days post transplant are 53.6% and 46.6%, respectively (Chakrabarti et al., 2002). Antiviral drug therapy is highly effective in controlling CMV viraemia in the majority of patients, but its prolonged use in patients with long period of viraemia or recurrent reactivations is associated with renal toxicity and myelosuppression. This leads to increased morbidity and management complexity of these patients. There is also evidence that first line antivirals such as ganciclovir and foscarnet may not be effective in the complete absence of CMV-specific T-cells (D. Lewis et al., submitted paper to BMT, 2016). There is therefore an unmet need for more effective and less toxic therapy for these more complex cases. Amongst different strategies for controlling CMV reactivation, adoptive cellular therapy with CMV-specific T-cells aims to restore the CMV-specific T-cell immunity in HSCT patients. To date, there are 11 published reports on phase I/II single armed clinical trials of cellular

therapy with CMV-specific $CD4^+$ and/or $CD8^+$ T-cells (chapter 1). These studies have demonstrated safety and provided evidence of efficacy for CMV-specific cellular therapy.

This PhD project comprised three experimental chapters. In chapter 3, CMV-specific T-cell responses were analysed following the randomised controlled phase II adoptive cellular therapy (ACE-ASPECT trial) of T-cell depleted allogeneic HSCT patients. This trial was one of the first randomised controlled cell therapy for CMV-specific T-cells and the primary end point was reconstitution of CMV-specific $CD8^+$ T-cells rather than viral control as this is covered by another ongoing trial. The primary end point was the recovery of CMV-specific $CD8^+$ T-cells to $1x10^4$ cells/ml of peripheral blood by 2 months post cellular therapy. Adoptive CMV-specific $CD8^+$ T-cell immunotherapy led to the rapid reconstitution of CMV-specific $CD8^+$ T-cells within 2 months post-ACT in 8 out of 17 patients (47.1%). In these patients, the mean frequency of CMV-specific $CD8^+$ T-cells within 2 months post-ACT was $35x10^3$ cells/ml, above the trial end point level of $1x10^4$/ml. The trial end point was achieved in 37% of the control group who were on the best available anti-viral drugs. Further analysis of the infused cell dose and serum Alemtuzumab levels by the clinical trial sponsor identified these as the two main factors influencing the reconstitution of immunotherapeutic cells. The failure to achieve the pre-specified trial end point (CMV- specific T-cell level) was related to the low number of infused cells (below $1x10^3$/kg) and high levels of Alemtuzumab (>0.15mg/ml). There were no statistically significant differences between the trial arms with regard to the frequency of patients who achieved the trial end point level and to the absolute number of CMV-specific $CD8^+$ T-cells at their peak. However, the rate of cell expansion shortly after cell infusion was considerably higher in the ACT group. At one month post-ACT, a greater cell expansion from baseline to peak was observed in the ACT group. These

data clearly showed that early cell expansion was more rapid in the ACT arm. Interestingly, T-cell expansion was not restricted to the specificity of the infused cells. Non-infused CMV-specific $CD8^+$ T-cells and CMV-specific $CD4^+$ T-cells also expanded alongside the whole $CD4^+$ and $CD8^+$ populations in the ACT group. This thesis is the first to report the boosting and supportive effects of the therapeutic cells in the reconstitution of non-infused $CD4^+$ and $CD8^+$ T-cells following adoptive cellular therapy. Although the trial's aim was not to explore the possible factors involved in the broader expansion of non-infused T-cells in the ACT group, it is reasonable to speculate on a number of possible mechanisms.

One explanation for the expansion of non-infused cells may relate to antigen spreading, which has been reported in other immunotherapy trials such as in vaccination of melanoma antigens which generated T-cells against non-vaccine tumour antigens and contributed to the regression of melanoma metastases (Corbiere et al., 2011).

