Biological and Clinical Significance Of Chronic Herpes Virus Infection in Patients Undergoing Treatment for Myeloid Malignancies

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A Thesis Submitted to

The University of Birmingham

For the Degree of

DOCTOR OF PHILOSOPHY

School of Cancer Sciences

College of Medical and Dental Studies

The University of Birmingham

January 2014

Abstract

Cytomegalovirus (CMV) is a β-herpes virus that infects the majority of the world's population.

Tyrosine kinase inhibitors (in particular imatinib, dasatinib and nilotinib) have been successfully used in the treatment of chronic myeloid leukaemia, as they target the *Abl* kinase, which is constitutively activated in the disease. Although thought of as targeted therapies, they have significant "off target" effects including inhibition of *Src* family kinases important in T cell receptor mediated activation.

I demonstrated that CMV infection is associated with significant alterations in the immune repertoire in imatinib-treated patients; in particular with expansions of differentiated CD8 T cells and Vδ1 γδ T cells. Furthermore, dasatinib treatment is associated with evidence of subclinical CMV reactivation and marked expansions of terminally differentiated CD8 T cells and Vδ1 γδ T cells. These atypical Vδ1 γδ T cells have activity against CMV infected fibroblasts, and sequencing of their TCRs demonstrated remarkable oligoclonality suggestive of antigen driven proliferation.

In a second group of patients that underwent reduced intensity allogeneic stem cell transplant for myeloid malignancies, CMV seropositivity of patient or donor is associated with increased lymphocyte counts at 3 months post transpant, particularly of CD8 and Vδ1 γδ T cell subsets. Survival analysis of these patients revealed that CMV seropositivity is associated with improved overall survival, due to a decreased relapse risk.

Dedication

Dedicated to Lisa, Olly and Lucy

Acknowledgments

Many thanks to my supervisors, Professor Paul Moss and Professor Charles Craddock, for all their invaluable advice and support throughout

I would like to thank everyone in the Moss group for all their helpful advice throughout this project, particularly Dr Annette Pachnio for her assistance and provision of HLA-peptide tetramers and CMV peptides, and to Dr Joanne Croudace for her assistance setting up the original panel for flow cytometric analysis.

The study would have been impossible without the help of the clinical trials staff at the Centre for Clinical Haematology, Queen Elizabeth Hospital in Birmingham for helping me to recruit patients and collect the sample – particularly Jane Nunnick and Shamyla Siddique.

Professor Ben Willcox and Carrie Willcox also provided much useful advice and encouragement.

Thanks to Christopher Holmes and Jasvir Virdee, who were excellent students to teach.

Dr Guy Pratt helped by helping to supply many clinical samples, as did Dr Beth Harrison and Yvonne Beadle at Coventry.

Finally, many thanks to all of the patients who donated blood for this study and without whom I would have no results.

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Introduction

The vertebrate immune system

The vertebrate immune system has evolved to eliminate foreign pathogens and recognize abnormal host antigens. It has been divided into "innate" and "adaptive" responses. Innate immunity is considered the most evolutionary ancient part of the immune system, and involves recognition of pathogens via invariant, germ line encoded receptors, which recognize conserved features of pathogenic organisms, or pathogen-associated molecular patterns (PAMPs). Cells of the innate immune system include neutrophils, monocytes, macrophages, eosinophils, mast cells and basophils. Natural killer (NK) cells monitor self-protein expression, which can be altered by viral infection or malignant transformation.

Because of the evolutionary capacity of pathogens to evade detection by the innate immune system, the adaptive immune system has evolved. It is composed of B- and T-lymphocytes. The T and B cell receptors are not germ line encoded, but are generated via a series of rearrangements of gene fragments as the cell develops. It has been estimated that 10^{15} unique T cell receptors may be generated (Davis and Bjorkman, 1988, Arstila et al., 1999). After gene rearrangement, self-reactive lymphocytes are deleted or become anergic. During an infection, cells that recognize antigen receive co-stimulatory signals, which allow them to replicate. This clonal expansion may take days to develop, but persists in significant numbers, allowing subsequent responses to be more rapid.

A common lymphoid progenitor in the bone marrow gives rise to the B-cells, NK cells and to both $\alpha\beta$ and $\gamma\delta$ T cells. In the peripheral blood, most lymphocytes appear as small featureless cells with few organelles and inactive nuclear chromatin. Indeed, until the 1960s it was thought that these cells were unimportant. Briefly, the two major subtypes of lymphocytes, B and T cells have different roles and antigen receptors. B lymphocytes

bind antigen via the B cell receptor (BCR), after which proliferation and differentiation into plasma cells occurs. Plasma cells secrete antibodies, which are a secreted form of the BCR with the same antigen specificity. T lymphocytes are activated via the T cell receptor (TCR). After encountering its antigen, T lymphocytes proliferate and differentiate into a functional T lymphocyte. Broadly, they can have a variety of different functions. Cytotoxic T cells are capable of killing cells infected with pathogen. Helper T cells can secrete cytokines in order to alter the behavior other cell subsets. Regulatory T cells suppress the activity of other lymphocytes.

Antigen Receptors

Adaptive immune cells are able to recognize foreign antigen via the expression of surface receptors. In the case of B cells, the BCR binding to its specific antigen causes intracellular signaling events leading to the production of secreted immunoglobulin (Ig) or antibodies. Binding of the TCR leads to T cell effector functions such as cytotoxicity or cytokine production.

Antigen receptors expressed by B and T cells are multimeric proteins, part of the immunoglobulin superfamily (IgSF). The huge potential variability in foreign antigens and requisite number of specific TCRs and BCRs is more than could possibly be encoded within the genome, therefore a process of gene rearrangement has evolved that enables highly diverse receptor repertoire generation from limited genetic material. Gene segments undergo rearrangement (Hozumi and Tonegawa, 1976) whereby DNA rearrangement causes antigen receptor genes to be recombined from individual gene segments. This process is dependent on the enzymes Recombination-activating gene (RAG)-1 and RAG-2. This process was first observed in B cells, where recombination of the immunoglobulin heavy locus (IgH) involves the joining of any of 250-1000 variable (V_H) gene segments to one of ten diversity (D_H) segments, and one of four joining (J_H)

segments. The rearranged segment is spliced to the constant domain in the mRNA. This leads to approximately 40,000 potential V(D)J joins. Diversity is further increased via the action of terminal deoxynucleotidyl transferase (TdT) which adds non-template nucleotides at regions of V(D)J joins. Rearranged IgH chains are paired with rearranged Immunoglobline light (IgL) chains, increasing the repertoire further. Initially the Ig is membrane bound as IgM. After clonal expansion, isotype switching occurs to other isotypes, which are expressed on the cell surface and can be secreted by plasma cells.

TCR structure is similar to Igs (Hedrick et al., 1984). In contrast to Igs they only exist in membrane bound form.

The majority of peripheral human T cells express a heterodimeric receptor comprised of α and β chains ($\alpha\beta$ T cells). $\gamma\delta$ T cells express γ and δ chains. Gene rearrangement facilitated by RAG-1 and RAG-2 is used to create a diverse TCR repertoire, similar to that used by B cell receptors. There are fewer V gene segments involved in the TCRα and β segments than IgH and IgL, but more J segments. The TCR β chain is also able to use D chain segments, and diversity can be increased by N-nucleotide addition (Davis and Bjorkman, 1988). Each chain consists of two extracellular Ig domains. It is composed of distal V domain, a proximal C domain, a transmembrane domain and a short tail. A disulfide bond usually connects the two chains near the transmembrane domain. Structures within the transmembrane domain allow association with the CD3 signalling complex.

There is great variation in the distal parts of the TCR complex; these are termed IgV domains. The proximal domains do not vary and are classed as IgC domains. There are three hypervariable regions (Kabat et al., 1977), defined as complementarity determining regions (CDR) They map to flexible loops in the structure which determine

specificity. In αβ T cells, the three CDR loops of the α chain combine with the CDR loops of the β chain, which forms the antigen-binding site.

T cells

The αβ T cell receptor interacts with peptide bound to Major Histocompatability Complex (MHC) molecules (Zinkernagel and Doherty, 1974). MHC molecules can bind and present peptide fragments derived from foreign antigens (Townsend et al., 1989). In humans, the MHC locus is termed the Human Leukocyte Antigen (HLA) locus.

αβ T cells are classified according to their surface expression of co-receptors, CD4 and CD8. CD8 T cells generally interact with complexes of peptides and MHC class I molecules (HLA-A, HLA-B and HLA C in humans), and generally have cytotoxic functions. CD4 T cells interact with peptide-MHC class II (HLA-DP, DQ and DR) molecule complexes, and have varying functions, depending on the pattern of cytokines that they produce (Mucida and Cheroutre, 2010). CD4 cells develop into T-Helper 1 (T_H1) cells in the presence of Interleukin-12 (IL12), and are capable of secretion of Interferon-gamma (IFNγ) and Tumour-Necrosis-Factor alpha (TNFα). These enhance cytotoxicity of NK and CD8 T cells. T_H2 cells develop in the presence of IL-4 and secrete cytokines, which aid B-cell activation and proliferation. T_H17 cells are important in the recruitment of innate immune cells such as neutrophils to sites of infection. They develop in the presence of IL6 and TGFB, and are capable of secreting IL17 and IL22 (Korn et al., 2009). Regulatory T cells (Tregs) can be either natural Tregs which develop in the thymus, or induced Tregs which are induced following stimulation in the presence of TGFB and IL2 (Vignali et al., 2008). When Tregs are activated they secrete immunosuppressive cytokines such as IL10 and TGFβ and hence reduce activation of other antigen specific T cells.

Thymic Selection of αβ T cells

T cell progenitor cells migrate from the bone marrow to the thymus. Notch signaling in the thymus commits these progenitors to the T cell lineage.

A series of VDJ rearrangements occurs in the thymus. The TCR β-chain locus is rearranged first. D_B to J_B rearrangements occurs first, followed by V_B to DJ_B rearrangements. If no productive rearrangement is made at this stage, the cells die (Shortman et al., 1990). If productive β chain rearrangements are made, the β chains pair with a surrogate pre-TCR α chain, allowing the assembly of a complete pre-TCR (Borowski et al., 2004). This associates with CD3 molecules at the cell surface to provide a complete CD3:TCR receptor complex that causes constitutive signaling. This signaling leads to proliferation, and the expression of CD4 and CD8 (double positive thymocytes). B chain rearrangements are halted at this stage. After double positive cells have ceased to proliferate, the α chain locus rearrangements begin. The α chain locus can rearrange multiple times, with the effect that most double positive T cells go on to produce functional αβ TCRs (Huang et al., 2005). Cells whose TCRs recognize self-peptide:self-MHC complexes are positively selected, go on to mature and express high levels of their TCR (Huesmann et al., 1991). They also lose expression of either CD4 or CD8, becoming single positive CD4 or CD8 T cells (Singer et al., 2008).

These single positive CD4 or CD8 T cells exhibit a wide range of affinities for MHC molecules. Cells that express TCRs that recognize MHC too strongly undergo negative selection, and die by apoptosis (Sprent and Kishimoto, 2002). This allows the survival of T cells that weakly recognize self-MHC molecules, but retain the potential for specificity for foreign antigen/self MHC complexes.

TCR gene rearrangement is capable of producing a repertoire of 10^{15} unique TCRs. Many of these possible recombinations are unable to recognize self-MHC, or may recognize self MHC too strongly (leading to possible autoimmune effects). They therefore undergo positive and negative selection. This process ensures that T cells expressing TCRs which are weakly self-reactive are positively selected, whereas TCRs which are strongly self reactive and likely to cause autoimmune phenomena are negatively selected (Stritesky et al., 2012). The vast majority of T-cell precursors that enter the thymus die there.

Clonal expansion of αβ T cells

Adaptive immune responses require activation. Naïve αβ T cells circulate via the bloodstream to the lymph nodes, spleen and mucosa-associated lymphoid tissues. They sample large numbers of peptide:MHC complexes on the surface of dendritic cells (Itano and Jenkins, 2003). When a T cell recognizes its specific antigen on the surface of a dendritic cell, it ceases migrating and undergoes clonal expansion.

After 5 days of proliferation, activated T cells differentiate into effector T cells, and migrate to their site of action. Effector T cells no longer require co-stimulation to activate on encountering their TCR target (Gudmundsdottir et al., 1999). A portion of these effector T cells are retained as part of a memory pool, which have no need for expansion prior to activation upon encountering their target antigen, and are able to mount a quick secondary response (Hayday 2009).

CD8 T cell differentiation

In response to viral infection, naïve CD8, MHC-class I restricted, virus-specific T cells expand clonally and differentiate into effector cells that kill the virus-infected cells, and memory cells that provide enhanced immunity to prevent re-infection, (Veiga-Fernandes et al., 2000, Kaech and Ahmed, 2001, Sprent and Surh, 2001). It is possible to distinguish functionally distinct CD8 T cell subsets by expression of several

combinations of key phenotypic markers such as CD45RO/CD45RA (Kuijpers et al., 2003), the co-stimulatory molecules CD28, CD27 and the chemokine receptor CCR7, a lymph node-homing chemokine receptor. Naïve CD8 T cells express the phosphatase CD45RA, CD28, CD27 and CCR7. Viral infection induces large expansions of antigen-specific CD8 T cells, with up-regulation of perforin and granzyme B (Roos et al., 2000). During an acute infection, CD45RO, CD38, HLA-DR, CD28 and CD27 are expressed, whereas later in infection CD28 and CD27 can be down-regulated, as costimulation is no longer required (Gamadia et al., 2003). A "primed" CD8 population which is CD8+, CD45RA+ and CD27- is capable of secreting IFN-γ and TNF-α, express perforin, granzyme B and CD95 ligand and shows high cytolytic activity (Baars et al., 2000), and this subset has been shown to expand in patients with CMV (Kuijpers et al., 2003). CD45RA is re-expressed in sub-populations of memory CD8 T cells. This subset is considered more highly differentiated, having undergone extensive cell division. These cells have short telomeres and limited replicative potential but still retain cytolytic activity with expression of granzyme and perforin (Appay et al., 2008), see Figure 1.1.

CD8 T cell differentiation and expression of cell surface markers

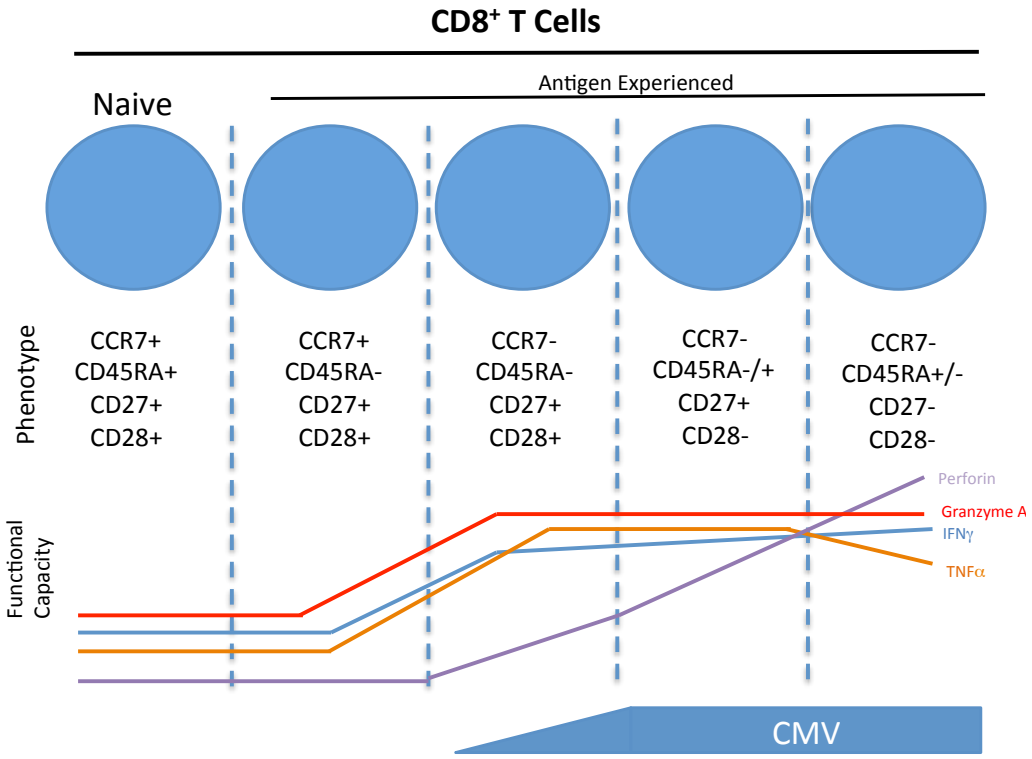


Figure 1.1 *As CD8 T cells become antigen experienced they express different cell surface markers and have different functional capacity.*

Adapted *From (Appay et al., 2008)*

Unconventional T cells

The conventional adaptive immune response is clearly very powerful and sufficient to eliminate many pathogens. However, it takes time for antigen-presenting cell (APC) migration, antigen presentation, clonal selection and differentiation into effector cells. Other immune responses are required to generate quicker responses to microbial challenges. Unconventional lymphocytes may fulfill some of this role, (Hayday, 2009b, Hayday and Viney, 2000), and may respond to early signs of infection or cellular transformation.

T cells that do not recognize classical class I or II MHC molecules and peptides are termed unconventional T cells. One class is specific for the MHC I related MR1 (Tilloy et al., 1999, Treiner et al., 2003). Some recognize lipids presented by another MHC related molecule, CD1. (Brigl and Brenner, 2004).

Gamma delta T cells

The four T cell receptor families (α, β, γ, δ) appear conserved amongst jawed vertebrates, across 420 million years of evolution (Hayday, 2000a). The TCR δ and α genes are closely related, indeed interspersed, in all modern mammals (Rast et al., 1997). γδ T cells are the first T cells to develop in many vertebrates (Hayday, 2009b).

Their discovery was unexpected. Saito et al (Saito et al., 1984) cloned a γ TCR cDNA whilst attempting to identify the α chain of the TCR. Further mouse studies followed, and the γ locus was identified on chromosome 13. It is arranged in four clusters, with one or more V segments, a single J and a single C gene (Hayday et al., 1985, Vernooij et al., 1993). The murine Vδ locus was subsequently identified after biochemical studies suggested that a fourth chain heterodimerised with γ (Brenner et al., 1986, Pardoll et al.,

1987, Maeda et al., 1987). The murine Vδ locus is embedded with the α chain locus on chromosome 14.

γδ TCR Recombination

The generation of γδ T cells is largely thymic, and the γδ TCR is generated via RAG mediated V(D)J recombination (Kazen and Adams, 2011). There are some overlaps with αβ T cells: the TCRD locus is embedded in the TCRA locus, and some TCR (V) regions are interchangeably used in γ and α chains.

Similar to the α and β chains, the γ and δ chain proteins are encoded by genes somatically rearranged during intrathymic development from a pool of variable germ line genes encoding V, D, J and C regions(Beetz et al., 2008). In humans, the available repertoire of γ and δ genes is small. There are only six available Vγ genes, five of which (Vγ2, 3, 4, 5, 8) belong to the VγI family, whilst the VγII family contains only Vγ9 (Arden et al., 1995a). There are only 8 functional Vδ genes, some of which are also used in TCRα rearrangement (O'Brien et al., 2007). Diversity of γδ T cells is increased relative to the αβ T cell lineage by two features (Davis and Bjorkman, 1988). In the recombination of the Vγ chain, D regions are not utilised, whereas in the δ chain, more than one Dδ chain can be used. In contrast, TCRβ chains can only use one. These Dδ chains can also be read in any frame. Potential diversity of the γδ TCR therefore exceeds that of either the BCR or αβ TCR (see Table 1.1).

Molecularly, the Vδ gene segments are homologous to the Vα segments. However, there are sequence features distinct from Vα chains used exclusively by Vδ chains (Vδ1, Vδ2 and Vδ3 in humans) (Arden et al., 1995b, Arden et al., 1995a, Clark et al., 1995). These features include increased length of CDR1δ and 2δ regions relative to CDR1α and 2α by

two amino acids. Certain highly conserved amino acids between Vα chains are not conserved in Vδ.

	Immunoglobulin		TCR αβ		TCR γδ	
	H	K	A	B	G	D
Variable Segments	250-1000	250	100	25	7	10
Diversity Segments	10	0	0	2	0	2
Ds read in all frames	Rare	-	-	Often	-	Often
N region added	V-D, D-J	None	V-J	V-D, D-J	V-J	$V\text{-}D_1$, $D_1\text{-}D_2$, $D_1\text{-}J$
Joining Segments	4	4	50	12	2	4
V region combinations	62,500-250,000		2,500		70	
J recombinations	10^{11}		10^{15}		10^{18}	

Table 1.1 Sequence Diversity in immunoglobulin genes and αβ/γδ TCRs (Davis and Bjorkman, 1988)

The decision to commit to a T cell of the αβ versus γδ T cell lineage depends upon which T cell receptor is expressed first by the T cell (Kang and Raulet, 1997). The γ, δ and β loci undergo rearrangement almost simultaneously within the thymus. Most T cells will become αβ T cells, as it is more likely that a productive B chain locus will pair with the pre-α TCR; for a γδ TCR to be expressed, a productive rearrangement of both the γ and δ loci needs to occur – there is no evidence of a functional pre-TCR for γδ T cells (Kang and Raulet, 1997, Lauritsen et al., 2006).

The majority of γδ TCRs can signal independently of thymic ligands in order to mature (Yamasaki et al., 2006). There is therefore less evidence for positive selection within the

thymus, with the exception of the mouse Vδ1 dendritic epidermal T cell (DETC) chain (Jensen et al., 2008) used by the DETC repertoire known to require skint1 expression in the thymus for development (Boyden et al., 2008).

Similarly, whereas in αβ T cells negative selection reduces overly self-reactive TCRs, this mechanism does not appear to be as important in the development of γδ T cells (Sherwood et al., 2011). Deep sequencing analyses comparing frequencies of in-frame and out-of-frame Vγ genes suggested that only low levels of negative selection occur. There is also some evidence for deletion of high tetramer staining γδ T cells in the mouse (Jensen et al., 2008).

Localisation of γδ T cells

In humans, approximately 1-10% of peripheral CD3+ve T cells are positive for the γ/δ TCR (Beetz et al., 2008). They are more prevalent in epithelia, where they make up up to 60% of T cells (Kalyan and Kabelitz, 2013), and are termed "intraepithelial γδ lymphocytes", or IELs. The most prevalent subtype of γδ T cell in the peripheral blood expresses the Vδ2Vγ9 receptor. In contrast, the γδ T cells in the intestinal mucosa rarely express Vδ2 and are often referred to as Vδ2 negative γδ T cells, which can associate with a wide variety of TCR γ chains.

In the mouse, γδ T cells appear in waves during foetal development, with successive waves populating different anatomical sites in the animal. It is not clear whether a similar process occurs in humans. In the mouse, the first wave of γδ T cells homes to the epidermis, the second to the genital tract, and subsequent waves to other tissues such as lung and gut (Hayday, 2009a). Vγ5Vδ1 DETC progenitors interact with SKINT+ mTECs in the foetal thymus, and acquire expression of CCR10 (Jin et al., 2010), directing them to the epidermis; successive waves of γδ T cells may acquire specific chemokine receptors.