$CD8^+$ T-cells have also been found to support the proliferation of $CD4^+$ T-cells *in vitro* through CD40 Ligand on activated $CD8^+$ T-cells (Xydia et al., 2011; Romagnoli et. al, 2013). It is possible to speculate that a similar scenario existed *in vivo* where the infused CMV-specific $CD8^+$ T-cells helped the $CD4^+$ T-cells response within the first month post ACT which led to the greater fold increase in the number of $CD4^+$ T-cells in comparison to the control group.

Cytokines and co-stimulatory molecules in the presence of CMV-derived antigens govern T-cell reconstitution post-HSCT. Following HSCT, high levels of cytokines such as IL-7, IL-15 and TNF-α are present (William and Gress, 2008). It is possible that because of the low number of CMV-specific T-cells at the beginning of the immune response in the control patients, other cells out-competed them in the consumption of

cytokines, whereas in the therapeutic arm, the larger number of infused CMV-specific T-cells dominated the consumption of cytokines and expanded rapidly.

Finally, it is possible that there were contaminating cells in the selected CMV-specific $CD8^+$ T-cell population that expanded following cell therapy. It should be mentioned that the purity of isolated CMV-specific T-cells was considerably less than 100% and therefore, contaminated cells might have contributed in the greater cell expansion observed in the ACT group.

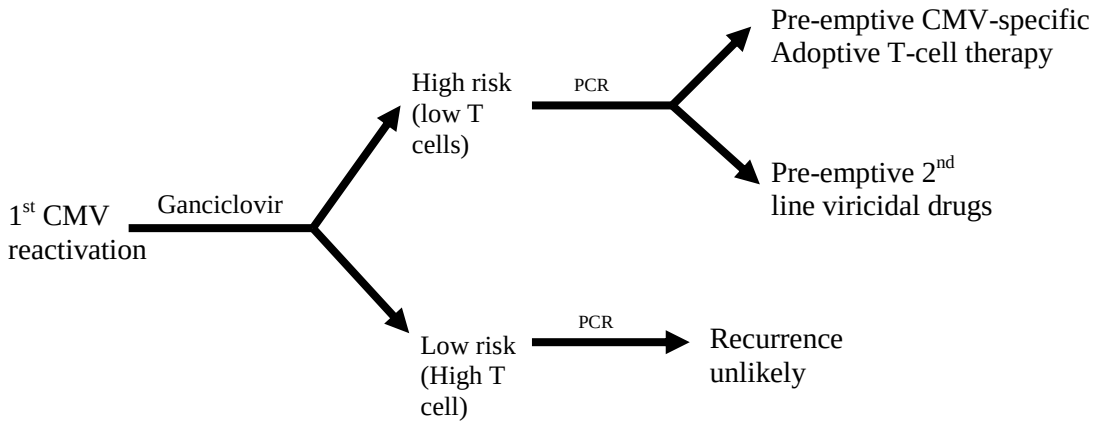
The rapid cell expansion of CMV-specific T-cells within the first month after cell therapy was followed by a subsequent retraction in cell number. This phenomenon differed from the reconstitution pattern in the control arm. The reason for this is not clear. T-regulatory cells have important role in the control of cell reconstitution post-HSCT (William and Gress, 2008). Alongside the global cellular reconstitution, the generation of T-reg may have also been quicker in the ACT arm, which in turn induced a sharp retraction phase.

At the time of writing this thesis, there were no further data regarding the secondary objectives of ACE-ASPECT trial including evaluation of the potential clinical benefit of cell therapy as measured by reduction in CMV, anti-viral drug treatment and number of in-patient days. However, the incidence of new onset grade II-IV GVHD after therapy did not differ significantly between the cellular therapy and control arms (17.6% vs 27.3% respectively) (Chen et al., 2014). Taken together, this first randomized trial of CMV- specific T-cell therapy post-HSCT proved that cell therapy was safe and resulted in the earlier and greater expansion of CMV-specific T cells. Further investigation is underway to determine if the observed increase in CMV-specific cells translates to

clinical benefit. Some of the results obtained in ACE-ASPECT trial published as an abstract (Chen et al., 2014).

The observation of concomitant CMV-specific $CD4^+$ and $CD8^+$ T-cells reconstitution warranted a more detailed analysis of CMV-specific $CD4^+$ T-cells through the usage of a novel HLA-class II restricted tetramer. Chapter 4 presented the results of first direct visualization of CMV-specific $CD4^+$ T-cells in human responses to CMV infection. In a cohort of non-trial HSCT patients, co-expansion of CMV-specific T-cells was observed and it was possible to directly quantify CMV-specific $CD4^+$ T-cells conferring protection against CMV reactivation. In line with some of the previously published data using indirect methods of CMV-specific $CD4^+$ T-cell detection (Lilleri et al., 2008), the minimum level of $CD4^+$ cells required for clearance of CMV viraemia, determined as $0.7x10^3$ cells/ml. The frequency of cells at levels lower than this resulted in multiple episodes of CMV reactivations. In this chapter, the frequency of CMV-specific $CD8^+$ T-cells was also studied. Despite having a relatively small cohort of patients, analysis of the data on CMV-specific $CD8^+$ T-cell numbers from 29 patients clearly showed the potential of HLA tetramer/multimers to identify patients who are able to control CMV viraemia versus those who cannot and end up suffering from multiple episodes of viraemia. These data showed that HLA-tetramers could be used as a clinical diagnostic assay where a CMV-specific $CD8^+$ T-cell level below $7.22x10^3$/ml after resolution of first CMV reactivation can predict recurrent CMV reactivations with sensitivity of 85.46% and specificity of 75.00%. Based on the previous data from the non-trial patients, this suggests that patients with more than $7.22x10^3$/ml CMV-specific T–cells would probably not have benefited from ACT. Therefore, in future trials, it would be important to stratify patients into high and low risk groups according to the level of

CMV-specific T-cells, and ACT offered only to those who have CMV-specific T-cells below the threshold level and thus at high risk of multiple CMV reactivations. The majority of patients develop CMV reactivation within 28 days of transplant. However, only half will go on to develop recurrent reactivations. This group of patients have low recovery of CMV-specific T-cells following the first episode of viraemia, and are at high risk of transplant-related morbidity. This group of patients are the ones most at need of nontoxic antiviral therapy, and adoptive cell therapy should in the future be targeted at this group. Risk-assessment of these patients based on immune monitoring of CMV-specific T-cell levels would help to select the most appropriate patients for ACT.



The study identified CD57 as a possible surrogate marker for CMV-specific $CD4^+$ T-cells. $CD4^+$ $CD57^+$ T-cells presented only in CMV-seropositive individuals and expanded in response to CMV reactivation in parallel with class II tetramer-staining CMV-specific $CD4^+$ T and $CD8^+$ T-cells. As such they were found to be as informative as tetramers in identifying patients with recurrent CMV reactivation, and thus be used as a biomarker for risk stratifying patients. As a diagnostic assay, $CD4^+$ $CD57^+$ T-cell level lower than $5x10^3$/ml can identify patients suffering from multiple reactivations

with 85% sensitivity and 85% specificity. For a diagnostic assay, these are good discriminatory values. Importantly, $CD4^+$ $CD57^+$ T-cell quantification, unlike tetramers, is independent of HLA restrictions. Therefore, CD57 expression could potentially serve as universal biomarker usable in assessment of CMV immunity in all patients. Further prospective studies will be required to validate its clinical utility.

The use of HLA-class I and II tetramers in this thesis permitted the immunophenotyping of antigen specific T-cells for additional information on their quality and function.

Of interest, $CD4^+$ T-cells were found to have an earlier differentiation phenotype ($CCR7^-$ $CD45RA^-$, EM phenotype) than CMV-specific $CD8^+$ T-cells which had mostly a highly differentiated 'exhausted' phenotype ($CCR7^-$ $CD45RA^+$, EMRA phenotype). In keeping with this, IL-7Rα (CD127) was down regulated in CMV-specific $CD8^+$ T-cells, though a considerable proportion of CMV-specific $CD4^+$ T-cells did express it, possibly indicating their better proliferative and survival potential over the $CD8^+$ counterparts.