In the mouse, IELs show restricted V-region usage and limited junctional diversity. Particular combinations are characteristic of anatomical sites. Intestinal murine γδ T cells predominantly use Vγ7, skin DETCs use an invariant Vγ5Vδ1 combination, and uterine γδ T cells utilise an invariant Vγ6Vδ1 TCR. Systemic murine γδ T cells are more diverse. It has been shown that dendritic epidermal γδ T cells have the capacity to engage only one or two antigens (Asarnow et al., 1988).

γδ T cells have been referred to as non-conventional, innate-like or transitional T-cells, because of features shared with innate cells; they rapidly activate and mobilize without a need for prior clonal expansion (Bonneville et al., 2010).

Effector Mechanisms of γδ T cells

Similarly to αβ T cells, γδ T cells can produce a wide variety of cytokines and perform cytolytic, helper or regulatory functions(O'Brien RL, 2010). In mouse models, it has been demonstrated that different Vγ and Vδ TCRs correlate with function. γδ T cells express cytolytic effector molecules such as perforin, granzymes and Fas/FasL (Hayday and Viney, 2000). In terms of cytokine production, studies have characterised γδ T cells as T_H1 (secreting IL-2 and IFNγ), or T_H2 (secreting IL4, IL5, IL6 and IL10). (Wen et al., 1998, Wen and Hayday, 1997).

TCR chain usage correlates with aspects of γδ T cell function. In an experimental model, Vδ2+ cells express more genes involved in promoting inflammation, including TNF-α, IFNγ, macrophage colony stimulating factor, IL17 and IL21. Vδ1+ cells expressed higher levels of regulatory genes such as IL10 and IL11(Kress et al., 2006). Vδ1+ cells also expressed higher amounts of CCR7 and L-selectin, important homing molecules. Both subtypes expressed high levels of NK receptors and toll-like receptors.

Ligands of γδ TCRs

A small number of ligands for γδ T cells have been identified. They are generally not MHC restricted. Phosphoantigens, pyrophosphomonoesters, representing metabolites of isoprenoid biosynthetic pathways have been shown to stimulate Vγ9δ2 cells expressing a particular junctional motif (Yamashita et al., 2003). Whilst some of these phosphoantigens are "foreign", for example γδ T cells reactive to a herpes simplex virus glycoprotein have been obtained from an infected mouse (Sciammas et al., 1994), some such as isopentenyl pyrophosphate, are normal intermediates of the mammalian mevalonate pathway(O'Brien et al., 2007).

Little is known about the specificities of γδ T cell TCRs. Few specificities have been deduced (see Table 1.2).

Subset	Antigen	References
Human		
Vδ1 (IELs)	MICA	(Xu et al., 2011)
Vδ2	ULBP4	(Kong et al., 2009)
Vδ2	BTN3A1	(Vavassori et al., 2013)
Vδ1 (clones)	CD1c	(Spada et al., 2000)
Vδ1 (blood γδ T cells)	CD1d Tetramers loaded with sulphatide	(Bai et al., 2012)
Vγ4Vδ5 (clone)	EPCR	(Willcox et al., 2012)
Vδ1 (clones)	Lipohexapeptides	(Vincent et al., 1998)
Various	Phycoerythrin	(Zeng et al., 2012)
Vγ9δ2	Phosphoantigens	(Constant et al., 1994)
Vγ9δ2	F1-ATPase	(Mookerjee-Basu et al., 2010)
Vγ1.3δ2	Histidyl-RNA synthetase	(Bruder et al., 2012)
Mouse		
Various	H2-T10, H2-T22	(Crowley et al., 1997),(Shin et al., 2005)
Vγ2Vδ5 (clone)	I-Ek	(Matis et al., 1989)
Vγ2Vδ8 (clone)	HSV glycoprotein 1	(Johnson et al., 1992)
Vγ1 (clones)	Cardiolipin, apolipoprotein H	(Born et al., 2003)
Various	Phycoerythrin	(Zeng et al., 2012)
Vγ1 (clones)	Insulin Peptide (B:9-23)	(Zhang et al., 2010)

Table 1.2 Ligands of γδ TCRs. Adapted from (Vantourout and Hayday, 2013)

Functions of γδ T cells

γδ T cells have the ability to participate in the early stages of an immune response, due to their capacity to recognize antigens that are rapidly displayed following infection and their ability to respond in large numbers with no need for clonal expansion. In contrast with αβ T cells, therefore, they are able to act in the early, afferent phase, of the immune response. There are similarities in this aspect of function with myeloid cells, in that an immune response can occur without clonal expansion or differentiation (Strid et al.,

2008). The phrase "lymphoid stress-surveillance" has been used to describe this ability (Hayday, 2009b).

The predominant γδ T cell subset in the peripheral blood of humans express the Vγ9Vδ2 TCR. This is a chain usage restricted to primates, and has no homologues in mice (Hayday, 2000a). In mice, they have been shown to expand in response to infectious challenges, including mycobacteria, salmonella, listeria, toxoplasmosis and malaria (Sutton et al., 2012). Their TCR recognise phosphoantigens, small non-peptide phosphorylated compounds derived from isoprenoid biosynthetic pathways (Pfeffer et al., 1990, Constant et al., 1994, Tanaka et al., 1994). HMB-PP is an essential metabolite for most pathogenic bacteria such as *Mycobacterium Tuberculosis* and is a potent stimulus of the Vγ9Vδ2 TCR (Davey et al., 2011).

Tyrosine Kinase Proteins in T cell signalling

A tyrosine kinase is an enzyme that can transfer a phosphate group from ATP to a tyrosine residue on a protein. They are a subclass of protein kinases. The phosphate group is attached to the amino acid, tyrosine. Tyrosine kinases are important in signal transduction and regulating cellular activity such as proliferation. Receptor tyrosine kinases function in transmembrane signalling, tyrosine kinases within the cell (non-receptor tyrosine kinases) function in signal transduction to the nucleus (Ruetten and Thiemermann, 1997). More than 90 tyrosine kinase proteins have been identified within the human genome, of which 58 are classed as receptor tyrosine kinases, and 32 are non-receptor tyrosine kinases. (Robinson et al., 2000) The 58 receptor tyrosine kinases are grouped into 20 subfamiles. They comprise an extracellular domain, able to bind a specific ligand, a transmembrane domain and an intracellular catalytic domain, able to bind and phosphorylate substrates (Hanks et al., 1988). Many receptor tyrosine kinases

are important in oncogenesis, by gene mutation, chromosomal translocation, or overexpression (Gunby et al., 2007).

Non-receptor tyrosine kinases (NRTKs) lack receptor-like features such as an extracellular ligand-binding domain and transmembrane spanning region, and are usually located in the cell cytoplasm (Neet and Hunter, 1996). They are important in signal transduction in T and B cells. Receptors that depend on NRTK proteins include TCRs, BCRs, IL2 receptors, Ig Fc receptors, erythropoietin receptors and prolactin receptors.

Abl Tyrosine Kinases and Src Family Kinases in T cell Signalling

The *Src* family kinases, *Lck* and *Fyn* are very important for downstream signaling and the initiation of T cell signalling (Germain and Stefanova, 1999). They are 56 and 59 kDa respectively (Salmond et al., 2009, Palacios and Weiss, 2004), and are constitutively localised to membranes. The unique domain of *Lck* contains a di-cysteine motif that is required for association with CD4 and CD8 co-receptors (Turner et al., 1990). *Lck* is necessary for optimal T cell development (Rudd et al., 2006). *Lck* and *Fyn* are both activated by TCR triggering by peptide/MHC. Confirmational changes arise from binding of ligands to SH3 and/or SH2 domains of the kinase (da Silva et al., 1997, Holdorf et al., 1999, Hofmann et al., 2005, Xu et al., 1999), and two critical tyrosine residues are phosphorylated (Palacios and Weiss, 2004).

Activated *Lck* phosphorylates tyrosine residues in the CD3 and mu-chain immunoreceptor tyrosine-based activation motifs (ITAMs), which recruit the 70kda protein ZAP-70 (van Oers et al., 1994). This phosphorylates the protein linker for activation of T cells (LAT) (Zhang et al., 1998), which goes on to nucleate the T cell "signalosome", activating further downstream kinases and enzymes.

Lck has been shown to be vital for T cell development. In a *Lck* deficient mouse model, there is a striking decrease in the numbers of double positive thymocytes, leading to a decrease in the peripheral T cell numbers, and a skewing in favour of CD8 T cells (versus CD4)(Molina et al., 1992). In the absence of *Lck*, it has been shown that T cell differentiation can proceed as normal (Salmond et al., 2009), but some effector functions are reduced (Lovatt et al., 2006), notably cytotoxic effector function (Tsun et al., 2011). In situations with persistent antigenemia, it has been shown that variations in *Lck* expression may alter the development of central memory (CM) and effector memory (EM) T cell populations (Salmond et al., 2009).

The role of *Fyn* is less clear; it may act as a negative regulator of TCR mediated signalling, and the levels of expression of *Lck* and *Fyn* may affect the balance of positive and negative signals following TCR stimulation(Salmond et al., 2009). It may directly associate with the CD3 subunits(Samelson et al., 1990).

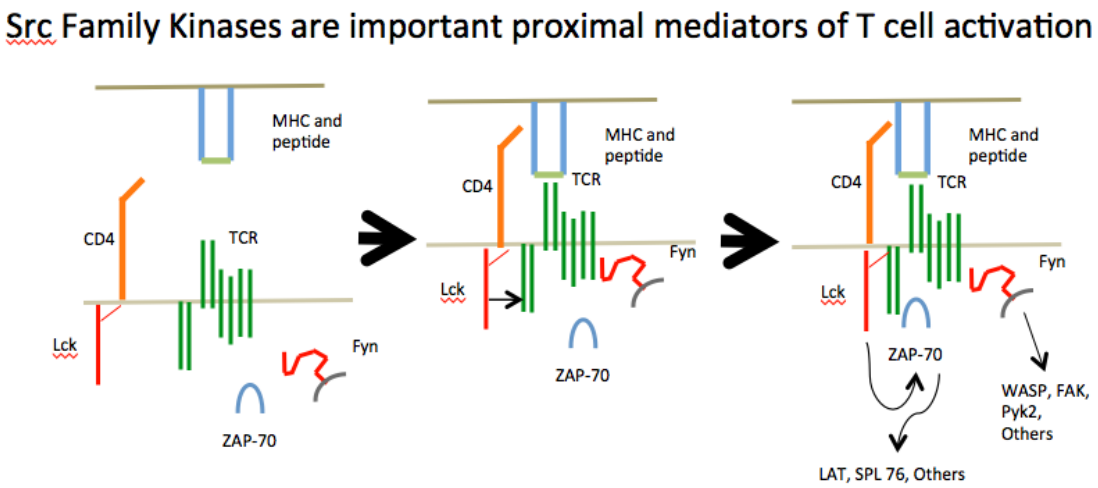


Figure 1.2:The SRC family kinases Lck and Fyn are important mediators in T cell signalling. Lck is associated with CD4 and CD8 co-receptors. They are both activated by TCR triggering by peptide/MHC complexes, two critical tyrosine residues are phosphorylated triggering downstream signalling cascades *Adapted from* (Palacios and Weiss, 2004).

There are two *Abl* kinases in vertebrates: *Abl1* and *Abl2* (Arg)(Goff et al., 1980, Kruh et al., 1990). They are non-receptor tyrosine kinases, and have important roles in cytoskeletal reorganisation, cell proliferation, survival and stress responses (Pendergast, 2002). They are activated by stimulation of growth factor receptor tyrosine kinases such as platelet derived growth factor (PDGFR), the epidermal growth factor receptor (EGFR), and the insulin-like growth factor (IGF-1R) (reviewed in (Gu et al., 2009)). Intracellular *Abl* kinases are also important in T cell activation. (Gu et al., 2009). Activated *Abl* kinases promote the phosphorylation of ZAP-70 and LAT.

A mouse model using the ablm1 mutation demonstrated deficiencies in the function of both B and T cells (Schwartzberg et al., 1991). Knockout mouse models disrupting the *Abl1* gene resulted in various phenotypes including runtedness, susceptibility to infections, splenic and thymic atrophy. (Tybulewicz, 1991). Abl non receptor kinases

are involved in cytoskeletal reorganisation and stress responses. They are activated by invasion of pathogens into mammalian cells. (Backert et al., 2008).

Natural Killer Cells

Natural killer cells comprise 10-15% of lymphocytes.(Robertson and Ritz, 1990) They have the morphological appearance of large granular lymphocytes (LGLs). Evolutionarily, NK-like cells are ancient and predate the adaptive immune system, which first evolved around 500 million years ago. (Caligiuri, 2008) As opposed to T- and B- lymphocytes, NK cells are controlled by a limited repertoire of germ line encoded receptors that do not undergo somatic recombination (Lanier, 2005).

Phenotypically they are defined by the lack of CD3 and the presence of CD56, a 140kDa isoform of the neural cell adhesion molecule (Lanier et al., 1989), on their cell surface. (Robertson and Ritz, 1990).

They are a functionally heterogenous group of cells (Caligiuri, 2008). Cells with a high density of CD56 on their surface ($CD56^{bright}$ NK cells) do not lyse tumour targets but are capable of producing a wide range of cytokines (Cooper et al., 2001). Cells with a lower concentration of CD56 ($CD56^{dim}$ NK cells) have less ability to produce cytokines but some of these are capable of spontaneously lysing target cells (Walzer et al., 2007). $CD56^{dim}$ NK cells express abundant CD16, the low affinity FCγ receptor IIIA (Caligiuri, 2008). The "missing self" hypothesis suggests that $CD56^{dim}$ NK cells are activated in response to the lack of MHC class I molecules on target cells, resulting in a lack of signal through various inhibitory receptors on the NK cell surface (Bryceson et al., 2006). Alternatively, NK cells can be activated through activating receptor binding to ligand. For example, NKG2D interacts with various ligands, such as MICA, MICB and ULBP 1-6

on target cells and activates NK cells (Bauer et al., 1999). NK cells, as opposed to T cells, have no naïve state and are always functional after licensing (Lanier, 2005),

NK cells have been implicated in an anti-tumour role, and may be associated with a graft versus leukaemia effect in the setting of allogeneic stem cell transplant (Ruggeri et al., 2002).

Cytomegalovirus

Cytomegalovirus (CMV) (Human Herpes Virus 5) is a β-herpes virus that has infected most of the human population. Herpes viruses are morphologically distinct from all other viruses; a linear, double-stranded DNA genome of 125-290kbp is contained within an icosahedral capsid, which is surrounded by a proteinaceous matrix (the tegument) and then by a lipid envelope containing membrane associated proteins (Davison et al., 2009). CMV is the largest of the known herpes viruses, with a 230 kb double-stranded, linear genome (Fields, Knipe et al 2007). It can cause serious disease in the immunocompromised host, such as in patients with AIDS (Gandhi and Khanna, 2004), following haematopoietic stem cell transplantation (Craddock et al., 2001), or if on immune suppression following solid organ transplant (Fisher, 2009).

Similarly to other herpesviruses, CMV establishes lifelong persistence in the infected host. It is thought that it causes sporadic reactivations, controlled by a cell-mediated immune response. The latent nature of CMV makes it difficult to study because of the low level of infected cells in individuals (Sinclair et al., 1992, Larsson et al., 1998). It is thought that CMV may establish latency/persistence in monocyte precursors and tissue stromal cells(Jarvis and Nelson, 2002), although this is not entirely clear (Sinclair and Sissons, 2006). The prevalence of CMV has been shown to vary depending on the populations studied, from 50% to 90%(Sinclair and Sissons, 2006). It is around 60% in

the US, and increases with age (Staras et al., 2006). It is higher in lower socio-economic groups.

Immune Control of CMV

Humoral immunity is established quickly after infection. IgG sero-positivity remains the standard test of prior infection (Adler et al., 2007). IgM appears first. Where anti-CMV IgG and IgM are present together, the IgG is usually of low avidity, as the response has not undergone avidity maturation and somatic hypermutation.

It is well established that once established infection has occurred, the cellular immune response is vital for control of CMV. CD4 and CD8 T cells as well as NK cells are thought to play a role, and the CD8 T cells seem particularly important (Moss and Khan, 2004). In immunosuppressed individuals, there is rapid reactivation of CMV, suggesting that constant immunosurveillance is required to keep CMV infection under control (Gandhi and Khanna, 2004). Lack of CD8 T cells following transplantation is associated with CMV disease (Li et al., 1994) and the reconstitution of CD8 T cells following haematopoietic stem cell transplant (HSCT) is associated with a lower risk of CMV reactivation following HSCT (Borchers et al.). Furthermore, adoptive transfer of CMV-specific CD8 T cells following HSCT is associated with improved control of CMV (Cobbold et al., 2005). It is known from studies of patients receiving bone marrow allografts that CD8+ T cells are very important in preventing CMV reactivation (Avetisyan et al., 2007).

CMV reactivation has been detected by PCR of blood from patients receiving conventional chemotherapy (Kuo et al., 2008, Mitchell et al., 2008, Lunghi et al., 2009).

CMV has profound effects on the immune system. These responses seem to accumulate over time. In normal CMV seropositive individuals, the CMV-specific CD4 and CD8 T cell response makes up approximately 10% of the CD4 and CD8 memory compartments

(Sylwester et al., 2005). In the elderly population up to 80% of T cells in CMV seropositive individuals are CMV-reactive (Moss and Khan, 2004), and are clonally restricted (Khan et al., 2002). This expansion is termed "memory inflation" (Karrer et al., 2003). There is evidence that in elderly populations high anti-CMV titres may correlate with mortality (Derhovanessian et al., 2009, Roberts et al., 2010, Pawelec et al., 2009, Strandberg et al., 2009). CMV infection has been shown to be associated with reduced telomere length in the circulating T-cell pool (van de Berg et al., 2010)

CMV specific T cells are characteristically of a late-differentiated phenotype; they have low expression of the co-stimulatory molecules CD27 and CD28, low expression of CCR7, a high number of CD45RA revertant cells, and high perforin expression (reviewed in (Moss and Khan, 2004)).

Innate Immune Responses To CMV

As well as αβ T cells, innate lymphocyte subsets such as NK cells appear to be important for the immune anti-CMV response(Biron et al., 1989).

In a mouse model, NK cells can directly recognise the MCMV protein m157 and kill virus-infected cells(Jackson et al., 2011, Brown et al., 2001). In humans, deficiencies in NK cells lead to recurrent CMV infections usually only seen in immunosuppressed patients (Biron et al., 1989, Gazit et al., 2004). Furthermore, NK cell function has been shown to be an important factor influencing survival from CMV infection following allo HSCT (Quinnan et al., 1982).

CMV infection causes downregulation of HLA class I molecules on the cell surface, which could lead to NK cell activation and killing(Biassoni et al., 2001). CMV has evolved mechanisms to avoid NK-mediated killing, including expression of a viral homologue of

HLA class I, UL18 (Beck and Barrell, 1988). NKG2D interacts with MICA/B, ULBP 1-3, Retinoic acid early transcript (RAET)1E, RAET1G and RAET1L (Jackson et al., 2011). UL16 downmodulates many NKG2D ligands to evade this pathway (Cosman et al., 2001).

γδ T cells and CMV

It has been shown that γδ T cells expand in immunosuppressed renal allograft patients with acute CMV infection (Lafarge et al., 2001), and that the expansion was associated with shorter duration of antigenaemia and lower maximum pp65 levels. Sorted Vδ2 –ve γδ T cells had activity against CMV-infected foreskin fibrolast monolayers.

The percentage of circulating Vδ1 γδ T cells is higher in CMV positive individuals compared to CMV negative people (Pitard et al., 2008). The Vδ2 negative γδ T cells also demonstrated a restricted repertoire in CMV infected people, measured by the immunoscope technique, estimating the quantity of different TCR sequences after PCR with Vδ1 and Vδ2 specific primers supplemented with a fluorescent tag, in order to quantify deviation of sample T-cell repertoires from controls and generate an index of clonality (Pitard et al., 2008). Other herpes viruses had no effect on the repertoire.

In cases of *in utero* CMV transmission, expansions of Vδ1 γδ Tcells have been seen which have highly conserved TCRs, suggesting a "public TCR", which may respond to a single epitope (Vermijlen et al., 2010).

γδ T cells in Tumour Immunology

Vγ9Vδ2 γδ T cells are thought to possess anti-tumour properties because host-produced phosphoantigens such as isopentenyl pyrophosphate (IPP) can stimulate Vγ9Vδ2 T cells, albeit 10^4-10^7 times less potently than HMB-PP. It is possible, therefore, that these cells may be involved in tumour surveillance by recognising up-regulated IPP on "stressed"

cells. Furthermore, studies have demonstrated that anti-tumour responses of Vγ9Vδ2 T cells may be increased by treatment with bisphosphonate agents. Some evidence of clinical efficacy has been demonstrated using these agents to stimulate anti-tumour responses (Wilhelm et al., 2003, Dieli et al., 2003).

Bisphosphonates inhibit enzymes in the mevalonate pathway, leading to increased IPP accumulation (Hewitt et al., 2005). The combination of zoledronate and IL-2 has been used to treat hormone-refractory prostate carcinoma (Dieli et al., 2007) in a phase I study. Zoledronate has been used to treat breast carcinoma, (Gnant et al., 2009). The mechanism of action is thought to be to an expansion and enhancement of activity of Vδ2 γδ T cells. Vγ9Vδ2 γδ T cells have been shown to effectively lyse CML cell lines pre-treated with zoledronate (D'Asaro et al., 2010).

Vδ2 negative γδ T cells in tumour immunology

Non Vδ2 γδ T cells have been postulated to have anti-tumour roles. γδ T cells have been shown to confer protection from chemical carcinogenesis and spontaneous tumours in a transgenic mouse model (Girardi and Hayday, 2005).

Vδ1 positive cells have been shown to have activity against epithelial tumour cell lines (Steinle et al., 1998, Groh et al., 1999), probably in these cases mostly via NKG2D. CMV-reactive Vδ2 negative clones exhibit dual reactivity against tumour cells, which suggests reactivity towards self "stress" antigens (Halary et al., 2005), and *in vivo* anti tumour effects have been demonstrated in a mouse model of colon cancer. (Devaud et al., 2009). Furthermore, following renal allograft and consequent immunosuppression there is an increased risk of skin tumours. Increased numbers of Vδ2 negative γδ T cells associated with CMV reactivation was associated with a lower risk of tumour development (Couzi et al., 2010).

Epstein-Barr Virus

Epstein-Barr Virus (EBV) is a gamma Herpes virus present in over 90% of the adult population. Similarly to other herpes viruses, it causes lifelong infection. Similar to CMV, it is known to be associated with oligoclonal populations of T cells (Strickler et al., 1990), and is known to cause pathology in immunosuppressed individuals (Fox et al., 2013).

Chronic Myeloid Leukaemia

Chronic myeloid leukaemia (CML) was first described in 1845 (Bennett, 1845). Chronic Myeloid Leukaemia is characterised by unregulated proliferation of myeloid progenitor cells. It was the first malignancy that was discovered to have a specific chromosomal aberration associated with it: the Philadelphia Chromosome (a 9,22 translocation) (Nowell, 1962). It was further characterised in 1973 as a t(9,22)(q34;q11)(Rowley, 1973), and this translocation is now recognised as the hallmark of the disease (Rowley, 1975).

The ABL gene encodes a membrane-bound tyrosine kinase. The (9,22) translocation juxtaposes the *BCR* (Breakpoint Cluster Region) region of chromosome 22 to the *ABL1* gene on chromosome 9. In CML, the usual size of the protein produced by *BCR-ABL* is 210 kda.