T-bet was expressed by the majority of CMV-specific $CD4^+$ and $CD8^+$ T-cells, demonstrating the Th1 profile. Additionally, up to one third of CMV-specific $CD4^+$ T-cells expressed both perforin and granzyme B, supporting a cytotoxic role for this subgroup of CMV-specific $CD4^+$ T-cells (Casazza et al., 2006). The frequency of these cytotoxic cells were significantly higher in the CMV-specific $CD4^+$ T-cells expressing CD57. This relation between CD57 expression and cytotoxic activity has also been described for CD8 population (Chattopadhyay et al., 2009).

As the reconstitution of T-cells following HSCT is dependent on exogenous factors such as cytokines (Melenhorst et al., 2012), it is important to examine the role of cytokine receptors on T cells, as their expression or non-expression provide an

indication of their function and responsiveness to the cognate cytokines, whether in terms of proliferation, apoptosis or effector function.

In this thesis, first, the baseline expression of cytokine receptors to IL-2, IL-7, IL-15, TNFR2 and IL-6, were analyzed in T-cells from healthy individuals (data presented in chapter 5). Distinct and stable patterns of cytokine receptor expression were found in each differentiation subset. Of interest, IL-7Rα was found to be expressed predominantly by naïve and early differentiated cells as previously demonstrated, whereas in a direct contrast, TNFR2 was uniquely found in late differentiated cells.

Then, in HSCT patients (Chapter 4), the cytokines receptors on reconstituting CMV-specific T-cells were investigated in greater detail. In these patients, CMV-specific $CD8^+$ T-cells showed low IL-2Rα, IL-7Rα expression and high levels of CD57, in keeping with highly differentiated T cells. A high proportion of cells also expressed TNFR2, but importantly there was also a rapid increase in the density (MFI) of TNFR2 expression at the single cell level during CMV reactivation. Following *in vitro* ligation of these cells with TNFR2 ligand, they went through apoptosis, suggesting that the increased TNFR2 expression by expanded cells in response to CMV reactivation could be a mechanism by which expanded and activated cells deflate following resolution of the infection, a phenomenon often referred to as Activation-induced Cell death (AICD). The importance of TNFR2 function in the contraction of activated T-cells will need further research, not only in the HSCT setting but also in other conditions where there is a chronic immune stimulation. In this regard, TNFR2 ligation might be important in the apoptosis of CMV-specific and other Th1 cells (with EM/EMRA phenotype) present in many inflammatory diseases like type I diabetes and IBD (Faustman and Davis, 2013; Punit et al., 2015).

In summary, studying cytokine receptor expression can help identify fundamental aspects of cytokine function involved in the expansion, survival and contraction of T-cell subsets, whilst providing critical information on the physiological and pathological properties of T-cells in disease states. The current study suggests a potential role of TNFR2 in the termination of terminal effector T-cells ($CCR7^-$ $CD45RA^-$ $CD57^+$ IL-$7R\alpha^-$ $TNFR2^+$) following immune response to antigens (following figure).

Different phases of an immune response to a virus which leads to the T-cell memory generation and effector T-cell retraction (from Kaech and Cui, 2012). Effector (IL-$7R\alpha^{low}$, BCL-2^{low}) and memory precursor T-cells (IL-$7R\alpha^{high}$, BCL-2^{high}) are shown as blue and red cells, respectively. Populations in other colours are T-cells with intermediate differentiation state with mixed phenotypes.