The fusion partners provide domains that promote oligomerization of the chimeric protein, leading to trans-phosphorylation and enhanced kinase activity (Pendergast, 2002).

The BCR-Abl is constitutively active, and activates a number of downstream cell cycle controlling proteins and enzymes as well as inhibiting gene repair. Bcr-Abl signalling prevents downregulation of cyclin-depedent kinase activity and cell cycle arrest in haematopoietic progenitor cells. Bcr-Abl expression is sufficient to induce G1-to-S phase transition, DNA synthesis and activation of cyclin-dependent kinases in cells that were arrested in G0 by growth factor deprivation. It also activates the Ras, Erk and Jnk pathways as a consequence of activation (Cortez et al., 1997).

The $P210^{bcr/abl}$ translocation can induce CML in transplanted marrow infected with the Philadelphia chromosome via a retrovirus (Daley et al., 1990).

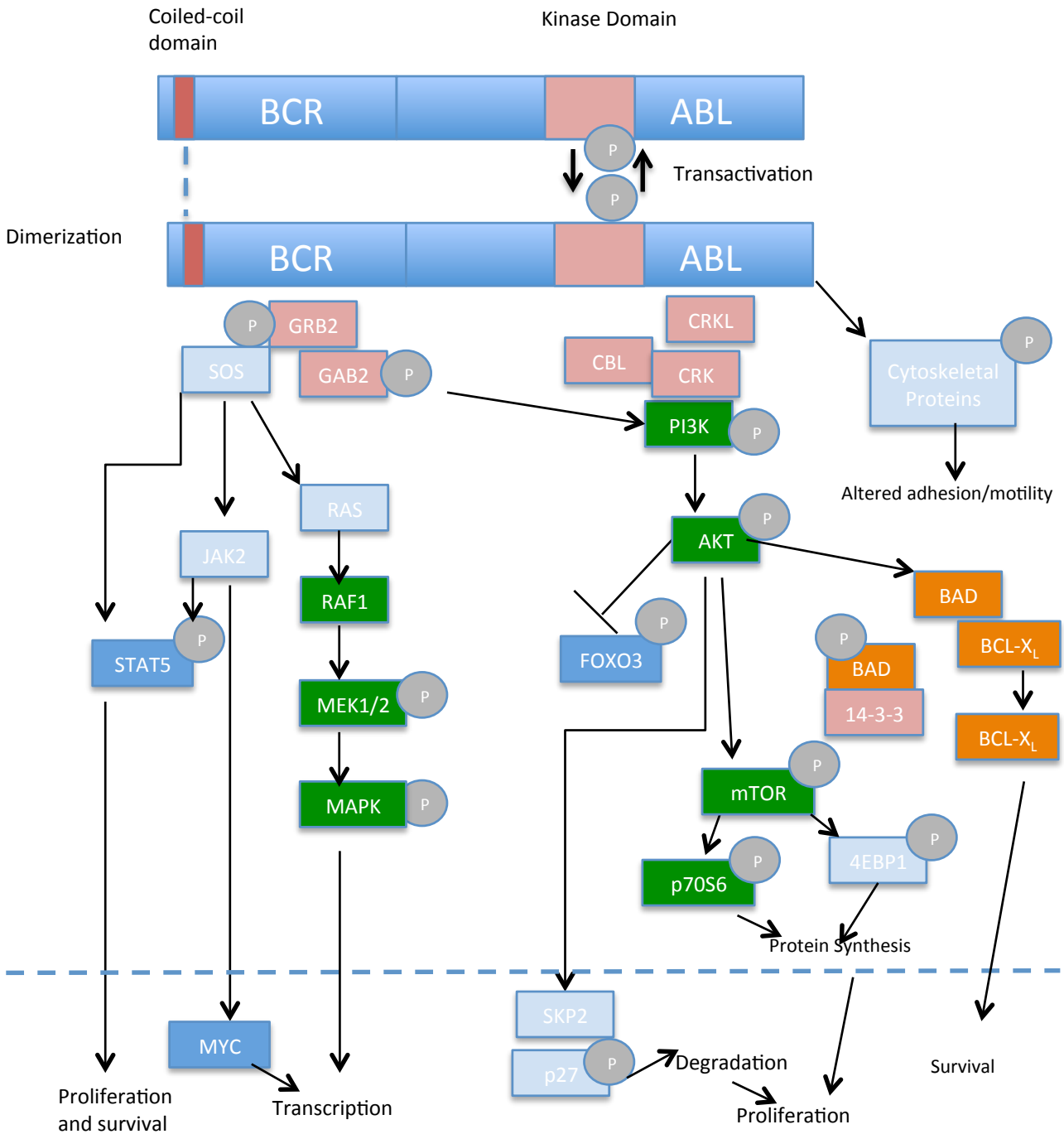


Figure 1.3: Bcr-Abl is constitutively active and activates a number of downstream cascades From (O'Hare et al., 2011) .

Natural course

The annual incidence of CML is 1-2/100,000 people. There is a slight preponderance of male patients. The risk of CML is higher in patients who have been exposed to high levels of ionising radiation.

90% of patients with CML are diagnosed in the *chronic phase.* Untreated patients progress through an accelerated phase, to a blast crisis. The majority of patients who progress to blast crisis die of the disease (Kantarjian et al., 1988). Prior to the emergence of effective TKI therapy the median survival was in the order of five years.

Phase	Chronic	Accelerated	Blast
Blast %age	<10	10-19	>=20
Basophils	<20	>20%	
Plt count	100-1000	<100 or>1000	

Table 1.3 WHO Definitions of Phases of Disease in CML (adapted from (Baccarani et al., 2013).

Accelerated phase is also characterised by increasing splenomegaly and overall white cell count, unresponsive to therapy, or thrombocytopenia $<100*10^9$/L unrelated to therapy. The blast crisis is defined as above in table 1.3 but can also be diagnosed by extramedullary blast proliferation, and is caused by an accumulation of genetic abnormalities that lead to progressive loss of differentiation of the abnormal clone (Calabretta and Perrotti, 2004), It is associated with poor survival.

Assessment of Response to Treatment

Chronic myeloid leukaemia is classified according to its *haematological response*, based on clinical examination of the patient and blood film, *cytogenetic response,* based on

conventional cytogenetic examination of bone marrow, and *molecular response,* based on real time quantitative PCR of *BCR/ABL* transcripts from peripheral blood samples (Baccarani et al., 2006).

A complete haematological response (CHR) is achieved when there is normalisation of the blood counts (Plt count <450 * 10^9/L, WCC < 10*10^9/L, no immature granulocytes on peripheral film, less than 5% Basophils and no palpable spleen.

A complete cytogenetic response (CCyR) is achieved when there is no detection of the Philadelphia chromosome, either by conventional karyotyping or by FISH when at least 20 cells are assessed.

Molecular responses are assessed by quantatitive PCR methods, comparing the *BCR-ABL* transcripts to a control gene, usually *ABL1*, according to the international scale (Baccarani et al., 2013). A major molecular response (MMR) represents a *BCR-ABL/ABL* ratio of <= 0.1%. Patients that achieve this are protected from progression to accelerated phase or blast crisis (Hughes et al., 2010). More recently, the definition of $MR^{4.5}$, with either detectible disease with <0.0032% *BCR-ABL/ABL* or undetectable disease with >32,000 *ABL* transcripts (Baccarani et al., 2013). This may define a subset of patients able to successfully discontinue treatment (Cross et al., 2012).

The main aim of treatment has been to maintain patients in chronic phase. The only treatment considered curative is an allogeneic stem cell transplant. Lack of achievement of time-dependent treatment milestones has been agreed to represent trigger points to make therapeutic decisions (Baccarani et al., 2009, Baccarani et al., 2013).

Current guidelines suggest that with tyrosine kinase inhibitor treatment, a failure to achieve CHR by 3 months, or a *BCR-ABL/ABL* ratio>10% at 6 months, or a lack of complete cytogenetic response at 12 months represents treatment failure. A loss of haematological response, loss of CCyR, loss of MMR or mutations in the *BCR/ABL* ATP binding site at any time represent failure (Baccarani et al., 2013). More recently, it has been suggested that using an aggressive intervention approach based upon earlier timepoints may be beneficial (Hughes et al., 2008). Strategies included dose escalation or early switching to nilotinib for failure to reach *BCR-ABL* < 10% at 3 months of therapy with imatinib . This level corresponds with a major cytogenetic response (Ross et al., 2006), and failure to reach this level at 3 and 6 months correlates with a poorer outcome (Hughes et al., 2010, Hanfstein et al., 2012, Marin et al., 2012).

Monitoring of response is continued even after achievements of milestones, because loss of response may be associated with tyrosinse kinase-inhibitor mutations appearing. The probability of a mutation arising is proportional to the number of cells at risk, and thus is highest in the first 3-4 years after commencement of therapy (Kantarjian et al., 2011).

Treatment of CML

Prior to the development of TKIs, treatments such as arsenicals, radiotherapy, busulphan, hydroxycarbamide and interferon-alpha were used as therapies for CML (Goldman, 2007). Until the development of imatinib, it was conventional to offer patients with CML in chronic phase treatment with allo HSCT which offered up to a 60-80% probability of five-year survival , (Gratwohl et al., 1998, Passweg et al., 2004) after which, the probability of relapse is very low (Mughal et al., 2001).

Imatinib

Imatinib is a 2-phenylaminopyridimine compound, with activity against BCR-ABL, but which also inhibits platelet-derived growth factor (PDGFR) and c-KIT. Early studies on

cell lines and material collected from patients demonstrated selective inhibition of CML cells (Druker et al., 1996, Deininger et al., 2000). BCR-ABL target cells and primary CD34+ve cells are inhibited by imatinib and undergo G1 cell cycle arrest (Andreu et al., 2005).

The IRIS study compared treatment of CML with imatinib 400mg od vs interferon 5million U/m^2 plus cytarabine 20mg/m^2. 1106 patients were recruited between June 2000 and January 2001. A six year follow up of the IRIS study (Hochhaus et al., 2009) showed 88% overall survival in the group randomised to imatinib. 66% of the patients randomised to imatinib were still taking it after six years. 82% of the patients randomised to imatinib achieved a CCyR, of which 84% maintained it. Imatinib has become accepted first line therapy for CML, as evidenced by the falling numbers of patients receiving allografts for CML (Gratwohl et al., 2006, Giralt et al., 2007).

Second Generation TKIs

Because of the approximately 25% of patients who fail first line therapy with imatinib, either due to disease resistance or intolerance of the drug, second line TKIs have been developed (Talpaz et al., 2006, le Coutre et al., 2008, Kantarjian et al., 2006). Two are currently widely used in the UK. *Nilotinib* (Novartis), and *Dasatinib* (Bristol-Myers-Squibb). They are more potent inhibitors of the BCR-ABL oncoprotein; for example, dasatinib has approximately 300 times more potent inhibition than imatinib *in vitro* (Kantarjian et al., 2010, Kantarjian et al., 2012). Current recommendations from the European Leukemia Network recommend commencement on a second generation TKI should treatment with imatinib fail, based on failure to achieve time-dependent outcomes, or intolerance to imatinib (Baccarani et al., 2013). As second line therapy, dasatinib therapy can achieve a progression-free survival of 56% at 5 years (Baccarani

et al., 2013); nilotinib is associated with a 57% progression-free survival at 4 years (Giles et al., 2013).

Second generation TKIs have also been used as primary therapy in CML; dasatinib induces higher proportions of CCyR at 12 months compared to imatinib. Deep molecular responses are more common in patients treated with dasatinib (Kantarjian et al., 2012). Nilotinib as primary therapy has similarly been associated with higher rates of CCyR at 1 and 2 years, and deeper molecular responses (Larson et al., 2012).

"Off Target" Effects of TKIs

As well as its inhibition of *Abl* Kinase proteins, imatinib also has inhibitory effects on other kinase targets, such as PDGFR and c-Kit. Nilotinib has a very similar profile of kinase targets, but is much more potent. Dasatinib has a much wider target kinase profile, and inhibits the SRC family of kinases as well (Giles et al., 2009)(See Table 1.4).

Drug	Kinase Targets
Imatinib	ABL, PDGFR, c-KIT
Dasatinib	ABL, PDGFR, c-KIT, FGR, FYN, HCK, LCK, LYN, SRC, YES, EPHB4
Nilotinib	ABL, PDGFR, c-KIT, ARG, EPHB4

Table 1.4 Kinase Targets of Imatinib, Dasatinib and Nilotinib (Adapted from (Giles et al., 2009)).

Although commonly thought of as "targeted" therapies, tyrosine kinase inhibitors are associated with significant side effects, due to their "off target" effects. Most common amongst these are asthenia, skin rashes, muscle cramps and hair loss (Darkow et al., 2007, Marin, 2012, Eliasson et al., 2011). These are the most common cause for poor compliance with TKI therapy (Darkow et al., 2007, Noens et al., 2009), which has been shown to be a major contributor to relapse in patients with CCyR (Marin et al., 2010, Ibrahim et al., 2011), and may well be important in guiding decisions to change to second line TKI therapy.

Dasatinib is recognized to be associated with pleural effusions. The incidence of pleural effusions has been reported to be as high as 54% (de Lavallade et al., 2008, Quintas-Cardama et al., 2007, Kim et al., 2011, Kantarjian et al., 2012, Porkka et al., 2010). It has also more recently been reported that dasatinib is rarely associated with pulmonary hypertension, which is usually reversible on discontinuation of the drug (Montani et al., 2012). The development of pleural effusions is associated with expansions of large granular lymphocytes in some patients (Tanaka et al., 2012, Nagata et al., 2010). The mechanism of action is not fully understood, but it has been hypothesized that dasatinib may augment IgE-dependent secretion of histamine in basophils (Herrmann et al., 2012).

In vitro immunosuppression

Imatinib inhibits a number of T cell functions in a dose dependent manner, including upregulation of CD69 to activation via CD3, and decreased proliferation of CMV-specific T cells when stimulated with CMV peptides, via decreased phosphorylation of ZAP70 and LAT (Seggewiss et al., 2005). It also inhibits T cell proliferation in an in vitro model, using isolated peripheral blood mononuclear cells (PBMCs) from healthy donors, as well as reducing IFNγ production from CD4 and CD8 T cells after activation via CD3/CD28 (Cwynarski et al., 2004). CD4+CD25+ Tregs (Chen et al., 2007b), and antigen-specific CD8 T cell responses (Chen et al., 2007a) have also been shown to be inhibited in vitro.

Dasatinib profoundly inhibits proliferation of T cells to phytohaemagglutinin (PHA) in a dose-dependent manner. The inhibition was at least 100 fold that of imatinib. The addition of dasatinib significantly downregulates expression of HLA-DR, CD25 and CD69 on stimulation, including to CMV-specific CD8 T cells (Fei et al., 2008). Dasatinib also has profound inhibitory effects on NK mediated cytotoxicity and IFNγ production of the MHC

class I deficient NK cell target, K562 , via inhibition of ERK and PI3K phosphorylation (Salih et al., 2010). Dasatinib causes a significant reduction of TCR-mediated signal transduction, proliferation, cytokine production and in vivo T cell responses in PBMCs from healthy donors (Schade et al., 2008), and is capable of inhibiting antigen-specific T cell responses in vitro and in a mouse model (Fraser et al., 2009).

Nilotinib can inhibit the Src family kinase LCK and thus inhibit T cell function in vitro (Blake et al., 2009). Nilotinib has been demonstrated to inhibit antigen peptide specific CD8 T cells in vitro, twice as strongly as imatinib, via inhibition of phosphorylation of ZAP-70, Lck, ERK1/2 and the NF-KappaB signalling pathway (Chen et al., 2008).

In vivo immune effects

In a mouse model the presence of imatinib significantly decreased secondary immune responses to OT-1 by 60% (Sinai et al., 2007). Measured Ig levels steadily diminished during treatment with imatinib in a group of patients commenced on imatinib. The proportion of CD19 positive B cells actually increased throughout the period of analysis. Despite the hypogammaglobulinaemia, there was no significant excess in infective complications (Steegmann et al., 2003).

In imatinib-treated patients, there are significant decreases in IL-2, IFNγ and TNFα from patients' CD4+ cells when the experiments were performed in the presence of autologous plasma. When the experiments were performed in RPMI supplemented with FCS, there was no difference between the responses from T cells of imatinib treated patients compared to healthy controls or IFNα treated patients (Gao et al., 2005).

Imatinib has been used as salvage therapy in chronic graft versus host disease (GvHD), GvHD occurs after allo HSCT when donor T cells demonstrate immunological

intolerance to host antigens (Ferrara et al., 2009). In this clinical situation imatinib inhibits PDGFR and TGFβ dependent pathways, which are thought to be important in the generation of steroid refractory chronic GvHD (McCormick et al., 1999). In one study, median duration of treatment was 5.9 months. Seven patients responded to the imatinib, with four of the patients showing dramatic clinical improvement (Magro et al., 2009). In a study of 39 patients with steroid refractory chronic GvHD, over half of patients treated with imatinib experienced at least patial responses as defined by NIH criteria, with the best responses seen in the lungs, gut and skin (Olivieri et al., 2013).

Dasatinib at 140mg/day has been reported to cause infectious complications such as EBV positive leucoplakia, pneumocystis pneumonia and skin tumours (Sillaber et al., 2009).

Can Tyrosine Kinase Inhibitors Cure CML?

Allo-HSCT has been considered the only treatment that can cure CML. This is based on the evidence that this treatment modality offers long-term freedom from cytogenetic or haematological recurrence of the disease without need for maintenance therapy (van Rhee et al., 1997). It has been demonstrated that stopping imatinib in patients that have achieved a complete molecular response is safe, and approximately 40% do not relapse. The remaining 60% could be safely retreated with imatinib. (Mahon et al., 2010). In the TWISTER study, cessation of imatinib in patients who had achieved undetectable minimal residual disease had a 47% chance of remaining disease free at 2 years. Persistence of the original CML clone was revealed in all patients by highly sensitive, patient-specific BCR-ABL DNA PCR (Ross et al., 2013).

"Operational cure" by TKIs in CML could potentially be achieved by stem cell depletion, stem cell exhaustion, and immune control (Melo and Ross, 2011). Strategies to identify

patients who might benefit from these strategies will improve outcomes for patients with CML in the future.

Evidence of Immune anti CML Effects

Results from allo HSCT have shown that CML is particularly susceptible to the graft versus leukaemia (GVL) effect. This effect is thought to be partly mediated by alloreactive T cells responding to minor histocompatability antigens (Riddell et al., 2002), and partly to NK cell activity (Hoyle et al., 1998, Hauch et al., 1990, Mackinnon et al., 1990) . Evidence comes from the particular efficacy of donor lymphocyte infusion (DLI) in CML (Pavlu et al., 2011). CML was the first disease in which DLI was shown to be effective a re-inducing remission following allo HSCT.

A vaccine-based approach using junctional sequences shown to induce T-cell responses against the e14a2 BCR-ABL junction (Bocchia et al., 1995) has been shown to be associated with a fall in disease burden (Rojas et al., 2007) post vaccination.

Lymphocytosis on treatment with dasatinib has been noted in a number of papers (Kim et al., 2009, Valent and Schiffer, 2011, Kreutzman et al., 2011a) and has been suggested to be associated with improved outcome to treatment (Mustjoki et al., 2009). Expansions of CD8 T cells and NK cells are seen most commonly in a subset of dasatinib-treated patients (Kreutzman et al., 2011a) These papers are described in more detail on pages 171-174.

Reduced Intensity Allo HSCT

AlloHSCT is potentially curative in a variety of haematological disorders. Reduced intensity alloHSCT (RIC alloHSCT) are increasingly used as alternative to conventional

myeloablative alloHSCT, due to decreased toxicity. RIC alloHSCT has been defined as a regimen that is not intended to eradicate host haematopoiesis, but still allow engraftment of donor stem cells, achieving a graft-versus leukaemia effect (Bacigalupo, 2004). They are usually based on a purine analogue such as fludarabine with an alkylating agent to provide various degrees of myelosuppression (Giralt et al., 2001, Nagler et al., 2001).

Immune reconstitution following RIC alloHSCT has been demonstrated to be broadly similar to that following myeloablative alloHSCT, with a rapid recovery of NK cells and CD8 T cells, with CD4 T cells slower to recover (Jimenez et al., 2007); in some studies the reconstitution of CD4 T cells has been shown to be faster following RIC alloHSCT compared to conventional myeloablative alloHSCT (Schulenburg et al., 2005). However, the use of RIC alloHSCT with the addition of Campath 1H (alemtuzumab) or ATG could significantly delay immune reconstitution. In a group of patients receiving RIC alloHSCT with 100mg alemtuzumab for myeloma, the total T cell count remained below normal for 21 months after transplant, and the CD4 count remained decreased throughout the entire follow up period (D'Sa et al., 2003). When the alemtuzumab dose is decreased there was more rapid reconstitution of CD4 and CD8 T cells in a group of 27 patients transplanted for a variety of haematological malignancies (Dodero et al., 2005).

Evidence of immune anti AML effects post alloHSCT

After RIC alloHSCT for AML or MDS it has been shown that the occurrence of GVHD is associated with a decreased relapse risk, suggesting a graft-versus-leukaemia role of the transplant (Martino et al., 2002).

Minor Histocompatability antigens, such as HA-1 have been demonstrated to be expressed on AML cells and are capable of eliciting an alloimmune response (Spierings et al., 2004). Anti-HA-1 CTLs are able to lyse AML cell lines in vitro and in a mouse

model were able to delay leukaemia progression (Hambach et al., 2006). Furthermore, after donor lymphocyte infusion, the emergence of HA-1 and HA-2 specific CD8 T cells has been demonstrated to coincide with complete remission from leukaemia (Marijt et al., 2003).

Post alloHSCT anti-LAA responses have been seen post alloHSCT in patients and correlated with ongoing remissions (Steger et al., 2014).

Aims and Objectives

The aim of this project was to investigate the immune profiles of patients undergoing treatment for myeloid malignancies, and investigate the immune changes associated with herpes virus infection, particularly CMV. I was particularly interested in assessing whether CMV was associated with changes in innate lymphoid subsets, which have been postulated as having a dual anti-infective and anti-tumour role.

As described above, innate-like subsets of T cells such as γδ T cells have been shown to have anti-viral effects and anti-tumour properties. I was interested in assessing whether an anti-herpes virus response by these lymphocyte subsets could be associated with anti leukaemia properties, and ultimately whether there was a measurable clinical effect induced by changes in the lymphocyte profile of patients associated with chronic herpes virus infection, particularly CMV.

I therefore measured *in vivo* effects of TKI inhibition in patients treated with the TKIs imatinib, dasatinib or nilotinib for CML.

I wanted to measure the effects of CMV infection on patients treated with tyrosine kinase inhibition, with particular emphasis on immune anti-CMV effects. I was

particularly interested in examining changes in γδ T cells in relation to CMV, and investigating whether changes in this less-studied lymphocyte subset could impact on responses to treatment in patients treated for CML.

I collected patients' blood samples from participating UK centres. PBMCs were isolated and a panel of lymphoid markers was used to assess the relative and absolute numbers of a number of lymphoid subsets. We wanted to assess the changes in lymphocyte subsets on initiation of TKI therapy, and particularly measure the effect of CMV on the changes in the lymphocyte subsets.

I also looked at the effects of CMV on the reconstituting immune system following reduced-intensity alloHSCT to see if any of the differences that we saw following TKI treatment were mirrored in patients undergoing allograft. Furthermore, CMV infection is currently considered to be associated with significant morbidity following allo HSCT. I was interested to investigate whether changes in some lymphoid subsets in relation to CMV may actually be beneficial.

Chapter 2
Materials and Methods

List of Media used

Wash media	RPMI 1640 (Gibco BRL)
	100 U/ml Penicillin (Gibco BRL)
	100 mcg/ml Streptomycin (Gibco BRL)
General Media	RPMI 1640 (Gibco BRL)
	100U/ml Penicillin (Gibco BRL)
	100mcg/ml Streptomycin (Gibco BRL)
	2mM Glutamine (Gibco BRL)
	10% Foetal Calf Serum (SBS Biologicals)
Freezing Media	Foetal Calf Serum (SBS Biologicals)
	10% DMSO (Sigma)
MACS Buffer	1 x PBS (University of Birmingham)
	0.5% Bovine serum albumin (Sigma)
	2mM EDTA (Sigma)
	Sterile Filtered
TBE Buffer	89nM Tris HCl (Geneflow)
	89nM Boric Acid (Geneflow)
	3mM EDTA (Geneflow)
Peptide Line Media	RPMI 1640 (Gibco BRL)
	100U/ml Penicillin (Gibco BRL)
	100 mcg/ml Streptomycin (Giboc BRL)
	2mM Glutamine (Gibco BRL)
	10% Human Serum (HD Supplies)

Ethical Approval

Ethical permission to perform this study was granted by the South Birmingham Research Ethics Committee (09/H1207/146). All samples were obtained with written, informed consent and were stored in compliance with the requirements of the Human Tissue Act (2004). Samples obtained from patients treated with allo HSCT were treated in accordance with ethics committee approval Q5/Q2070/175.

Cohort of Patients with Chronic Myeloid Leukaemia

Between September 2009 and December 2012 patients with CML were recruited from clinics around Birmingham. Initial patient recruitment, particularly of patients on second line tyrosine kinase inhibitors, was slow, and so the study was opened at other centres. Patients were recruited from University Hospital Birmingham, Birmingham Heartlands Hospital, Worcester General Hospital, University Hospital Coventry, Stafford General Hospital, Gartnavel General Hospital, Glasgow and St James University Hospital, Leeds. Serial samples were obtained from patients at University Hospital Birmingham, Birmingham Heartlands Hospital and University Hospital Coventry.

Patients recruited post allogeneic stem cell transplant

Lymphocyte subsets at 3 months post allo HSCT were analysed from a cohort of patients that underwent transplant at the Queen Elizabeth Hospital in Birmingham, for a variety of clinical indications. In order to assess outcome in patients receiving allo HSCT for AML, patient data was collected from University Hospital Birmingham, Heartlands Hospital and University Hospital London.

Sample Preparation

Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

Preparation of peripheral blood mononuclear cells was performed under sterile conditions by density gradient centrifugation. 20ml of patient blood was collected into heparinized vacutainers. Blood was first mixed with an equal volume of wash media. It was then layered onto lymphoprep media (Nycomed, Sweden), and spun at 758g for 30 minutes. The centrifuge was allowed to slow down with no brake. Layers of lymphocytes were identified and were transferred into 20ml of wash media. This was then spun at 2000rpm for 10 minutes with the brake on the centrifuge. The cell pellet was identified and the supernatant removed by pouring. After resuspension in a further 20ml of wash media, the cells were spun down at 1500rpm, and the cell pellet was again identified and the supernatant removed. The cells were then resuspended in 20ml "general media" 20 μl of cell suspension was placed onto a heamocytometer, and the cells counted. After one more spin at 1200rpm for 10 minutes, the supernatant was removed.

Cryopreservation of Peripheral Blood Mononuclear Cells

The pellets were agitated and an appropriate amount of freezing media was added. In order to calculate the amount of freezing media, the number of cells in the sample was first calculated. The cells were frozen down in cryovials (Thermo Scientific, US) at a concentration of 5-10 million cells per cryovial. 1ml of freezing media was added per cryovial required, and the cell/freezing media mixture was transferred to cryovials. It was subsequently frozen down at -80ºC in a freezing container (Nalgene, Thermo Scientific, US) to ensure cooling at 1ºC per minute.

Recovery of Cells from storage

After being defrosted in a water bath at 37C for two minutes, cells were transferred into 10ml of general media and centrifuged for 5 minutes at 300g. After another wash step, viability was confirmed by adding Trypan blue (Sigma) and counting.

Preparation of Serum

5 ml of patient blood was collected into a serum tube. This was allowed to rest for at least an hour in order to ensure adequate clotting of the samples. The straw coloured serum was removed and transferred to an appropriate cryovial with a transfer pipette. Serum samples were frozen and stored at -80ºC.

Preparation of Buffy Coats

4 ml of patient blood was collected into a vacutainer (vacuette) containing EDTA. This was spun at 2000rpm for 5 minutes. The buffy coat was identified, and removed using a transfer pipette. This was frozen at -80 and stored at -80 until use.

DNA Extraction

DNA was extracted using a QIAGEN DNA extraction kit, and the method adapted from the manufacturer's instructions. 20 μl of proteinase K was added to a 2ml microcentrifuge tube. To this, 200 μl of the buffy coat sample was added. This was mixed with 200 μl of buffer AL, and thoroughly vortexed. The mixture was then suspended in a water bath pre-heated to 56C for ten minutes. 200 mcl of ethanol was added to the sample mixture after 10 minutes. The mixture was then transferred into a DNeasy mini spin column placed in a 2 ml collection tube, and centrifuged at 8000 rpm (>= 6000 g) for 1 minute. The flow through was discarded and the spin column placed in another 2 ml collection tube. After the addition of 500 μl buffer AW1, the column and collection tube was spun at 8000 rpm (>= 6000 g) for 1 minute. The flow through and collection tube were discarded.

After placing the column in a new 2 ml collection tube, 500 μl of buffer AW2 was added and the mixture spun at 14,000 rpm (20,000 g) to dry the DNeasy membrane. The collection tube and flow through were discarded. The column was subsequently placed in a 2 ml microcentrifuge tube. 200 μl buffer AE was placed directly onto the DNeasy membrane. After incubation for 1 minute, the sample was centrifuged at 8000 rpm (>=6000 g) for 1 minute. The column was discarded. The extracted DNA sample was stored at -80C until use.

Measurement of DNA concentration

The concentration of DNA in the sample mixture was determined by UV-spectrophotometry at 260nm using the Nanodrop spectrophotometer (Thermo Scientific, US). All DNA concentration measurements were performed on a Nanodrop. The Nanodrop machine was first prepared by adding 1μl DNA-free water to the platform, and wiping the excess away. A blank sample was first added and "measure blank" performed. 1 μl of the sample was added to the platform and "measure" selected on the software. Acceptable range for purity was above 1.6 on the 260/280 ratio.

Cell Lines

K562 cell lines were used for functional analysis. They were grown in "general media" in an incubator kept at 37ºC and 5%CO_2. They were monitored by assessing them under a microscope and split when required.

Before use, they were spun down at 2000 rpm for ten minutes, resuspended in general media and used for functional analysis after counting.

Generation of CMV Virus

Ten 150 cm^2 flasks with fibroblasts (HFFF-2) at 70% confluence were used. 10ml Fibroblast growth media (fGM) was added to each bottle, and CMV virus was added at a MOI of 0.1. They were subsequently placed into an incubator at 37ºC and 5% CO_2 for 3 hours, after which the media was discarded and replaced with 20 ml of fresh fGM. Fibroblasts were examined daily for cytopathic effect (CPE), and if significant CPE seen, media was removed, the sample was spun for 10 minutes at 1500rpm to remove cell debris, and supernatant decanted into autoclaved 250ml sorvall pots which were frozen at -80ºC. 20ml of fresh media was added, and supernatant was removed after 2-3 days.

When the last harvest was complete, the previous ones were defrosted, and spun at 12,000 rpm in GSA Sorvall for 120 minutes at room temperature. Supernatant was discarded, and the residual pellet resuspended in 1ml RPMI. This was aspirated through a 21G needle, and aliquots of 100µl were frozen at -80ºC.

CMV Infection of Fibroblasts

Human fibroblast cell lines were cultured in DMEM, plus 10% FCS and penicillin/streptomycin. They were seeded into a 24 well plate at a concentration of $6*10^4$/well. They were left overnight at 37C and were checked microscopically the next day, in order to check that they had achieved 70% confluence. After removal of the supernatant, the cells were infected with the AD169 CMV strain (courtesy of Dr Annette Pachnio). Virus was added at a concentration of 500µl/well. Two wells per sample were left as uninfected controls. The plate was spun at 1500rpm for 10 minutes and then left in an incubator for 2 hours at 37C. The supernatant was then exchanged for fresh media.

CMV IgG ELISAs

CMV IgG ELISAs were performed using BIOCHECK kit BC-1089. This method is adapted from the manucfacturer's instructions. (Biocheck Inc, USA). Calibrator samples at four

known concentrations of CMV IgG were provided with the kit, as well as a low control and a high control. Serum samples were defrosted at room temperature. 1:40 Dilutions of samples, calibrators and controls were prepared by the addition of 5 mcl of sample to 200 μl of sample diluent (provided with the kit). The diluted samples were mixed well. 100 μl of the mixture was transferred to the microtiter wells, which had been pre-coated with purified CMV antigen. Bubbles in the sample were removed by tapping on the plate. The plate was incubated at 37C for 30 minutes. The liquid was subsequently removed from all the wells. The plate was rinsed and flicked 5 times with wash buffer.
100 mcl of enzyme conjugate was added to each well, and mixed gently for 10 seconds. The plate was then incubated for a further 30 minutes at 37C. The enzyme conjugate was removed from all wells, and the wells washed with wash buffer a further 5 times as described above. 100 μl of tetramethylbenzidine (TMB) reagent was added to each well, and mixed for 10 seconds. A further 15-minute incubation step followed. 1M HCl (stop solution, BioCheck Inc, USA) was added to all the wells at the end of this incubation. The samples were mixed gently for 30s, until all wells had changed colour. The plate was then read on at 450nm on a microwell reader .

Calculation of Absolute CMV IgG Titres from Standard Curve

The Calibrator samples were used to create a standard curve. The raw data was transferred to PRISM for Mac (Version 5.0c). The standard curve was transformed using the x = log(x) and y = log(y) function, generating a straight line. The line was used as the basis of transformations for the ELISA sample results, by calculating y value using interpolation from the standard curve. The calculated y values were then transformed into absolute CMV IgG titres by using the $y = 10^{y}$ function.

CMV IgM ELISAs

CMV IgM ELISAs were performed using the Biocheck kit BC 1091 (Biocheck Inc, USA). The manufacturer's instructions were followed, in a very similar fashion to the IgG ELISA as above.

Luminex Assay

A 14 plex luminex assay was performed on serum samples. A MILLIPLEX MAP kit (Merck Millipore, US) was used. The method described below was adapted from the instructions supplied with the kit. Serum samples were defrosted at room temperature prior to use. Quality control samples were reconstituted with de-ionised water. Wash buffer was diluted appropriately. Serum matrix was prepared by addition of 1 ml de-ionised water. Human cytokine standard, at concentration of 10,000 pg/ml, was prepared by reconstituting with 250 mcl of de-ionised water. Working standards were produced by serial dilution of the cytokine standard. 200 mcl of assay buffer was put into five microfuge tubes. 50 mcl of the cytokine standard was added to the first of these and serial dilutions were then carried out to give concentrations of 2,000 pg/ml, 400 pg/ml, 80 pg/ml, 16 pg/ml and 3.2 pg/ml.

All wells in the luminex plate were first washed with 200 mcl wash buffer per well. They were subsequently shaken for 10 minutes and then decanted. 25 mcl of Standard and Control samples (supplied with kit) were then added to appropriate wells. 25 mcl of assay buffer was added to background and sample wells. 25 mcl of Matrix solution (supplied with kit) was added to backgrounds, standards and control wells. 25 mcl of each serum sample was then added to a well. Subsequently, 25 mcl of the bead mixture was added to each well. The plate was incubated overnight at 4C on a shaker plate. Subsequently, the plate contents were removed and the plates washed twice. 25 mcl per

well of detection antibodies were added. The plate was sealed, covered with foil and incubated at room temperature for one hour. 25 mcl of Streptvadin-Phycoerythrin was added to each well, and the plate subsequently sealed, covered with foil and incubated at room temperature on a plate shaker for 30 minutes at room temperature. The contents of each well were removed and the plates washed twice as previously described. 150 mcl of sheath fluid was added to each well, and the beads in the well resuspended by putting plate on a plate shaker for 5 minutes. The plate was then analysed on a Luminex 100 (Luminex, US). Calibration was performed with xMAP Reporter calibration microspheres (Luminex), and Starstation Luminex software (version 3.0) was used to calculate the concentrations of each cytokine from the standard curve generated from the calibration beads.

PCR for HLA type

HLA type was required for my patients in order to assess the CMV-specific $CD8^+$ T cell response. HLA-peptide tetramers were available for HLA types HLA-A1, HLA-A2, HLA-B7, HLA-B8 and HLA-B35, and therefore only these HLA types were tested for.

The 6 primer mixes were prepared at appropriate concentrations. For primers 1-4, 0.85 mcl of sense primer was added to 0.85 mcl of antisense primer. 2.3 mcl of PCR grade water was added to this. For primer mixes 5 and 6, 2 mcl of sense primer was added to 2 mcl of anti-sense primer. The amount of DNA to add to the sample was calculated using the calculation DNA required (80 ng)/DNA concentration of patient sample = x mcl of DNA sample. The volume of PCR grade water required per patient sample was calculated using the equation 23 – x mcl DNA mixture = y mcl PCR grade water. One "master mix" stock volume was made up so as to be sufficient for the number of patient samples to be run. Master mix was composed of 40.5 mcl of TDMH, y mcl of PCR grade water, 4 mcl of sense control primer, 4 mcl of anti-sense control primer, x mcl of patient

DNA sample and 0.43 mcl of Taq polymerase. 9 mcl of "master mix" was dispensed into each PCR tube. 4 mcl of primer mix was added to each of these. The PCR tubes were subsequently run on the GeneAmp PCR System 9700 ThermalCycler.

The cycle used was 1 minute at 96C, 5 cycles of 25 seconds at 96C, 45 seconds at 70C, 45 seconds at 72C. There were then 21 cycles of 25 seconds at 96C, 50 seconds at 65C, 45 seconds at 72C, followed by 4 cycles of 25 seconds at 96C, 60 seconds at 55C and 120 seconds at 72C.

Gel Electrophoresis

A 1% Agarose gel was prepared, by dissolving 1 g of agarose (Bioline, US) in 100 mls of TBE. Boiling it in a microwave dissolved the agarose. After cooling, ethidium bromide (Sigma) was added to the solution at 1 mcl per 20 ml of solution. The liquid agarose solution was poured into a gel tray with a gel comb placed in it, and allowed to cool and set for 45 minutes. After setting and removal of the comb, the tank was filled with TBE buffer until the gel was submerged. The PCR amplified samples were then loaded into the wells. 4 mcl of hyperladder IV (Bioine) was added to the first well. The gel was then run at 120 volts for one hour. The gel was photographed using DC290 capture software, on the UV gel reader (Kodak TVC312A).

Quantitative PCR for EBV Genome Load

Primers to detect the EBV POL (BALF5) gene were used (see Appendix). The β-2microglobulin gene was used as endogenous control gene. DNA standards were prepared by dilutions of Namalwa BL, which then had DNA isolated as described above. The sample was then adjusted to a final concentration of 132 ng/mcl, corresponding to 40,000 EBV copies/mcl. Serial dilutions of this stock were made. The primers were resuspended at 100 mcM in PCR grade water. Taqman probes were resuspended at 50

mcM. They were then diluted to appropriate working concentrations. PCR mastermix was prepared, containing 12.5 mcl Taqman Universal 2 x mastermix, 2.5 mcl (2 mcM) POL forward primer, 2.5 mcl (2 mcM) POL reverse primer, 1 mcl (5 mcM) POL probe, 0.5 mcl (3 mcM) B2m forward primer, 0.5 mcl (4 mcM) B2m reverse primer and 0.5 mcl (5 mcM) B2m probe.

A 96 well PCR plate was then prepared: 20 mcl of the PCR mastermix was pipetted into each well, plus 5 mcl of sample (either water, standard, or test DNA). The plate was then loaded onto an ABI7500 PCR machine. The data was analysed using 7500 system v1.4 software.

Quantitative PCR for CMV Viral load

The CMV UL132 gene was amplified using the applied biosciences Gene Expression Assay (Pa03453400_s1, Applied Biosciences), according to the manufacturer's instructions. 10-fold dilutions of DNA extracted from cultured CMV (strain AD169) were prepared; these were used to make a standard curve in every assay performed. In each assay, two positive controls from patients with known viraemia, two negative controls that were CMV IgG seronegative and two water controls were used. The PE-ABI 7500 sequence detection system was used. Amplification was carried out over 40 cycles.

Flow cytometry

Flow cytometry was conducted on an LSR II flow cytometer (BD Biosciences). The instrument was set up with a blue laser (488 nm), violet laser (405 nm) and a red laser (633 nm). Filters were as follows: 488nm; 695/40, 610/20, 530/30, 780/60, 660/20, 575/26, 480/10. 450 nm; 605/12, 560/20, 450/50, 655/8., 585/15, 525/50. 633 nm; 780/60, 660/20, 730/45

Compensation and Acquisition

The LSR II was calibrated using cytometer setup and tracking beads (BD Biosciences). Single colour compensation control beads (BD Biosciences) were included for each experiment and offline automated compensation was used. Negative and positive fluorescence was obtained separately for each single colour compensation.

The following antibody/flurochromes were used.

Antigen	Fluorochrome	Company	Catalogue number
Streptavadin	APC	InVitrogen	SA1005
CD107a	FITC	BD-Pharmingen	555800
CD3	APC	InVitrogen	MHCD0305
Pan γδ	PE	Beckman-Coulter	IM1418U
CD19	FITC	BD-Pharmingen	535412
CD4	Percp5.5	eBioscience	45-0049.41
CD45RA	AF700	Biolegend	304120
IL17A	Pacific Blue	Biolegend	512312
IFNγ	AF700	Biolegend	502520
Vδ1	FITC	Thermo Scientific	TCR2730
Panγδ	PE	BD Biosciences	333141
CD28	Percp5.5	eBioscience	45-0289.42
TNF	PeCy7	BD Biosciences	557647
Goat-anti-mouse IgG	PE	Invitrogen	P852
D2	FITC	Beckman Coulter	PN 1464
CD16	PeCy7	BioLegend	302016
CD38	PeCy7	Biolegend	303516
CD56	Texas Red	Invitrogen	MHCD5617
Tcr Vδ2	PE	Biolegend	331408
CD27	APC-780	Ebioscience	47-0279
CCR7	FITC	R+D Systems	FAB197F
CD45RO	ECD	Beckman Coulter	?52
CD8	AmCyan	BD Biosciences	24561
CD4	PeCy7	Ebiosciences	25.0049.42

Table 2.1 List of antibodies/fluorochromes used for flow cytometric analysis

Gating Strategy

In all experiments, unstained controls and single antibody stained controls were used to define positive and negative populations. Two antibody panels were used in all patient samples in both the TKI-treated cohort and the patients post allo HSCT. A "dump channel" was created, and dead cells were excluded. Cells were subsequently gated according to expression of CD3 versus CD19. CD3, CD19 dual negative cells were gated

according to expression of CD56 and CD16 in order to measure the $CD56^{bright}$ and $CD56^{dim}$ NK cell populations.

CD3 positive cells were selected and subpopulations gated around according to expression of CD8, CD4 and γδ TCR. These populations were selected and subpopulations measured according to expression of CD45RA and CD27 as detailed in the introduction, in order to measure memory subsets of naïve, effector memory (EM), central memory (CM) and revertant effector memory (TEMRA) subsets.

A second panel was also used to measure subsets of γδ T cells. The "dump channel" and strategy for gating CD3 and CD19 cells was identical to the previous panel. CD3 positive cells were selected and subpopulations selected according to the expression of Vδ1, Vδ2 and Vγ9. Different γδ T cell populations were measured as described above based upon their expression of CD27 and CD45RA.

Panel	Antibody	Fluorochrome
1	CD3	APC
	CD4	Percp5.5
	CD8	Amcyan
	Pan γδ	PE
	CD16	PECy7
	CD56	Texas Red
	CD27	APC-780
	CD45RA	AF700
	CD19	FITC
2	CD3	APC
	Vδ1	FITC
	Vδ2	PE
	Vγ9	PECy7
	CD27	APC780
	CD45RA	AF700

Table 2.2 Antibodies used in panels for flow cytometric analysis of lymphocyte subsets

Measurement of CMV-Specific T cell responses using MHC-peptide Tetramers

Due to negative selection in the thymus, TCRs have been selected to bind self-peptide/MHC complexes weakly. It is therefore challenging to identify antigen-specific T cell responses due to the short interaction time. MHC-peptide multimers were developed to solve this issue (Altman et al., 1996). Multimers increase the probability of successfully identifying antigen specific T cells, as the probability of all of the multimers dissociating from the TCR at the same time is low. The overall avidity exceeds the combined avidities (Laugel et al., 2005), which allows sufficient time to identify antigen specific T cells for surface staining. The most common peptide-MHC multimer is a tetramer.

Manufacture of peptide-MHC tetramers

Class I HLA peptide tetramers were synthesized according to published methods (Altman et al., 1996). HLA-A heavy chain and B2M proteins were refolded around the CMV peptides in the table below

Peptide	HLA restriction	CMV peptide
YSEHPTFTSQY	HLA-A1	pp65
VTEHDTLLY	HLA-A1	pp50
NLVPMVATV	HLA-A2	pp65
VLEETSVML	HLA-A2	IE-1
TPRVTGGGAM	HLA-B7	pp65
RPHERNGFTVL	HLA-B7	pp65
ELKRKMIYM	HLA-B8	IE-1
QIKVRVDMV	HLA-B8	IE-1
ELRRKMMYM	HLA-B8	IE-1

Table 2.3 CMV peptides used in manufacture of HLA-peptide tetramers

During the refolding process they were continually stirred at 4C in a refold buffer containing 400mM L-Arginine, 100mM Tris.HCL pH 8.0, 2mM EDTA, reduced

glutathiones, oxidized glutathiones and 0.1mM PMSF. 30mg heavy chain, 5mg peptide and 10mg β2M were used for the 500ml refold. The refold solution was subsequently concentrated using a miniplate device (Amicon "Stir cell" model 8400) with a Millipore 10,000 MW filter and buffer exchanged using PD-10 Sephadex G-25M desalting columns (Amersham pharmacia, UK) into the biotinylation reaction buffer (100mM Tris.HCL pH 7.5, 5mM $MgCl_2$, 20mM NaCl, 0.1 mM PMSF). Biotynilation was carried out overnight at room temperature in a volume of 2ml containing 5mM ATP, 5mM D-biotin (Sigma) and BirA enzyme. Complexes were purified by fast protein liquid chromatography (FPLC) using gel filtration and ion exchange columns (Amersham Pharmacia, UK).