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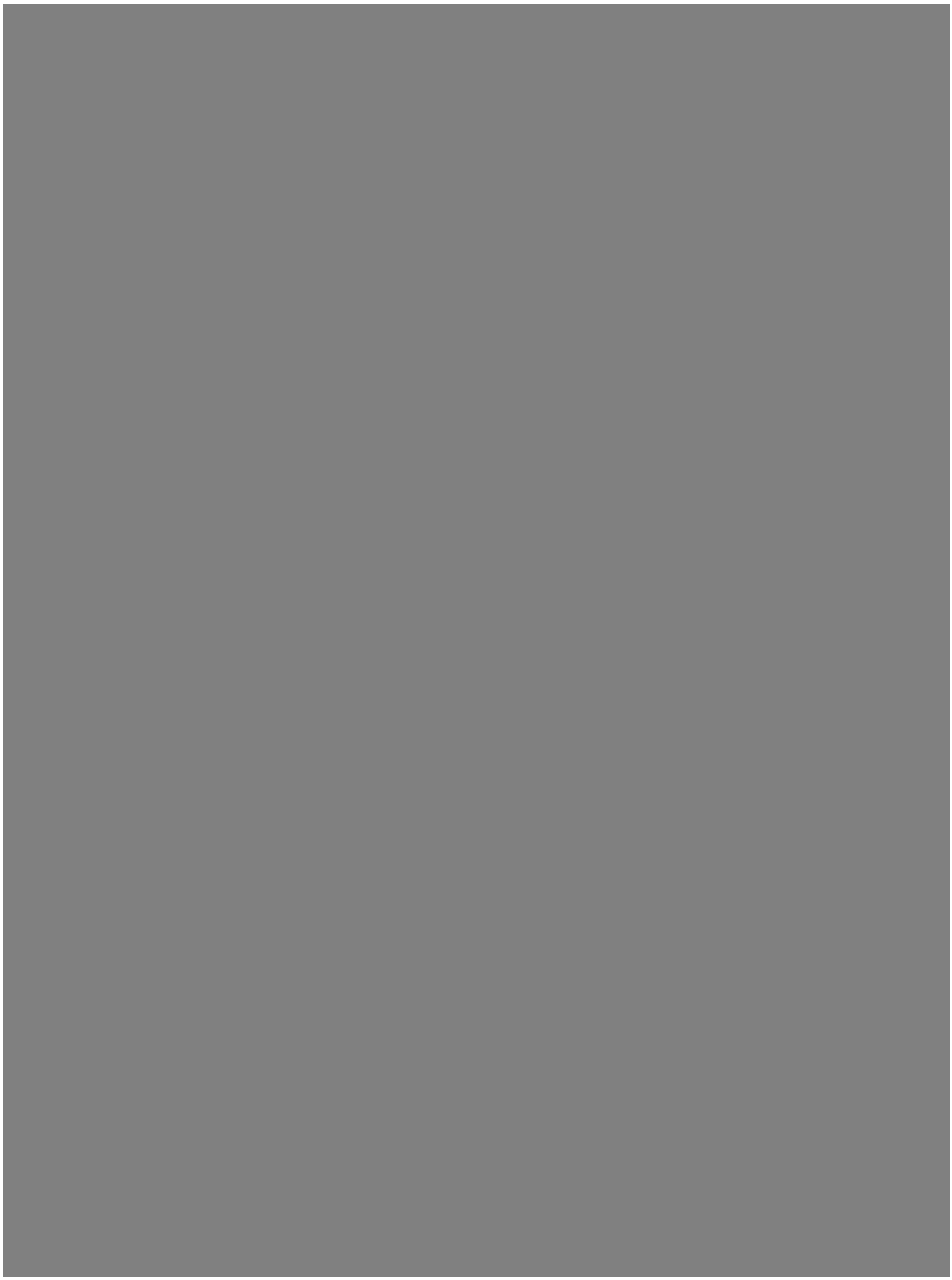
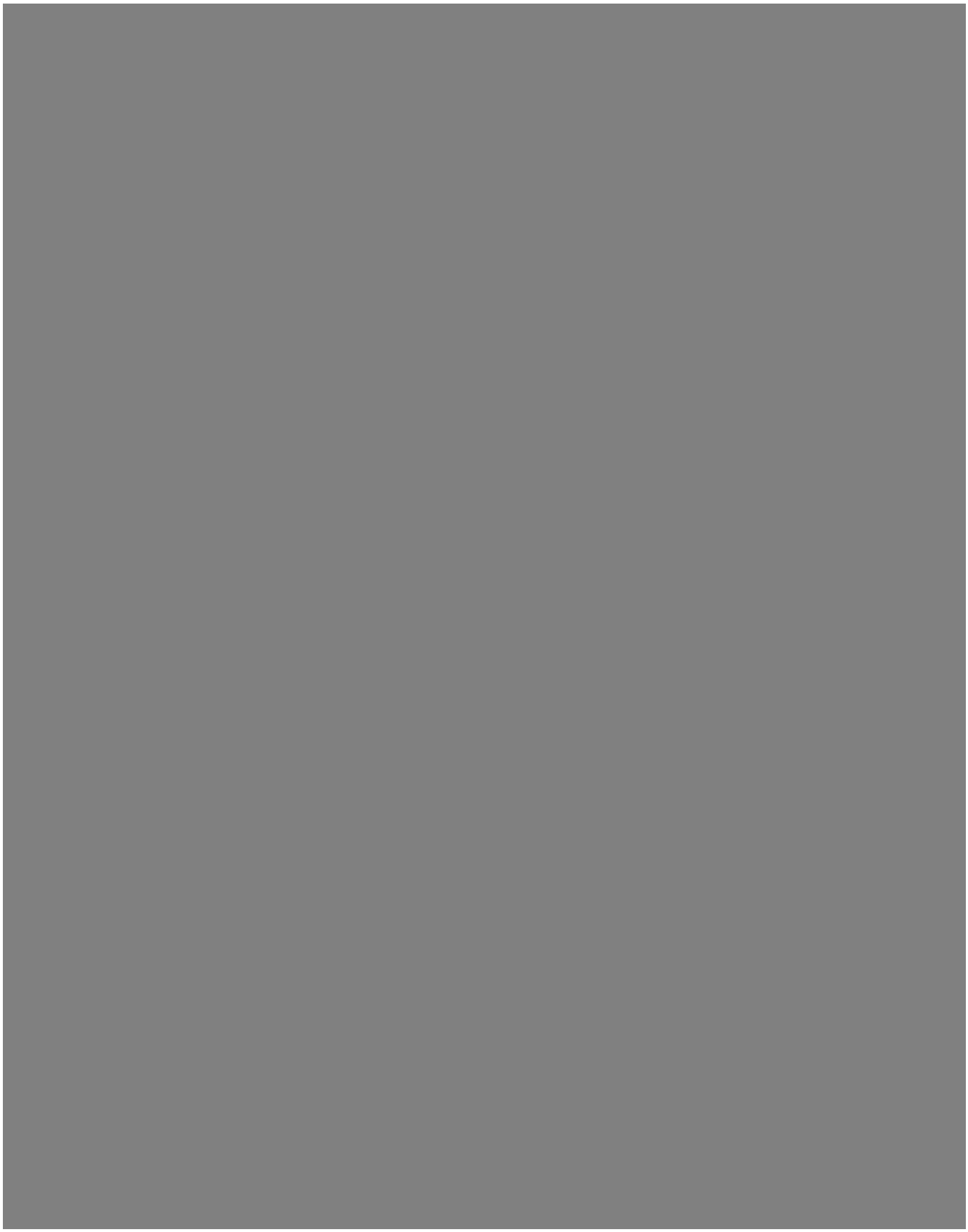
APPENDICES

Appendix 1



Appendix 2: Results of adoptive cellular therapy of CMV-specific T-cells Trial



Appendix 3: letter published in Haematologica

Characterization Of CMV-Specific CD4^{+} T-Cell Reconstitution Following Stem Cell Transplantation Through The Use Of HLA Class II-Peptide Tetramers Identifies Patients At High Risk Of Recurrent CMV Reactivation

Mohammad Raeiszadeh, Annette Pachnio, Jusnara Begum, Charles Craddock, Paul Mos s, Frederick E. Chen

Haematologica August 2015 100: e318-e322; Doi:10.3324/haematol.2015.123687

Appendix 4: Abstract on cytokine receptor expression pattern presented at 56th American Association of Haematologist, San Francisco, 2014