Protein concentration of the refold was then calculated, by calculating a standard curve with known concentrations of BSA in triplicate alongside the refolded proteins. 100mcl of Bio-Rad protein assay reagent (Bio-Rad) was added to each well. They were left at room temperature for 10 minutes and absorbances were read at 595mM with a platereader. The standard curve was used to calcualate the protein concentration of the refolded proteins. Biotinylation was confirmed with a biotinylation ELISA. Serial dilutions of the refold were added to a 96 well plate, and left for 1 hour at 37C. The plate was washed with PBS containing 0.05% Tween 20 (Sigma, UK). 100 mcl of a 1/1000 dilution of extravidin-peroxidase conjugate (Sigma, UK) in PBS with 0.1% BSA was added to each well; this only bound biotynilated proteins. After incubation at room temperature for 15 minutes the plate was washed in PBS with 0.05% Tween 20 (Sigma, UK). 100 mcl of TMB substrate solution (3,3'-5.5'-tetramethylbenzidine liquid substrate system, Sigma, UK) was added to each well. Blue reaction indicated the presence of biotynilated proteins, which were subsequently stored at -80C until use. Prior to use, tetrameric complexes were made by the addition of APC-conjugated streptavidin (Invitrogen), and subsequently kept at 4C in the dark until use.

HLA-peptide Tetramer Staining

Defrosted cells were washed and resuspended in 100 mcl of PBS. 1 mcl of the tetramer was added and the cells were incubated at 37C for 15 minutes. They were then washed in MACS buffer and surface stained with antibodies as described elsewhere.

MACS Sorting of γδ T cells

γδ T cells were sorted using a negative selection kit (Miltenyi biotech, Germany) Briefly 10^7 fresh cells were suspended in 100mcl MACS buffer. 20 mcl of primary antibody mix was added to the cell suspension and this was incubated in the dark at 4C for 10 minutes. The cells were subsequently washed and again resuspended in 100 mcl MACS buffer. 20 mcl of anti-biotin microbeads were added and the mixture incubated at 4C in the dark for 15 minutes. Cells were washed, and subsequently resuspended in 500 mcl MACS buffer. A MS column (Miltenyi Biotech, Germany) was placed in a MACS separator (Miltenyi Biotech, Germany). The column was first rinsed through with MACS buffer, and the cell mixture was subsequently run through the column. The unlabeled fraction was collected in a MACS tube. The column was then washed through by adding buffer three times.

Functional Analysis of γδ T cells

Sorted γδ T cells were plated out in a 96-well plate, in RPMI media plus BSA, at 100,000 cells per well. One well was left unstimulated. The positive control for each sample was with PMA at 25ng/ml and ionomycin at 400ng/ml (Sigma). K562 cells were suspended in GM media, and added at a effector:target ratio of 1:1.

Anti-CD107a (BD-Pharmingen) was added at 20mcl/well. The wells were mixed well and left for 30 minutes. Monensin (Sigma) was then added to each well at a

concentration of 1μM well (Maecker and Maino, 2004). Cells were subsequently incubated in the dark at 37C for 4-6 hours.

Cells were subsequently washed, resuspended in 100 mcl MACS buffer and stained with surface antibodies anti-CD3, anti Vδ2 and anti Vδ1. Cells were then washed, fixed and permeabilised as described elsewhere, and stained with the intracellular antibodies anti-IFNγ, anti-TNFα and anti-IL17.

Measurement of CD8 T cell Responses to CMV Peptides

Freshly separated cells were suspended in 500 μl general media (GM). Peptide mixes were added to each tube at 30 mcl in each 500 μl tube. The composition of the peptide mixes is described in Table 2.3. A positive control well was set up with the addition of PMA at 25ng/ml and ionomycin at 400ng/ml. One well for each sample was left unstimulated. Ionomycin is an ionophore produced by *Streptomyces congloblatus,* which raises the level of intracellular Ca+, and causes cells to secrete cytokines (Abramov and Duchen, 2003). PMA (phorbol 12-myristate 13-acetate) is a diester of phorbol and a potent activator of protein kinase C. Brefaldin A (Sigma) was used in the media in order to keep the cytokines intracellular. It inhibits the transport of proteins from the endoplasmic reticulum to the Golgi apparatus and induces retrograde transport from the Golgi to the Endoplasmic Reticulum, leading to protein accumulation within the endoplasmic reticulum. The mixtures were left at 37C for 6 hours, after which they were washed, and resuspended in MACS buffer. Surface staining for anti-CD3, CD4 and CD8 was performed as described elsewhere. Wash steps were subsequently performed, and intracellular staining was performed on each sample as described elsewhere. Anti-IFNγ and anti-TNFα antibodies were added to each tube after fix/permeabilisation steps had been carried out.

Table 2.4: List of Peptides used in CMV peptide mixes

Mix 1: IE-1

Peptide sequence	HLA restriction	CD4/CD8	Elispot pool
ATTFLQTMLRK	A68	CD8	**2**
KEVNSQLSL	B40	CD8	**2**
QIKVRVDMV	B8	CD8	**4**
DELRRKMMY	B44	CD8	**8**
ELRRKMMYM	B8	CD8	**8**
ELKRKMIYM	B8	CD8	
KRKMMYMCY	B27	CD8	
FPKTTNGCSQA	B55	CD8	**9**
CVETMCNEY	A1/B18	CD8	**12**
CRVLCCYVL	B7	CD8	**13**
YVLEETSVML	A2 (contains 2 epitopes: YVL & VLE)	CD8	**13**
RRIEEICMK	B27	CD8	**14**
EEAIVAYTL	B44	CD8	**16**
VLEETSVML	**A2**	**CD8**	**13**

Mix 2 : pp65

Peptide sequence	HLA restriction	CD4/CD8	Elispot pool
DTPVLPHETR	A68	CD8	**2**
QPSLILVSQY	B35	CD8	**3**
YTPDSTPCHR	A68	CD8	**3**
CPSQEPMSIY	B35	CD8	
VYALPLKML	A24	CD8	**4**
IPSINVHHY	B35	CD8	**5**
FVFPTKDVALR	A68	CD8	**7**
FPTKDVAL	B35	CD8	**7**
QYVKVYLESF	A24	CD8	**9**
RPHERNGFTVL	B7	CD8	**11**
QAIRETVELR	B35	CD8	**13**
QYDPVAALF	A24	CD8	**14**
YSEHPTFTSQY (HPTFTSQY)	A1 (also spans a B35 epitope)	CD8	**14, 15**
TPRVTGGGAM	B7	CD8	**17**
NLVPMVATV	A2	CD8	**20**
RIFAELEGV	A2	CD8	**21**
PDVYYTSAFVFP	DR7	CD4	**7**
IIKPGKISHIMLDVA	DR4	CD4	**11**
PQYSEHPTFTSQYRI	DR11	CD4	**14, 15**
FTSQYRIQGKLEYRHT	DR11	CD4	**15**
AGILARNLVPMVATV	DR ?	CD4	**20**
KYQEFFWDANDIYRI	**DR52**	**CD4**	**20**

Mix 3

Sequence	HLA restriction (and protein)	CD4/CD8
VTEHDTLLY	A1 (pp50)	CD8
DYSNTHSTRYV	DR7 (gB)	CD4
VFETSGGLVVFWQGI	DR7 (gB)	CD4
CMLTITTARSKYPYH	DR4 (gB)	CD4
HELLVLVKKAQL	**DR11 (gH)**	**CD4**

Intracellular staining protocol

Cells were suspended in 100 mcl MACS buffer and surface antibodies added at the required concentration. After incubation at 4C they were washed in MACS buffer and resuspended. 4% paraformaldehyde was added to each tube, and cells were left at room temperature in the dark for 15 minutes. After a further wash step in MACS buffer, 100mcl of saponin 0.5% was added to each tube. The tubes were incubated at room temperature in the dark for 10 minutes, and then intracellulat antibodies added at the required concentration. Cells were then washed again in MACS buffer, resuspended in 200 mcl MACS buffer and then run on the flow cytometer.

FACS Sorting of γδ T cells

The lymphocytes were gated according to forward/side scatter. A "dump channel" was created, and cells expressing CD19, CD11 and dead cells were excluded. They were sorted according to expression of Vδ1, Vδ2 and Vγ9, into Vδ2Vγ9, Vδ1Vγ9 and Vδ1nonγ9 subsets. A minimum of 5000 cells were sorted into 1.5 ml microtubules (Sarstedt AG and Co., Germany) containing 100 mcl RNAlater (Applied Biosystems by Life Technologies, USA).

T cell Receptor Repertoire Analysis

Analysis was performed on sorted γδ T cells, sorted using the protocol above. This work was performed in collaboration with David Price and Kristin Ladell in Cardiff University. The method is adapted from that published by Quiqley et al (Quigley et al., 2011).

Isolation of mRNA from sorted T cells

Purification of mRNA was performed with the Oligotex Direct mRNA Mini kit (Qiagen), with small deviations from the manufacturer's instructions (see Appendix). Contaminants are removed by washing with ethanolic wash buffer NT3, and pure DNA is eluted under low ionic strength conditions with the alkaline buffer NE (5mM Tris CL, pH 8.5).

cDNA was then manufactured using a SMARTer PCR cDNA Synthesis kit (Clontech), (see Appendix)

PCR Amplification of Rearranged TCR Products

Touchdown PCR was performed on the cDNA using TCR-specific and anchor-complementary primers. A PCR mastermix was first prepared. This contained: 5 mcl of 10*PCR buffer, 5 mcl of 10*UPM, 1 mcl of MBC2 primer, 1 mcl of dNTP mix, 7 to 13 mcl of cDNA, 1 mcl of AdvanTaq2 and water to make the total volume 50 mcl. The cDNA template was mixed with the mastermix in a 0.2 ml PCR tube, and this was centrifuged at 14,000 rpm for 30 seconds, 18 to 25C. The PCR reactions were run in a thermal cycler with the following conditions:

1 cycle at 95C for 30 seconds
5 cycles at 95C for 5 seconds, followed by 2 minutes at 72C
5 cycles at 95C for 5 seconds, followed by 10 seconds at 70C and 2 minutes at 27C
30 cycles at 95C for 5 seconds, followed by 10 seconds at 68C and 2 minutes at 72C.

The entire product was run on a 1% agarose gel made with TAE buffer, with an empty well between each sample to minimize the chance of cross-contamination. The gel was then stained with SYBR Gold and visualized with a low intensity UV light. The band of

interest was then carefully excised using a scalpel. The amplified TCR products were extracted from the gel and cleaned up for sub-cloning.

Amplicons were subsequently ligated into plasmids designed to capture adenine overhangs for sub cloning and sequencing.

The following samples were added to a 1.5 ml microcentrifuge tube: 5 mcl extracted DNA product, 1 mcl pGEM-T Easy Vector, 1.5 mcl T4 ligase, and 7.2 mcl 2* buffer 1. A negative control tube was set up, which was identical apart from the addition of 5 mcl of water in place of the DNA. A positive control was created with 2 mcl of control insert and 3 mcl of water in place of the DNA. This mixture was vortexed and spun briefly at 14,000 rpm for 30 seconds at room temperature. The mixture was incubated overnight at 4C.

Vector Transformation into Competent *E Coli*

The pGEM-T Easy vector contains an ampilillin resistant gene. Only transformed bacterial colonies will grow on agar plates in the presence of ampicillin. 7.5 mcl of each ligation reaction, including positive and negative controls, was pipetted into separate 1.5 ml microcentrifuge tubes. *E. Coli* cells were thawed gently on ice. 50 mcl was then pipetted into each tube, and the mixture was incubated on ice for 30 minutes to allow the DNA to adhere to the bacteria. The *E Coli* cells were then heat-shocked by placing in a 42C heat block for 50 seconds. The samples were then placed on ice for 2 minutes. 950 mcl of SOC medium (Invitrogen) was then added to each tube, and the samples were incubated at 37C in a thermomixer at 750 rpm for 2 hours.

200 mcl of the bacterial culture was transferred to a labeled LB plate on a plate spinner. The glass spreader was flamed in ethanol and allowed to cool for a few seconds, after

which the bacteria were spread evenly over the plate. The LB plates were incubated overnight at 37C.

Colony PCR and Sequencing

Single bacterial colony plasmid inserts were amplified in a 96-well plate using primers flanking the insertion site.

The PCR mastermix was first prepared in a 15 ml polypropylene tube: 250 mcl of 10* HiFi buffer (Invitrogen) was added to 100 mcl of $MgSO_4$, 50 mcl of dNTPs, 100 mcl of M13F primer, 100 mcl of M13R primer, 14 mcl of HiFi *Taq* and 1886 mcl of water. 25 mcl of mastermix was pipetted into each well of a 96 well plate. The restreak plate was prepared and labeled: a 96-well grid was drawn onto a LB plate and labeled with date and sample identification. The PCR place was placed on ice. Individual white colonies were harvested by spearing with a sterile toothpick. A gentle imprint was made on the restreak plate, after which the toothpick was placed in the first well of the PCR plate and twirled around to dislodge the bacteria. This was continued with separate colonies for each of the remaining wells. A dark blue colony (plasmid without insert) was used as a control colony for the remaining well. The restreak plate was placed in an incubator at 37C, then stored at 4C. Snap-cap lids were placed on the PCR plate, and the plate sealed with a cap installing tool. The PCR plate was briefly vortexed and centrifuged at 140 g, room temperature, for 30 seconds. The PCR plate was then run in a thermal cycler as follows:

5 minutes at 95C
35 cycles of 30 seconds at 95C, followed by 30 seconds at 57C and 90 seconds at 68C.
The sample was held at 4C at the end of the cycle.

The PCR products were then prepared for sequencing. The plate was removed from the thermal cycler and centrifuged for 1 minute at 140 g, room temperature. 25 mcl of DNase/RNase free water was added to each well. 25 mcl of the diluted PCR product was then transferred to a new 96-well plate. Plates were stored at -20C until ready for sequence.

5 mcl of several individual products from the back-up plate were run on an agarose gel to assess the efficiency of amplification. If colony PCR failed, then a new PCR was performed using colonies from the restreak plate. One plate per sorted T cell population was sent for Sanger sequencing in a high-throughput facility.

Statistical Analysis

Graphpad Prism version 5 for Mac OS X (Graphpad) was used for illustrations and univariate analysis. SPSS version 20 for Mac (IBM) was used for multivariate analyses, as well as survival analysis and Cox regression.

Mann-Whitney tests were used when proportional values were compared, t-tests when absolute values were compared. A general linear model (SPSS) was used to test significance when there was greater than one fixed factor that was being compared between subgroups.

Chapter 3: The effect of Cytomegalovirus on Immune Profiles in Imatinib-treated Patients

Introduction

Cytomegalovirus is a beta herpes virus that causes chronic infection in infected individuals, and infects up to 70% of the population. It is a highly immunodominant virus, and is known to dramatically alter the proportions and phenotype of CD8 T cells and, to a lesser extent, CD4 T cells (Chidrawar et al., 2009)..

CMV infection has been shown to increase the relative proportion of CD8 T cells, which express a terminally differentiated phenotype, expressed as CD45RA+ CCR7- and CD57+ (Pawelec et al., 2009) . This is believed to be due to accumulation of CMV-specific memory cells.

TKIs have become the standard of care for CML since the IRIS study demonstrated markedly improved outcomes compared to IFN- α , the previous standard therapy (O'Brien et al., 2003). Patients with CML can now be expected to live healthy lives for many years, taking TKI therapy to keep their disease in remission.

Imatinib causes dose-dependent haematological effects, including neutropenia and thrombocytopenia (Druker et al., 2001, Sneed et al., 2004). Hypogammaglobulinaemia and lymphopenia have been noted to occur in the course of imatinib treatment (Steegmann et al., 2003)

It is known that imatinib is capable of inhibiting TCR mediated activation of T cells in vitro (Seggewiss et al., 2005) including antigen-driven proliferation of T cells responsive to immune-dominant CMV and EBV peptides (NLV and GLC peptides used in A2 individuals), at concentrations of imatinib higher than is achieved in vivo.

More recently, there has been particular interest in the changes in lymphocyte subsets seen in the peripheral blood of patients treated with dasatinib, with a suggestion of improved outcomes in these patients (Mustjoki et al., 2009). It is possible that some of these expansions are associated with CMV seropositivity (Kreutzman et al., 2011a). However, there has currently been no detailed analysis of the effect of imatinib on the CMV-specific immune response during therapy.

In this chapter I will describe the effects of CMV serostatus on lymphocyte subsets within patients on imatinib, assess the changes of these subsets over time whilst on therapy and the potential alteration in CMV-specific T cell responses whilst on imatinib. I will go on to assess whether any of these alterations in lymphoid profile could alter the response to therapy.

Panel of Antibodies

Lymphocyte subsets were measured from patients recruited at participating centres. All flow cytometry experiments were performed from cryogenically stored samples, as described in the methods section. All of the patients that had their lymphocyte subsets measured had been established on treatment and were in at least a haematological remission. A panel comprising CD3, CD4, CD8, CD56, CD16, pan γδ, CD45RA and CD27, and CD19 was initially used to measure lymphocyte subsets.

The pattern of changes in lymphocyte subsets following initiation of TKI treatment

Twelve imatinib treated patients had samples taken at repeat timepoints at least one year apart. Within these patients we were able to follow the proportions of different lymphocyte subsets over time, to assess if there were cumulative changes to the immune profile associated with imatinib treatment.

Initially, I set out to assess the pattern of lymphocyte changes after initiation of imatinib therapy. Samples were available that allowed me to measure lymphocyte subsets within the first two weeks of commencing treatment with imatinib and then a year later. Paired samples were compared from each patient, comparing the early sample with the later one. Paired t-tests were used for analysis.

The overall lymphocyte count did not change (Figure 3.1a). The median numbers of T cells, both of CD8 (Figure 3.1b) and CD4 (Figure 3.1c), as well as Vδ1 γδ T cells (Figure 3.1d) subsets were increased over time, although these changes were not statistically

significant. The Vδ2 γδ T cell numbers were 2.75 times greater in the late sample (p = 0.02)(Figure 3.1e). The median counts are illustrated in Table 3.1.

Subset	Early	Late	p value
Lymphocyte count	1.85	2.15	0.21
CD4 count	0.61	0.81	0.15
CD8 count	0.29	0.54	0.13
Vδ1 γδ T cell	0.013	0.0089	0.50
Vδ2 γδ T cell	0.016	0.044	0.02

Table 3.1 Comparison of lymphocyte subsets over time in imatinib-treated patients (all $*10^9/L$)

Measurement of major memory subsets (naïve, central memory, effector memory and revertant memory, defined by CD45RA and CD27 expression) of CD8 T cells showed no changes over time, with no accumulation of more differentiated CD8 T cells, in either proportion or absolute number (Figures 3.1f).

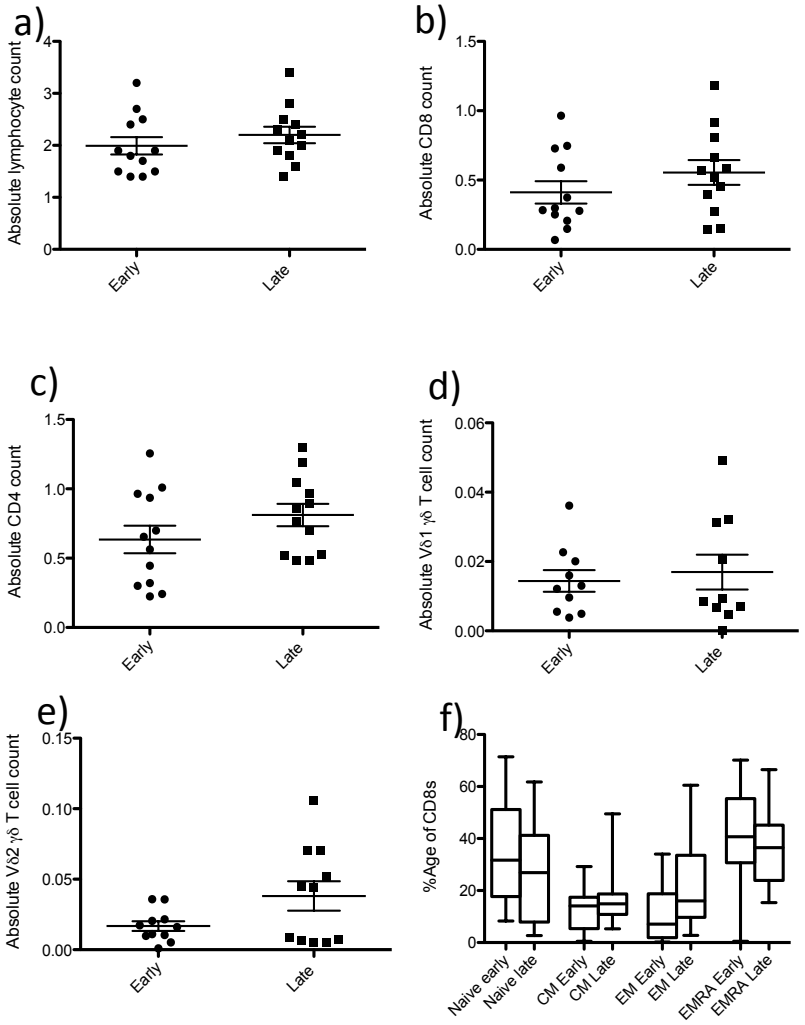


Figure 3.1*: *A Comparison of lymphocyte subsets from patients on imatinib over time

Comparing early (n = 12) post treatment lymphocytes to those taken 1 year later (n = 12), there was no significant difference in a) absolute lymphocyte count, b)absolute CD8 count, c) absolute CD4 count or d) absolute vδ1 γδ T cell count. e) The Vδ2 γδ T cell count was 2.8 times higher in the late sample (p = 0.02). There was no difference seen in f) the proportions of naïve, central memory, effector memory or EMRA CD8 T cells.

The effect of CMV serostatus on lymphocyte subsets within patients on Imatinib

CMV serology was assessed by IgG ELISA, as described in methods. This assay was performed on at least two occasions, and patients were defined as CMV seropositive or seronegative according to the calibration samples provided. A quantitative measurement of CMV IgG was calculated as described.

Only one patient had a borderline CMV serostatus (QE07). This was retested five times, and a mean IU/ml calculated all of the recalculations were consistent with CMV seropositivity. The median calculated titre in the CMV seropositive patients was 16.84 IU/ml (range 2.012 to 21.3).

The association of CMV serostatus with lymphocyte count and response to therapy

There were 37 (69.8%) CMV seropositive patients, and 16 (30.2%) CMV seronegative imatinib treated patients. The median age of the CMV seronegative group was 61 years old (95% CI 53.6 to 71.2), and of the CMV seropositive group was 67 years old (95% CI 60.4 to 70.5).

The median lymphocyte count in the CMV negative patients was $1.51*10^9$/l versus $1.50*10^9$/l in the CMV seropositive patients ($p = 0.85$, ns).

Where the molecular response was known, 7/13 (53%) CMV seronegative patients had achieved a major molecular response compared to 21/33 (64%) CMV seropositive patients (ns).

CD4:8 ratios in CMV seropositive *versus* seronegative patients

The CD4 and CD8 levels were measured by gating around the lymphocyte population on forward and side scatter, then gating around the live CD3 positive fraction, and determining the subpopulation of CD4 and CD8 T cells. The CD4:8 ratio was calculated from these values.

The CD4:8 ratio was significantly lower in CMV seropositive patients; the mean value in CMV negative patients was 5.27 compared to 2.03 in CMV seropositive patients ($p<0.01$ by Mann Whitney) (Figure 3.2a.); CD8 T cells made up 22% of the T cells in CMV-seropositive patients versus 11% in CMV seronegative patients($p = 0.0007$, Figure 3.2b). The CD4 count was relatively decreased ($p = 0.0059$), from 53% to 40% (Figure 3.2c).

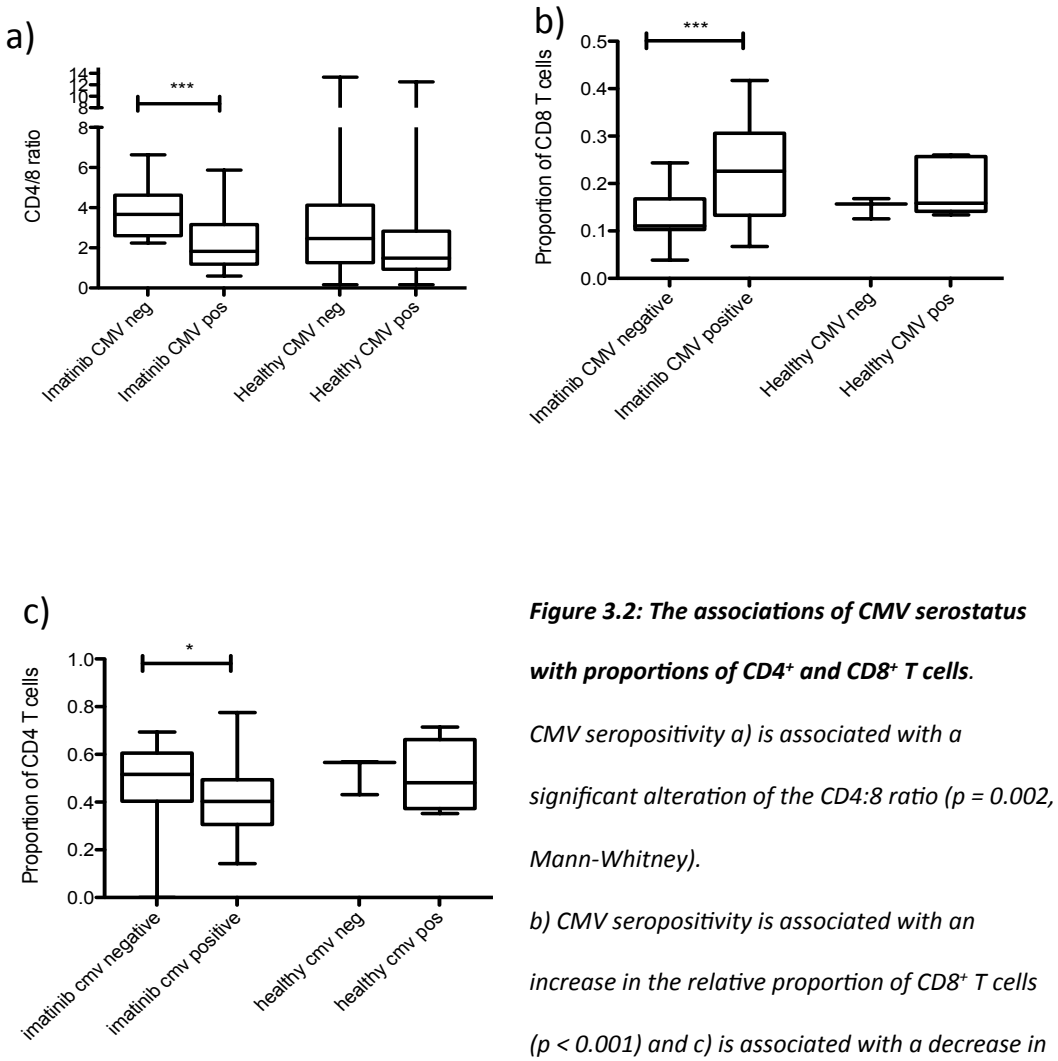


Figure 3.2: The associations of CMV serostatus with proportions of CD4⁺ and CD8⁺ T cells.

CMV seropositivity a) is associated with a significant alteration of the CD4:8 ratio (p = 0.002, Mann-Whitney).

b) CMV seropositivity is associated with an increase in the relative proportion of CD8⁺ T cells (p < 0.001) and c) is associated with a decrease in the proportion of CD4⁺ T cells (p = 0.006)

CD4 and CD8 numbers

The relative proportion of CD8 and CD4 T cells was then calculated as a proportion of the overall lymphocyte population. The absolute number of each subset was then calculated by multiplying the proportion of CD4/CD8 T cell by the absolute lymphocyte count. There was no difference in the absolute lymphocyte count between CMV seropositive and seronegative patients (Figure 3.3a)

CMV seropositivity was associated with a significantly higher absolute $CD8^+$ T cell count compared to CMV seronegative patients (median 0.17 vs 0.30, p=0.022, t test). The range of CD8 T cell numbers varied markedly from individual to individual. Within the CMV seronegative group, the range was from $0.084*10^9$/l to $0.40*10^9$/l. Within the CMV seropositive group, the absolute CD8 count ranged from $0.036*10^9$/l to $0.91*10^9$/l (Figure 3.3b and Table 3.2).

	Minimum	**25th percentile**	**Median**	**75th Percentile**	**Maximum**
CMV -ve	0.084	0.14	0.17	0.29	0.40
CMV +ve	0.036	0.21	0.30	0.52	0.91

Table 3.2 CD8 T cell numbers in CMV seropositive versus CMV seronegative patients

Higher $CD4^+$ T cell numbers were observed in the CMV seronegative group ($0.79 *10^9$/l vs 0.68, $*10^9$/l p=0.01 by t test), Figure 3.3c and Table 3.3.

	Minimum	**25th percentile**	**Median**	**75th Percentile**	**Maximum**
CMV -ve	0.41	0.70	0.79	1.13	1.41
CMV +ve	0.15	0.40	0.66	0.86	1.10

Table 3.3 $CD4^+$ T cell numbers in CMV seropositive versus CMV seronegative patients($*10^9$/L)

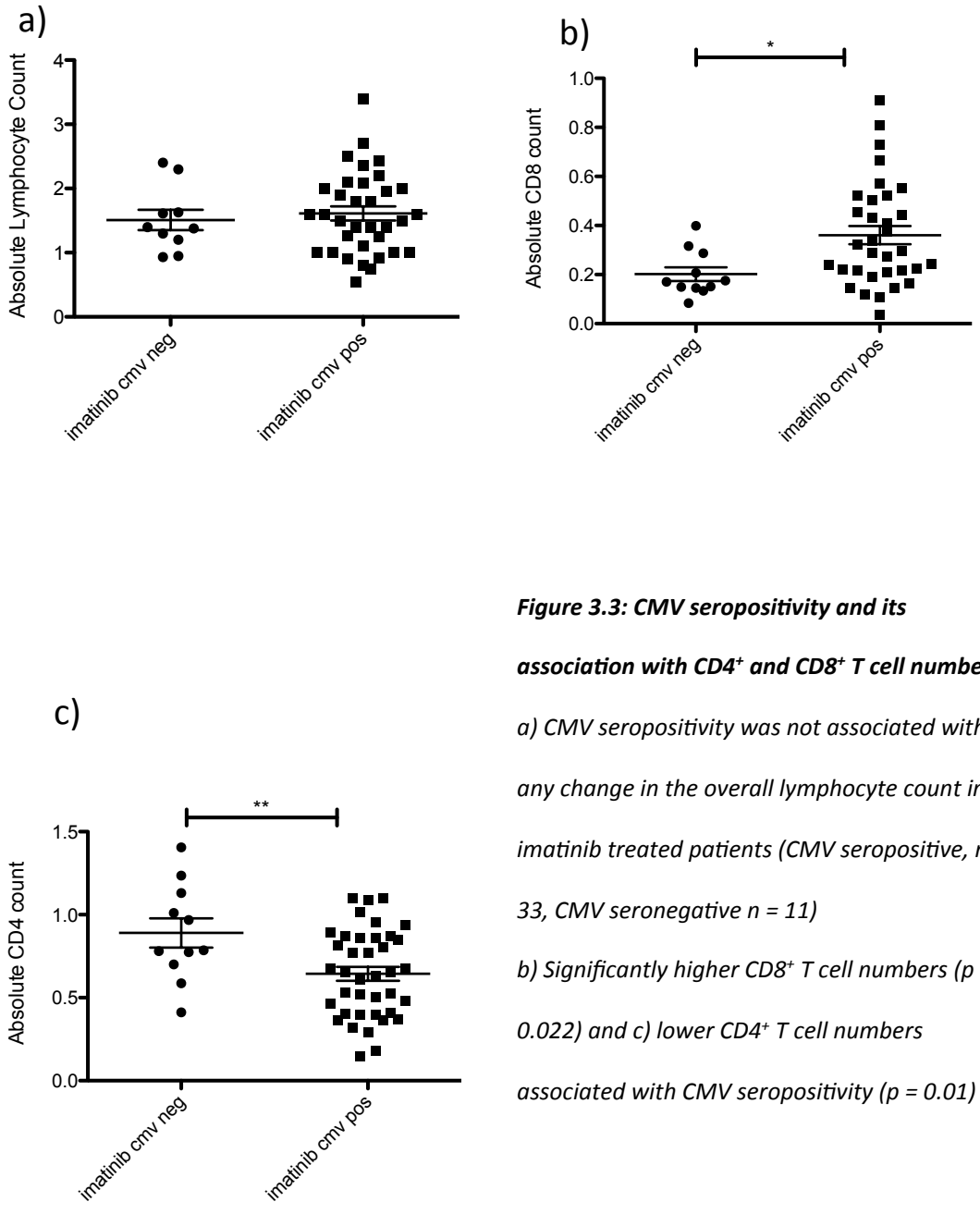


Figure 3.3: CMV seropositivity and its association with CD4⁺ and CD8⁺ T cell numbers

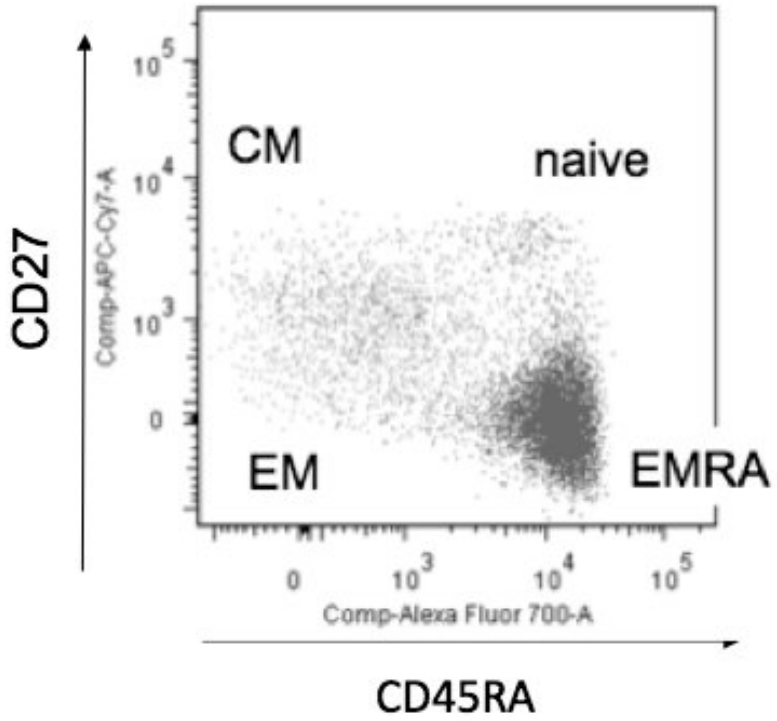
a) CMV seropositivity was not associated with any change in the overall lymphocyte count in imatinib treated patients (CMV seropositive, n = 33, CMV seronegative n = 11)

b) Significantly higher CD8⁺ T cell numbers (p = 0.022) and c) lower CD4⁺ T cell numbers associated with CMV seropositivity (p = 0.01)

The Effect of CMV serostatus on CD8 T cell memory subsets

Chronic infection with CMV is thought to cause an expansion of terminally differentiated CD8 T cells, which accumulate with age (Chidrawar et al., 2009). I therefore measure the memory phenotype of CD8 T cells in imatinib treated patients, comparing CMV seropositive with CMV seronegative patients.

Memory subsets were defined using the surface markers CD27 and CD45RA, as shown in the figure below. Briefly, CD8 T cells were defined as naïve if they expressed CD27 and CD45RA. Central memory (CM) cells were defined as CD27+, CD45RA-. Effector memory (EM) cells were defined as CD27-, CD45RA-. Terminally differentiated CD8 T cells (TEMRA cells) were defined as CD27-, CD45RA+ T cells. In order to maintain consistency, the gating strategy was kept consistent between samples.



CMV Seropositivity is associated with decrease in the proportion of CD45RA+ CD27+naïve CD8 T cells and an increase in CD45RA+ CD27- TEMRA T cells

CMV seropositivity has a dramatic effect on the memory phenotype of the CD8 T cells (Figure 3.4a). The proportion of CD45RA+ CD27+ naïve CD8 T cells is significantly decreased ($p=0.0006$, Mann-Whitney), whereas the proportion of CD45RA+ CD27- TEMRA CD8 T cells is increased ($p<0.0001$, Mann-Whitney). The proportion of CD27+ CD45RA- CM CD8 T cells is also decreased in CMV seropositive patients ($p=0.0041$, Mann-Whitney). The proportion of CD27- CD45RA- EM CD8 T cells is not significantly altered by CMV serostatus ($p=0.1748$, Mann-Whitney)

The absolute increase in CD8 T cells in CMV seropositive patients is mainly due to an increase in CD45RA+ CD27- EMRA CD8 T cells

Having demonstrated an increase in absolute CD8 numbers and a skewing of the memory phenotype of these cells in favour of CD45RA+ CD27- T cells, we next analysed the effect of CMV serostatus on absolute numbers of naïve, CM, EM and TEMRA CD8 T cells (Figure 3.4b).

There was no significant difference in the absolute number of naïve CD8 T cells between the CMV seronegative and seropositive patients ($0.098 *10^9$/l vs $0.093*10^9$/l, $p=0.79$, t test). The numbers of CM CD8 T cells was also not significantly different between the two groups (mean $0.039*10^9$/l vs $0.053*10^9$/l, $p= 0.19$). There was a non-significant trend towards an increase in absolute numbers of CD8 EM T cells (mean $0.027*10^9$/l vs $0.010*10^9$/l, $p=0.066$, t test). However, the absolute numbers of TEMRA CD8 T cells

were significantly increased in the CMV seropositive patients ($0.043*10^9$/l vs $0.19*10^9$/l, p=0.0013, t test).

The Effect of CMV serostatus on CD4 memory subsets

There was a significant decrease in the proportion of naïve CD4 T cells in the CMV seropositive patients (p=0.033, Mann-Whitney). There was no significant difference in the proportion of CM CD4 T cells (p=0.57, Mann-Whitney). CMV seropositivity was associated with a higher proportion of EM CD4 T cells (p=0.0012, Mann-Whitney) and TEMRA CD4 T cells (p=0.0042, Mann-Whitney) (Figure 3.4c).

Within the CD4 populations, there was a significantly lower absolute number of naïve CD4 T cells (CD45RA+, CD27+) in CMV seropositive compared to seronegative patients (p=0.046, t-test). CMV serostatus had no significant effect on the absolute numbers of other memory subsets of CD4 T cells (Figure 3.4d).

The Effect of Age in CMV seropositive patients.

Because ageing is associated with an increased number of CMV-specific CD8 T cells (Pawelec et al., 2009), I went on to examine whether this was a significant factor within this patient population. The CMV seropositive group was divided into 2 groups of equal size around the median age of 67. The older patients had significantly fewer naïve CD8 T cells compared to the younger ones (median 0.13 10^9/L versus $0.057*10^9$/L, p = 0.033). There was no significant difference in the overall lymphocyte count, or overall CD8 or CD4 numbers and there was no difference in other memory subsets' frequencies.

These results show that CMV seropositivity in imatinib treated patients is associated with an absolute increase in CD8 T cells, which is predominantly of the terminally differentiated CD45RA+, CD27- subset.

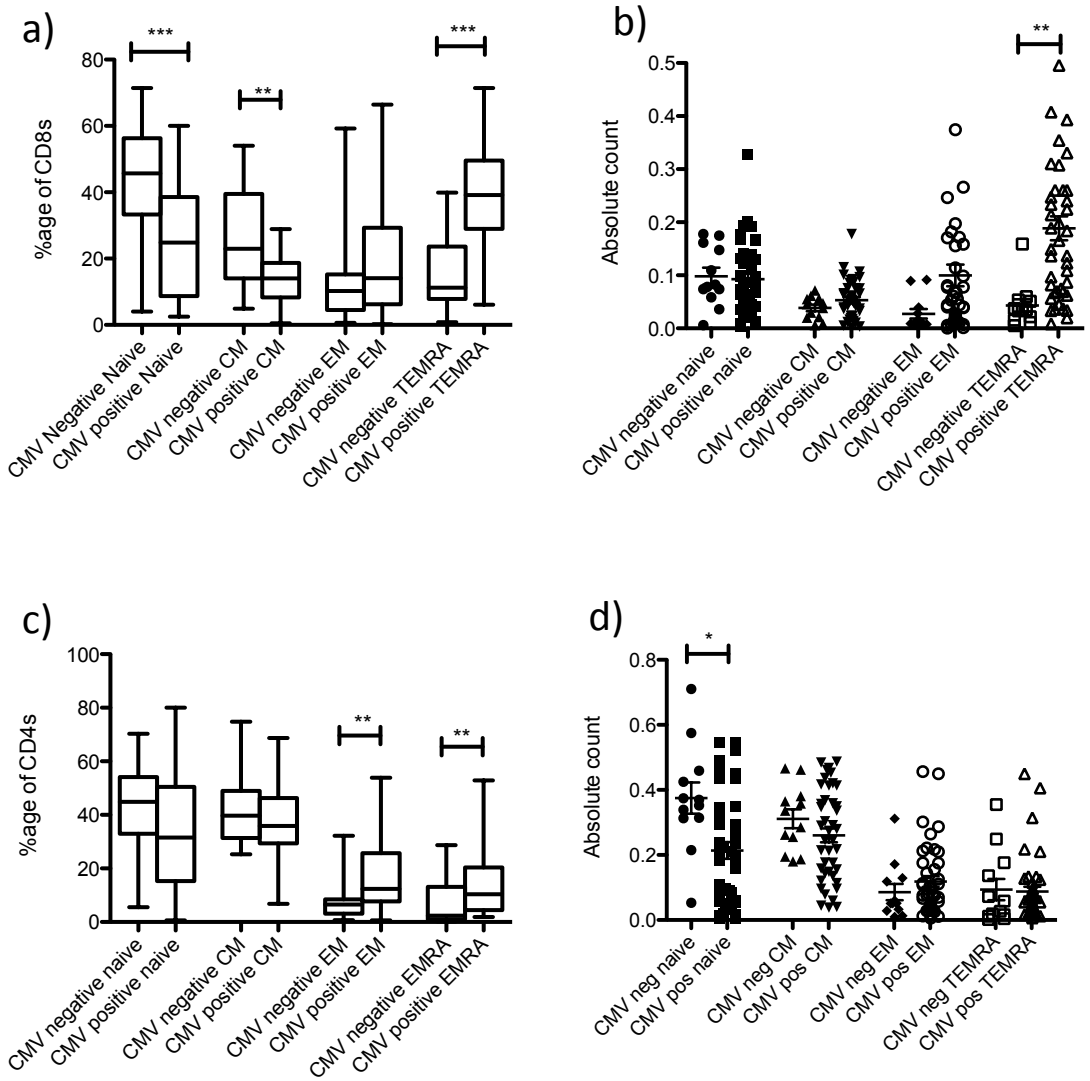


Figure 3.4: The effect of CMV serostatus on memory subsets of CD4 and CD8 T cells in imatinib-treated patients

a) CMV seropositivity is associated with significant decreases in the proportions of naïve CD8 T cells, with increases in EMRA and TEMRA CD8 proportions. b) There is an increase in the absolute numbers of TEMRA CD8 T cells. c) CMV seropositivity is associated with an increased proportion of EM and EMRA CD4 T cells, but d) a decrease in absolute numbers of naïve CD4 T cells and no significant effect on absolute numbers of more differentiated CD4 T cells.

CMV specific T cell responses in imatinib-treated patients

CMV-specific T cells were measured in a group of patients treated with imatinib, in order to assess if the CMV specific T cell response remained stable over time. The total of all individual HLA-peptide tetramer populations was added together in each patient and an absolute tetramer-positive lymphocyte count was calculated. The tetramer responses of individual patients were measured one year later, to assess if the magnitude of the response had changed. The memory phenotype of the tetramer positive cells based on CD45RA and CD27 expression was also ascertained.

There was a small amount of variation in the magnitude of the response over time, but comparing the samples in a paired t-test did not show any significant differences over the time period measured (n=7, Figure 3.5a, p = 0.64).

	Minimum	**25th percentile**	**Median**	**75th Percentile**	**Maximum**
Early	0.00030	0.00081	0.0079	0.023	0.057
Late	0.00032	0.0035	0.0084	0.023	0.029

Table 3.4 Absolute measurements of tetramer-positive $CD8^+$ T cells at early and late timepoints on imatinib treatment

The majority of the CMV-specific CD8 T cells had a terminally differentiated CD45RA+ CD27- phenotype (Figure 3.5b).

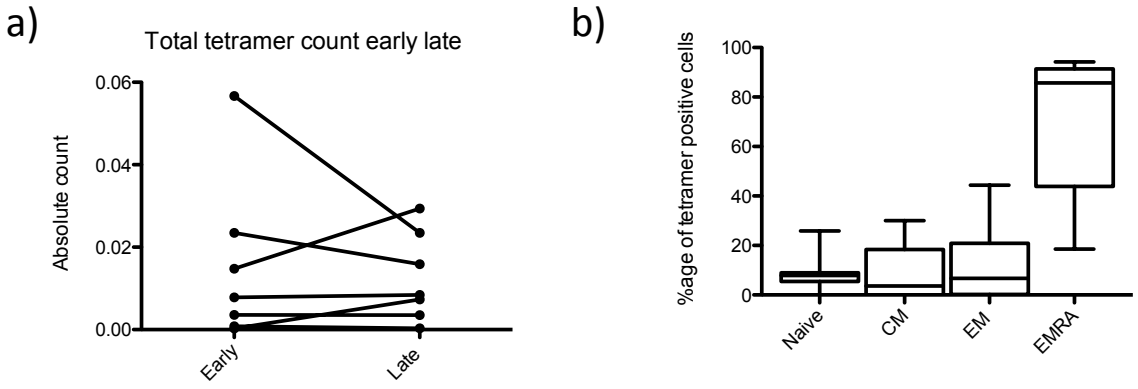


Figure 3.5: The CMV specific T cell response is stable over time

a) The measured CMV-specific T cell response is stable over time. b) The majority of CMV-specific T cells in imatinib-treated patients have a terminally differentiated, CD45RA+, CD27-, phenotype

γδ T cell measurement

The Vγ9δ2 subset is the predominant subset in the peripheral blood in healthy individuals (Hayday, 2009a) and accounts for up to 90% of γδ T cells. Most investigators have divided γδ T cells into Vδ2γ9 vs non-Vδ2γ9 subsets. This is obviously an oversimplification, as there is a considerable potential for heterogeneity within the non-Vγ9δ2 subset.

Two panels were used, one looking at the overall proportion of γδ T cells using a pan-γδ antibody, and a second panel looking in more detail at subsets of γδ T cells, utilizing a anti-Vδ1 antibody, an anti-Vδ2 antibody and an anti Vγ9 antibody, to identify and characterize populations of γδ T cells in these patients with unusual/unconventional chain usage.

No difference was observed in total γδ T cells in CMV seropositive versus seronegative patients

Initially, I measured total γδ T cell numbers, both as a proportion of total lymphocytes, and as an absolute number, in imatinib treated patients and compared the results within CMV seronegative patients with CMV seropositive patients.

Whilst there was no significant difference in the relative proportions (Figure 3.6a) or absolute numbers (Figure 3.6b, Table 3.5) of γδ T cells (median 1.59% of lymphocytes versus 1.58% CMV negative versus CMV positive), there were some patients within the CMV seropositive group with markedly higher numbers of total γδ T cells. We therefore analysed γδ subsets according to their expression of Vδ1, Vδ2 and Vγ9 chains.

	Minimum	25th percentile	Median	75th Percentile	Maximum
CMV -ve	0.0030	0.013	0.028	0.042	0.069
CMV +ve	0.0019	0.011	0.019	0.068	0.37

Table 3.5 The overall numbers of γδ T cells in CMV seropositive and negative patients (all10^9/l)*

As a proportion of overall lymphocytes, there was a non-significant trend for a decrease in the proportion of Vδ2 γδ T cells in CMV seropositive patients (p=0.0702, 0.94% CMV negative versus 0.44% CMV seropositive, Figure 3.6c), however no significant difference was seen in the absolute number of Vδ2 γδ T cells in the peripheral blood of either group (p=0.25, t test, Figure 3.6d, Table 3.6). A non-significant trend was observed for a lower proportion Vδ2 γδ T cells as a proportion of CD3 cells in the CMV seropositive group (p=0.0511, Mann-Whitney, median 1.75% in CMV seronegative group versus 0.8% in CMV seropositive group).

	Minimum	25th percentile	Median	75th Percentile	Maximum
CMV -ve	0.0059	0.0089	0.028	0.052	0.058
CMV +ve	0.00050	0.0044	0.0092	0.022	0.11

*Table 3.6 Absolute numbers of Vδ2γ9 γδ T cells in CMV seropositive vs seronegative patients, all *10^9/l*

FACS analysis showed that the Vδ2 γδ T cells all co-expressed the γ9 TCR chain.

Therefore, CMV serostatus had no effect on the proportion or absolute numbers of Vδ2γ9 γδ T cells seen in the peripheral blood of imatinib-treated patients.

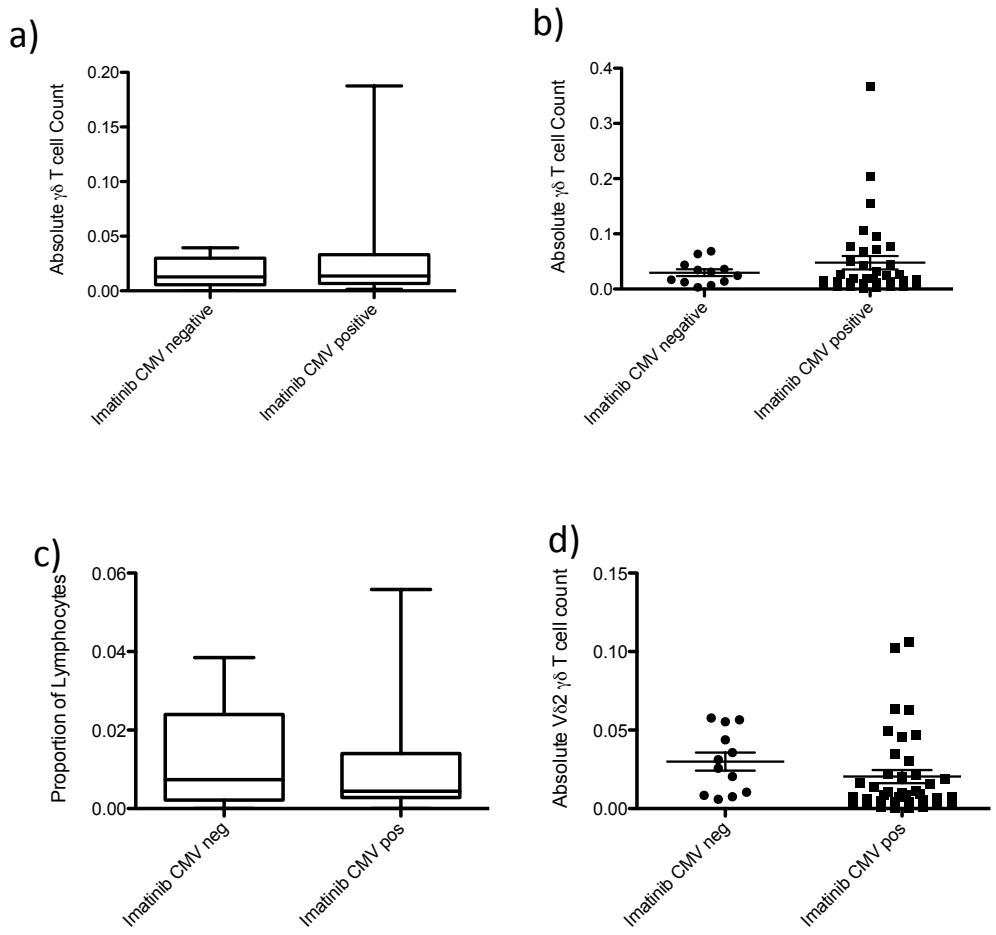


Figure 3.6: Relationship between CMV serostatus and γδ T cell count

There was no statistically significant difference between CMV seropositive (n=35) and seronegative (n= 13) patients in relation to a) relative proportion or b) absolute count of total γδ T cells in the peripheral blood, although some CMV seropositive patients have markedly increased numbers. There is no difference in c) the proportion or d) number of Vγ9δ2 γδ T cells in the peripheral blood of CMV seropositive versus CMV seronegative patients on imatinib

CMV Serostatus and Vδ1 γδ T cells

Since Vγ9δ2 subset is regarded as the predominant subset of γδ T cells in peripheral blood, one would therefore expect the Vδ2:Vδ1 γδ T cell ratio to be greater than one in normal individuals. Whilst this is true of the CMV seronegative imatinib treated group (median ratio 4.75), in the CMV seropositive group the median ratio is 0.90. This difference is statistically significant (p=0.0006, Mann-Whitney) (Figure 3.7a).

The proportion of Vδ1 γδ T cells as a proportion of total T cells was significantly greater in the CMV seropositive group (median 0.008 vs 0.003, p=0.0018, Figure 3.7b, Table 3.7).

	Minimum	**25th percentile**	**Median**	**75th Percentile**	**Maximum**
CMV –ve	0.001	0.002	0.003	0.005	0.012
CMV +ve	0.002	0.004	0.008	0.02	0.32

Table 3.7 Vδ1 T cells as a proportion of T cells in CMV seropositive vs seronegative patients

The Vδ1 γδ T cells therefore made up a significantly greater proportion of the lymphocyte population in the CMV seropositive imatinib treated patients (p=0.0027, Mann-Whitney). Within the CMV seropositive group, the median proportion of lymphocytes was 0.68% vs 0.22% in the CMV seronegative group (Figure 3.7c, Table 3.8).

	Minimum	**25th percentile**	**Median**	**75th Percentile**	**Maximum**
CMV –ve	0.00069	0.0010	0.0022	0.0047	0.0096
CMV +ve	0.00064	0.0031	0.0068	0.014	0.19

Table 3.8 Vδ1 γδ T cells as a proportion of the lymphocyte population

A significant increase in the absolute Vδ1 γδ cell count in CMV seropositive patients was also seen in comparison to CMV seronegative patients (Figure 3.7d, Table 3.9). The count was more than doubled such that the median number in the CMV negative group was 0.0038 $*10^9$/l compared to 0.010 $*10^9$/l in the CMV positive group (p=0.027, Mann-Whitney).

	Minimum	**25th percentile**	**Median**	**75th Percentile**	**Maximum**
CMV –ve	0.00064	0.0035	0.0038	0.0069	0.013
CMV +ve	0.00057	0.0040	0.010	0.026	0.37

Table 3.9 Absolute Vδ1 γδ T cell count ($*10^9$/L) according to CMV serostatus

Within the CMV seropositive group there were also a number of patients with large absolute increases in Vδ1 γδ T cells, which was not seen within the CMV seronegative patients.

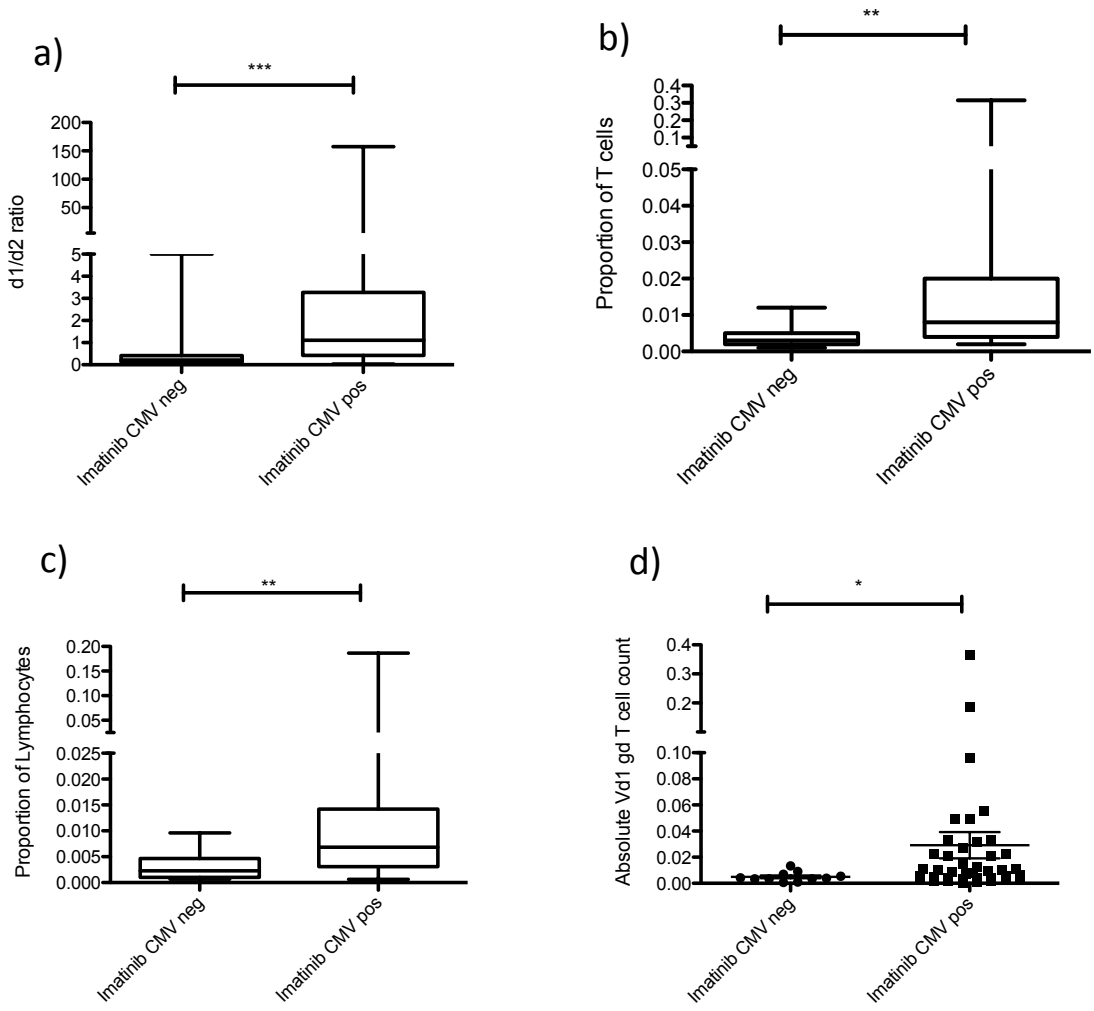


Figure 3.7: Vδ1 γδ T cell numbers are expanded in CMV seropositive patients

a) The ratio of Vδ1 to Vδ2 expressing γδ T cells is frequently reversed in CMV seropositive patients (p = 0.0006) b) The proportion of Vδ1 γδ T cells of CD3 positive cells is greater in CMV seropositive patients (p = 0.0018). c) The proportion of Vδ1 γδ T cells as a proportion of the overall lymphocyte population is increased (p = 0.0027). d) The absolute number of Vδ1 γδ T cells is increased in CMV seropositive patients (p = 0.0271)

CMV Seropositivity is associated with a higher proportion and absolute number of CD45RA+ CD27- Vδ1 γδ T cells

I then went on to define memory subsets of γδ T cells in the same way as with $CD8^+$ T cells by the expression of CD45RA and CD27. Using this analysis there was a very obvious skewing of the Vδ1 population towards a terminally differentiated CD27- CD45RA+ phenotype, but no effect on the Vδ2 γδ T cell subset (Figure 3.8a and b).

The median proportion of naïve (CD27+, CD45RA+) Vδ1 γδ T cells in CMV negative, imatinib-treated patients was 36.5% compared to 10.1% in CMV seropositive patients ($p = 0.0082$, Mann-Whitey). The median proportion of central memory (CD27+, CD45RA-) Vδ1 γδ T cells was 16.35% in CMV seronegative patients compared to 3.70% in CMV seropositive patients ($p = 0.0009$, Mann-Whitney). The median proportion of TEMRA (CD27- CD45RA+) Vδ1 γδ T cells in CMV negative patients was 33.8% versus 64.9% in CMV seropositive patients ($p=0.0022$, Mann-Whitney) (Figure 3.8c).

When comparing absolute numbers of different memory subsets, the only significant difference was in the TEMRA group of Vδ1 γδ T cells. The CMV positive patients had significantly more CD27-, CD45RA+ TEMRA Vδ1 γδ T cells than the CMV seropositive group. The median number of TEMRA Vδ1 γδ T cells detected in CMV seronegative patients was $0.0021*10^9$/l (range $0.00019*10^9$/l to $0.0052*10^9$/l) vs $0.0049*10^9$/l (range 0 to $0.36*10^9$/l) in CMV seropositive individuals ($p = 0.022$). Whilst there was no difference in the median number of EM (CD27-, CD45RA-) Vδ1 γδ T cells between the two groups, there were a number of patients within the CMV seropositive group with markedly expanded CD45RA- CD27- EM δ1 γδ T cells (Figure 3.8d).

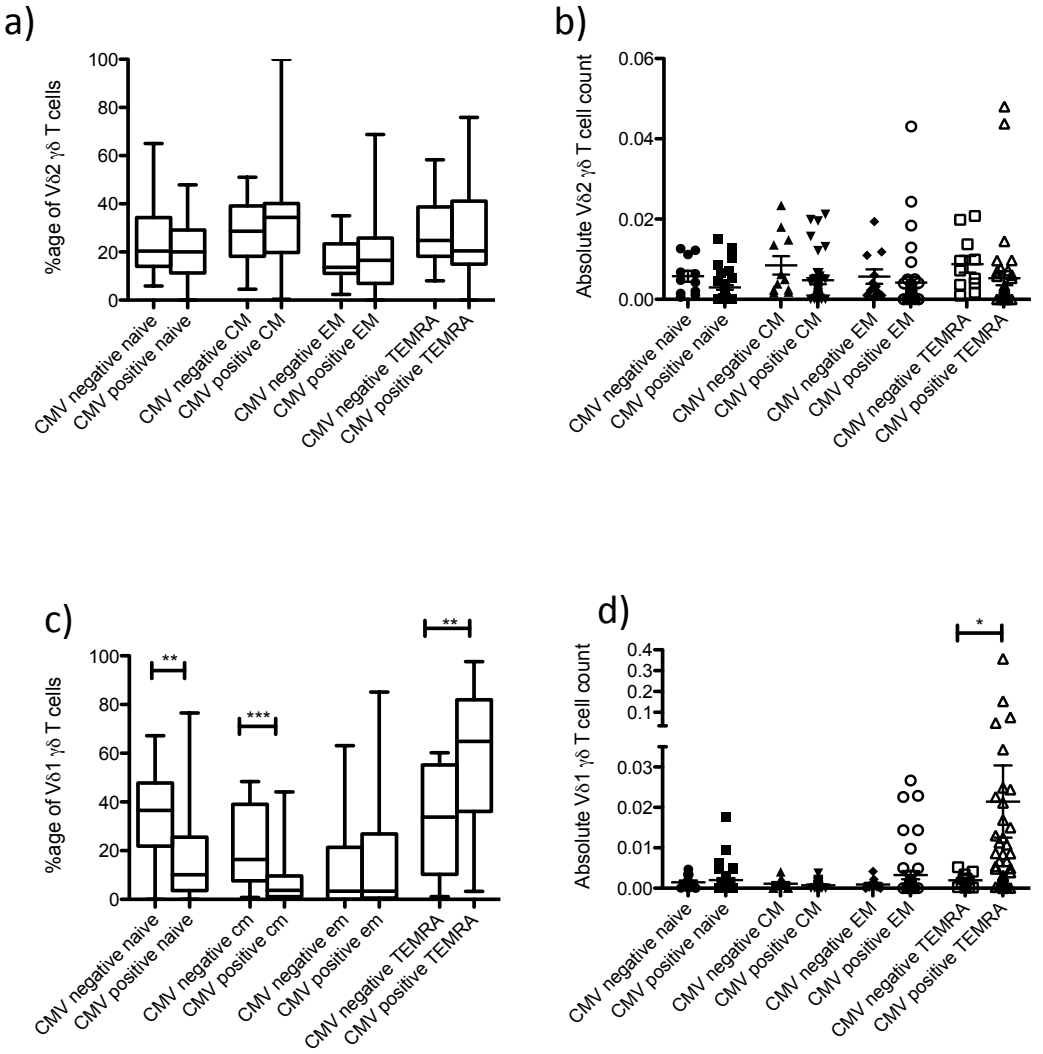


Figure 3.8: The impact of CMV seropositivity on the memory phenotype of γδ T cell subsets

a) CMV seropositivity has no effect on proportion of individual memory subsets of Vδ2 γδ T cells, or b) on absolute numbers of different memory subsets. c) CMV seropositivity is associated with a lower proportion of naïve and CM Vδ1 γδ T cells, and an increase in the proportion of TEMRA Vδ1 γδ T cells. d) In terms of absolute numbers, CMV seropositivity is associated with an increase in TEMRA Vδ1 γδ T cells, but no significant differences in the other memory subsets

Effect of CMV serostatus on NK Cells in imatinib treated patients

Natural killer cells can be categorized according to CD56 and CD16 expression, into $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells. These subsets have been shown to be functionally different (Sun and Lanier, 2011); the $CD56^{bright}$ subset has more capacity to secrete cytokines, whereas the $CD56^{dim}$ subset has more cytotoxic activity.

CMV serostatus did not affect the proportion (Figure 3.9a) or absolute number (Figure 3.9b) of the $CD56^{dim}$ NK cells. CMV serostatus did also not affect the numbers or relative proportion of $CD56^{bright}$ NK cells.

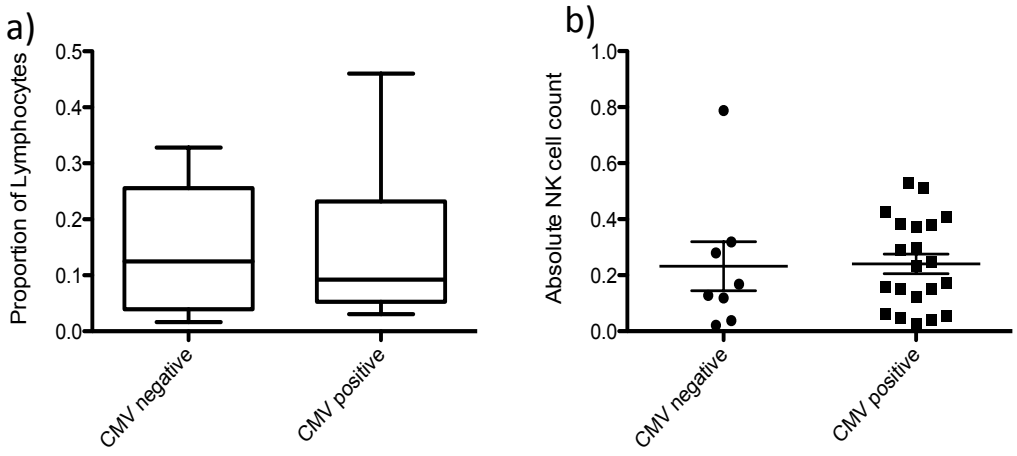


Figure 3.9: The relationship between NK cell numbers and CMV serostatus

56^{dim} natural killer cells were defined as CD3-, CD16+ and CD56 dim. a) There was no difference in the proportion of NK cells relative to the overall lymphocytes, or b) the absolute NK cell count, between the CMV seropositive group of patients and the CMV seronegative group

Analysis of Lymphocyte Subsets in relation to the depth of clinical response

It is recognized that the achievement of a MMR is desirable in patients treated with TKIs (Baccarani et al., 2013), and is associated with superior long-term clinical outcomes (Press, 2010). Because of the heterogeneity of the population studied (in terms of drug treatment, length of time on drug etc), we analysed patients treated with the TKI imatinib for the duration of their care, in order to identify differences in lymphocyte subsets between patients that achieved a MMR versus those that had not. All patients had been on TKI treatment for at least 18 months at the time of analysis, and had achieved a CCyR.

The status of achievement of molecular response was known in 45 patients. 31/45 (69%) of the patients were in MMR at the time of analysis of the lymphocyte subset analysis, compared to 14/45 (31%) that were not. The median age of both groups of patients was 65 years old. There was no difference in age between the groups (p = 0.45, t test).

	MMR	**Not in MMR**
n	25	14
Age	65.7	62.7
Sokal Score: High (%)	16%	14%
Medium	20%	21%
Low	20%	29%
Not known	44%	36%
Time on treatment	6 years	3.5 years

Table 3.10: Patient Characteristics of patients assessed for depth of response whilst on imatinib

No difference in CD4, CD8 or NK cell count was observed between patients that achieved MMR and those that did not

There was no significant difference in absolute lymphocyte count between the two groups ($1.55*10^9$/L in MMR versus $1.40*10^9$/L not in MMR, Figure 3.10a). Furthermore, there were no differences in the numbers of CD8 (Figure 3.10b) or CD4 T cells, or NK cells. As an example. the median CD8 T cell count in patients that achieved an MMR was $0.26*10^9$/L versus $0.21*10^9$/L in patients that did not. There was also no difference in the absolute count of $CD56^{dim}$ NK count between the two groups. Median count was $0.29*10^9$/l in MMR group versus $0.24*10^9$/l in the group that did not achieve an MMR (ns). The median CD4 count was $0.66*10^9$/L in patients were in a MMR at the time of the sample versus $0.74*10^9$/L in patients that did not; this difference was not statistically significant (Figure 3.10c).

Patients in MMR had a higher γδ T cell count

The total γδ (Vδ1 plus Vδ2) count in patients that had achieved an MMR was significantly higher compared to patients that did not ($p = 0.0097$, Mann-Whitney, Figure 3.10d). Specifically, the median total γδ T cell count in patients that achieved an MMR was $0.031*10^9$/L versus $0.014*10^9$/L in patients that did not. The median number of Vδ1 γδ T cells in patients that had achieved an MMR was $0.0063*10^9$/L versus $0.0055*10^9$/L in patients that did not. Some patients that achieved an MMR had significantly more Vδ1 γδ T cells (Figure 3.10e). The median Vδ2 γδ T cell count in patients that had achieved an MMR was $0.017*10^9$/L versus $0.0060*10^9$/L in patients that did not ($p = 0.0238$ Mann-Whitney, Figure 3.10f).

There was no difference in the memory phenotypes of Vδ1 or Vδ2 γδ T cells in patients that achieved an MMR versus those that did not.

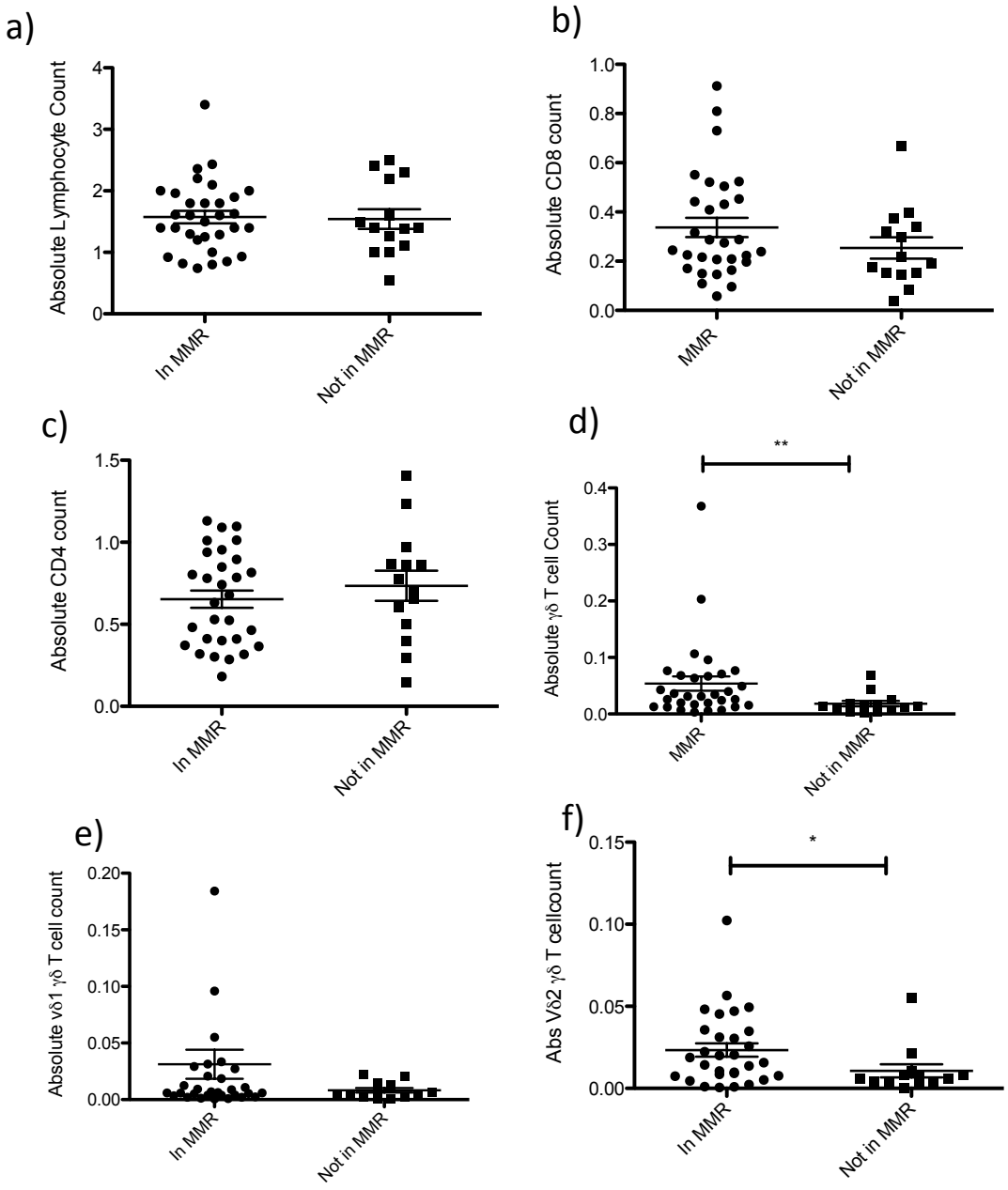


Figure 3.10: Association of changes in lymphocyte subsets with depth of response to imatinib

a) No difference in absolute lymphocyte count between patients that achieved MMR (n = 25) versus those that did not (n = 14) b) no difference in absolute CD8 or c) CD4 count. d) Significant increase in total γδ T cell count in patients in MMR at time of sample versus those not. e) Non-significant trend for increased Vδ1 γδ T cells in patients in MMR and f) significantly increased Vδ2 γδ T cells in patients in MMR versus those not in MMR

CMV serostatus, depth and speed of response to treatment

We have demonstrated that CMV has profound effects on the immune phenotype of imatinib-treated patients, and that specific subsets of lymphocytes, particularly γδ T cells, may be associated with deeper responses to imatinib. We therefore measured CMV serostatus in 100 patients in whom the time to disease response was known, and calculated the time to CCyR and time to MMR in both CMV seropositive and CMV seronegative patients.

Kaplan-Meier estimates of the median time to CCyR in the CMV seropositive group was 0.69 years versus 0.82 years in the CMV seronegative group. This was not a statistically significant difference. The Kaplan-Meier estimate for 18-month achievement of CCyR was also not significantly different, at 77% in the CMV seropositive group versus 65% in the CMV seronegative group (Figure 3.11a).

The median time to MMR in the CMV seropositive group was 2.6 years versus 2.0 years in the CMV seronegative group (Figure 3.11b), a non-significant difference.

These data show that, in this retrospective study, CMV serostatus did not affect the time to achievement of major treatment milestones for patients on imatinib therapy for CML.

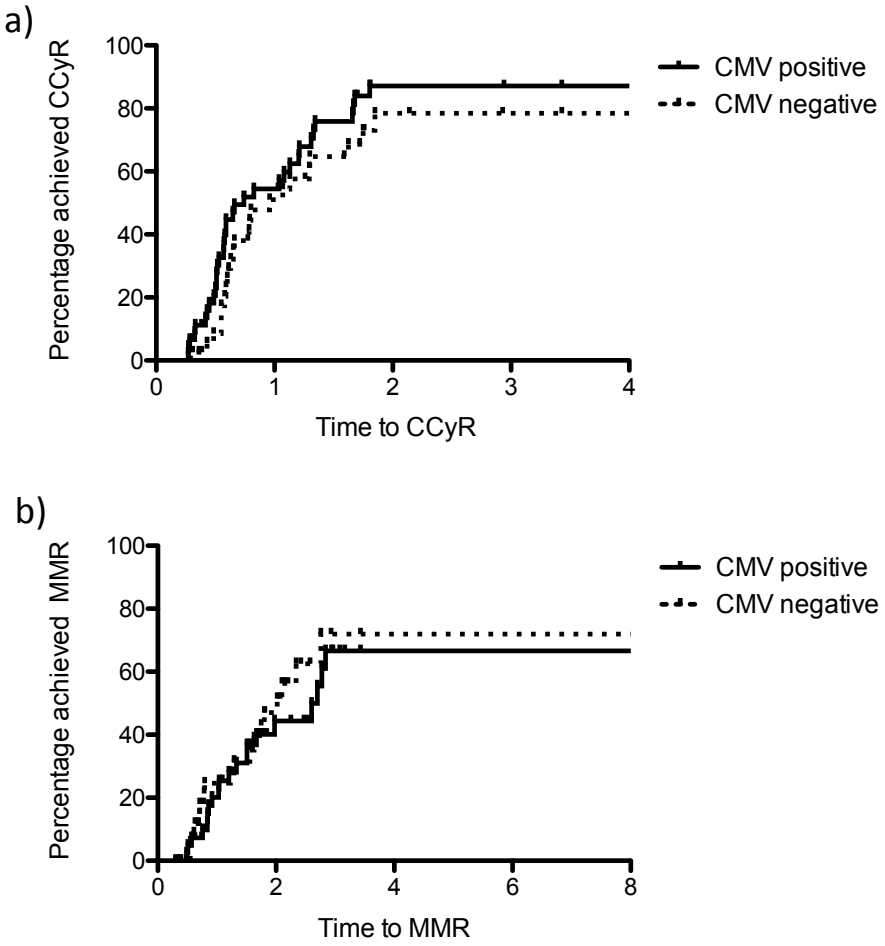


Figure 3.11: The relationship between CMV serostatus and achievement of response milestones whilst on imatinib

Comparing CMV seropositive patients to CMV seronegative patients, there was no difference in time to achieve, or the proportions of patients achieving a) a CCyR or b) a MMR in patients treated with imatinib for CML.

Discussion

It has been shown that CMV seropositivity is associated with an increase in the resting memory pool of CD8 T cells (Chidrawar et al., 2009). The CD4:8 ratio is known to increase in age, but in the presence of CMV this is reversed. It has also been shown that CMV seropositivity increases the numbers of $CD56^{dim}$ NK cells in elderly CMV seropositive individuals.

In ageing populations the concept of "memory inflation" of differentiated CD8 T cells has been described (Beswick et al., 2013, Pawelec et al., 2009), which is thought to be caused by repeated, sub clinical episodes of CMV reactivation.

Imatinib has revolutionized the treatment of CML , to the point that it is being suggested that some patients may be effectively cured by TKI therapy (Mahon et al., 2010, Ross et al., 2013). Changes in lymphocyte profiles of patients treated with TKI therapy have been implicated with the depth and speed of response (Mustjoki et al., 2009).

In vitro, imatinib is known to cause immunosuppressive effects, such as decreased proliferation of T cells to CMV peptides (Seggewiss et al., 2005) and other antigen-specific CD8 T cells (Chen et al., 2007a, Cwynarski et al., 2004). It might be expected that these immunosuppressive effects may cause subclinical CMV reactivation and changes over time of CMV-specific CD8 T cells.

In the cohort of patients studied, the CMV-specific T cell response as measured by tetramers appeared broadly stable over time. We did not see evidence of memory inflation in our imatinib-treated cohort of patients. In particular, there was no accumulation of CD45RA+ CD27- CD8 T cells over the time period measured. However, we only measured samples one year apart and it is possible that the effects of memory

inflation take longer than this to appear. Also, within our cohort of CMV seropositive patients there were already significant expansions of terminally differentiated CD8 T cells even at the early timepoint; it has been suggested that once expansion of the terminally differentiated CD8 T cell compartment has occurred, it remains stable (Snyder et al., 2008).

Cytokine release by CD4 T cells has also been shown to be inhibited by the addition of imatinib *in vitro* (Gao et al., 2005). We did not detect any changes to the number or memory phenotype of the CD4 T cells during treatment with imatinib in our patient cohort, suggesting that the changes seen in *in vitro* may be caused by higher than achievable in vivo concentrations used in the experiments, or that the inhibition due to imatinib is transient and does not significantly influence CD4 activation and maturation.

In the patients studied here, CD4 T cells also had a more differentiated phenotype in CMV seropositive patients. This is in keeping with published data on the phenotype of CD4 T cells in healthy individuals (Chidrawar et al., 2009).

We have demonstrated that CMV serostatus, more than treatment with imatinib itself, or age of the patients, profoundly influences the proportion of CD8 T cells and their memory phenotype. Furthermore, there is a profound increase in the terminally differentiated CD8 T cell compartment.

Vδ1 γδ T cells are also increased in number in CMV seropositive patients compared to CMV seronegative patients. Others have noted expansions of Vδ1 γδ T cells in patients in conjunction with CMV reactivation (Knight et al., 2010, Lafarge et al., 2001). We noted significant increases in Vδ1 γδ T cells in CMV seropositive patients without evidence of CMV reactivation, in a clinical setting not thought to be at risk of CMV reactivation.

Similarly to the memory inflation seen in CD8 T cells, the Vδ1 γδ T cells in CMV seropositive patients are CD27-ve CD45RA+ve well differentiated lymphocytes.

This is consistent with others' findings, with CMV seropositivity being associated with CD45RA+ve, CD27-ve nonVδ2 γδ T cells in CMV seropositive healthy blood donors, but with a largely CD45RA+ve, CD27+ve naïve phenotype in CMV seronegative populations (Pitard et al., 2008).

These innate-like T cells have been implicated in immune anti-tumour responses in a number of different settings (Devaud et al., 2009, Knight et al., 2012).

An increase in γδ T cells was noted in patients that had achieved a MMR to imatinib. Others have noted that increased γδ T cell numbers correlate with a better outcome in other clinical scenarios, such as patients treated with allogeneic stem cell transplant for acute leukaemia (Godder et al., 2007). The Vδ2 subset was increased significantly; this subset has been shown to have anti-tumour properties in prostate carcinoma (Dieli et al., 2007) and breast carcinoma (Gnant et al., 2009). In patients that have had previous IFN-α therapy, successful discontinuation was associated with increased NK cells and increases in the Vγ9 γδ T cell population (Kreutzman et al., 2011b).

I postulated that the changes in the numbers of γδ T cells with CMV serostatus may lead to differences in depth of response. However, we did not see any changes in outcome to TKI therapy in terms of depth and speed of response to imatinib according to CMV serostatus.

There has been recent interest in the peripheral blood lymphocyte profiles of patients treated with tyrosine kinase inhibitors for CML (Kreutzman et al., 2011b, Mustjoki et al.,

2009, Mustjoki et al., 2013). We have demonstrated that CMV seropositivity alone accounts for a large amount of the variation in lymphocyte subsets in the peripheral blood, particularly the $CD8^+$ T cell numbers, and the Vδ1 γδ T cell subset.

Conclusion

In imatinib-treated patients, CMV seropositivity accounts for a large amount of the variation in numbers of lymphocyte subsets, particularly of CD8 T cells. There is, however, little change in these subsets as treatment goes on, suggesting that the expansion of differentiated CD8 T cells seen with CMV infection has already occurred by the time the patients are commenced on imatinib, and has reached a stable level.

γδ T cells are innate-like lymphocytes with anti-tumour and anti-infective properties. I demonstrate that within this population, CMV seropositivity is associated with significant increases in γδ T cell subsets. Vδ2Vγ9 γδ T cells, which are the most common γδ T cell subset usually found in peripheral blood, are not altered by CMV seropositivity,. However, there is a dramatic increase in Vδ1 γδ T cells in conjunction with CMV seropositivity.

I demonstrate that improved response to imatinib is associated with higher numbers of γδ T cells, particularly of the Vδ2Vγ9 subset, although with some increase in the Vδ1 γδ T cell subset. Although CMV seropositivity has a dramatic effect on the frequencies of different lymphocyte subsets, there was no obvious difference in time to response to imatinib in a cohort of patients studied retrospectively.

Chapter 4: The Pattern of Lymphocyte changes according to CMV serostatus and TKI treatment

Introduction

Despite the success of imatinib a significant number of patients develop resistance, or intolerance to the drug. Second generation tyrosine kinases were developed following the success of imatinib, and are frequently used in patients who fail imatinib according to guidelines such as EUTOS (Baccarani et al., 2013). Second generation TKIs are also used as initial treatment of CML, particularly in clinical trials. They have been shown to induce faster, deeper responses to treatment compared to imatinib (Kantarjian et al., 2010, Larson et al., 2012).

The two most commonly used second generation TKIs in the UK currently are dasatinib and nilotinib. Nilotinib has a target profile similar to that of imatinib, targeting the Abl kinases as well as PDGFR and C-Kit, but is a much more potent agent than imatinib. (Larson et al., 2012)Dasatinib is a potent inhibitor of Abl kinases, but has a wider target profile than the other TKIs used in the treatment of CML, particularly in terms of "off-target" effects on *Src* family kinases, which are vital in initiating downstream effects of T-cell-receptor mediated signaling.

In vitro studies have demonstrated dasatinib to be profoundly immunosuppressive, blocking T cell receptor mediated activity via blockade of *Src* family kinases (Fei et al., 2008, Schade et al., 2008).

It has recently been demonstrated that in some patients treated with dasatinib a lymphocytosis develops (Kreutzman et al., 2010). This consists mainly of NK cells and CD8+ T cells. Interestingly, patients that developed the lymphocytosis had improved outcomes on the dasatinib compared with other patients, suggesting a possible anti-tumour effect of this lymphocyte expansion. A retrospective analysis of the DASISION trial (Schiffer et al., 2010), comparing dasatinib to imatinib in untreated CML patients,

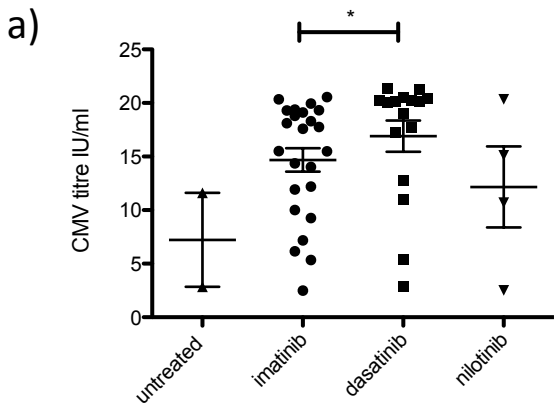
demonstrated improved outcomes in patients that had higher overall lymphocyte counts. No attempt was made to measure subsets of lymphocytes in this study.

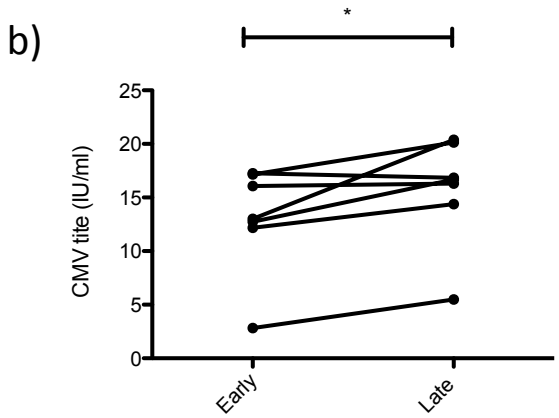
I hypothesized that these changes in lymphocyte counts were largely due to changes induced by altered immune responses to CMV. I was particularly interested to investigate whether the effects of CMV on the immune system were exaggerated in dasatinib-treated patients, and particularly whether CMV reactivation could be associated with atypical lymphocyte expansions in patients treated with second generation TKIs, perhaps due to inhibition of classical adaptive αβ T cell immunity, with a consequent increase in the innate immune response driven by CMV reactivation.

Higher CMV IgG Titres are seen in dasatinib treated patients compared to imatinib treated patients

In initial studies I investigated the humoral responses to CMV to see if there was any evidence of increased immune responses during dasatinib treatment. As well as measuring CMV seropositivity, a quantitative value for serum anti-CMV IgG was measured. Anti-viral antibodies are increased after episodic reactivation thought to be due to impaired cellular responses and upregulation of viral expression (Stowe et al., 2007). For this comparison, only patients that had been on TKI treatment for longer than 6 months and that were in at least a haematological remission were included. CMV seronegative individuals (with anti-CMV specific IgG <1 IU/ml) were excluded. The median age of the CMV seropositive dasatinib-treated patients was 61.5 years (34 – 78) compared to 63.0 years in the imatinib-treated patients (34 – 90). This was not a significant difference ($p = 0.413$). Untreated/early treatment patients were classed as those who were newly diagnosed, or had fewer than 6 months' treatment on TKI.

The median anti-CMV IgG titre in CMV seropositive dasatinib treated patients (n = 16) was 20.1 IU/ml (range 2.8 IU/ml -21.4 IU/ml). For imatinib treated patients (n = 24), the median was 16.1 IU/ml (range 2.0 IU/ml – 21.3 IU/ml), compared with 10.7 IU/ml (range 2.5 IU/ml – 15.0 IU/ml) for those on nilotinib (n = 4). Untreated/patients early in treatment (n =2) had a median anti-CMV titre of 5.5 IU/ml. There was a significant difference between the imatinib and dasatinib treated patients ($p = 0.048$, Mann-Whitney), but all of the other differences did not reach statistical significance (Figure 4.1a). Furthermore, the quantitative measure for anti-CMV IgG increased as treatment with dasatinib went on (Figure 4.1b). There was no correlation between age and CMV specific antibody titre by linear regression analysis ($p = 0.7$).





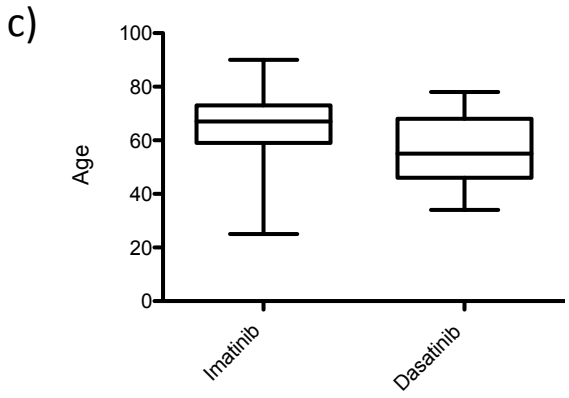


Figure 4.1: CMV IgG Titre and treatment

a) Dasatinib (n = 16) treated patients have significantly higher anti-CMV IgG titres compared to patients on imatinib (n = 24) (p = 0.048).

b) In patients treated over time (n = 7), patients treated with dasatinib generated stronger IgG anti-CMV during 12 months' follow up (p = 0.031 by paired t-test)

c) Dasatinib-treated CMV seropositive patients were not significantly different in age compared to imatinib-treated, CMV seropositive patients

Changes in serum cytokines were seen in dasatinib-treated patients depending on CMV serostatus

I went on to measure serum cytokine levels to assess if there were overt differences in inflammatory cytokines in the different groups. The same patient serum samples used to obtain CMV IgG was used as detailed previously. Patients' serums were analysed using a luminex plate, using a 13-plex panel as detailed in the methods section, for TNFα, IL1b, GM-CSF, IFNγ, IL10, IL4, IL12p70, IL17a, IL2, IL5, IL6, IL8 and IL9. Univariate analysis was initially performed with Mann-Whitney tests and an ANOVA model was used to test significance in a multivariable model.

IL-9 was higher in CMV seropositive (n = 37) compared to CMV seronegative (n = 19) patients (p = 0.0137, Mann-Whitney, Figure 4.2a). There was a non-significant trend for increased IFNγ (Figure 4.2b), IL10 (Figure 4.2c) and IL6 (Figure 4.2d) in CMV-seropositive versus CMV-seronegative patients.

There was a non-significant trend for increased IL2 in dasatinib-treated (n =20) patients versus imatinib-treated patients (n=36) (p = 0.085). Within the dasatinib-treated patients, there were significant increases in IFNγ (p = 0.0479, Mann-Whitney, Figure 4.2b), IL17a (p = 0.0384, Mann-Whitney, Figure 4.2e), and GMCSF (p = 0.0464, Mann-Whitney, Figure 4.2e) in CMV seropositive (n = 9) versus CMV seronegative (n = 11)patients. There were non-significant trends for increased TNFα, IL1b, IL10, IL2 and IL6 (p = 0.094) in CMV seropositive versus CMV seronegative patients. A general linear model (ANOVA) was constructed in SPSS using CMV serostatus and treatment type as fixed factors, and the different cytokine levels compared between these groups. There was no significant difference detectible between any of the groups for any in any of the cytokines using this method, taking either CMV serostatus or treatment as a fixed factor. There was considerable variation from patient to patient in each cytokine measured.

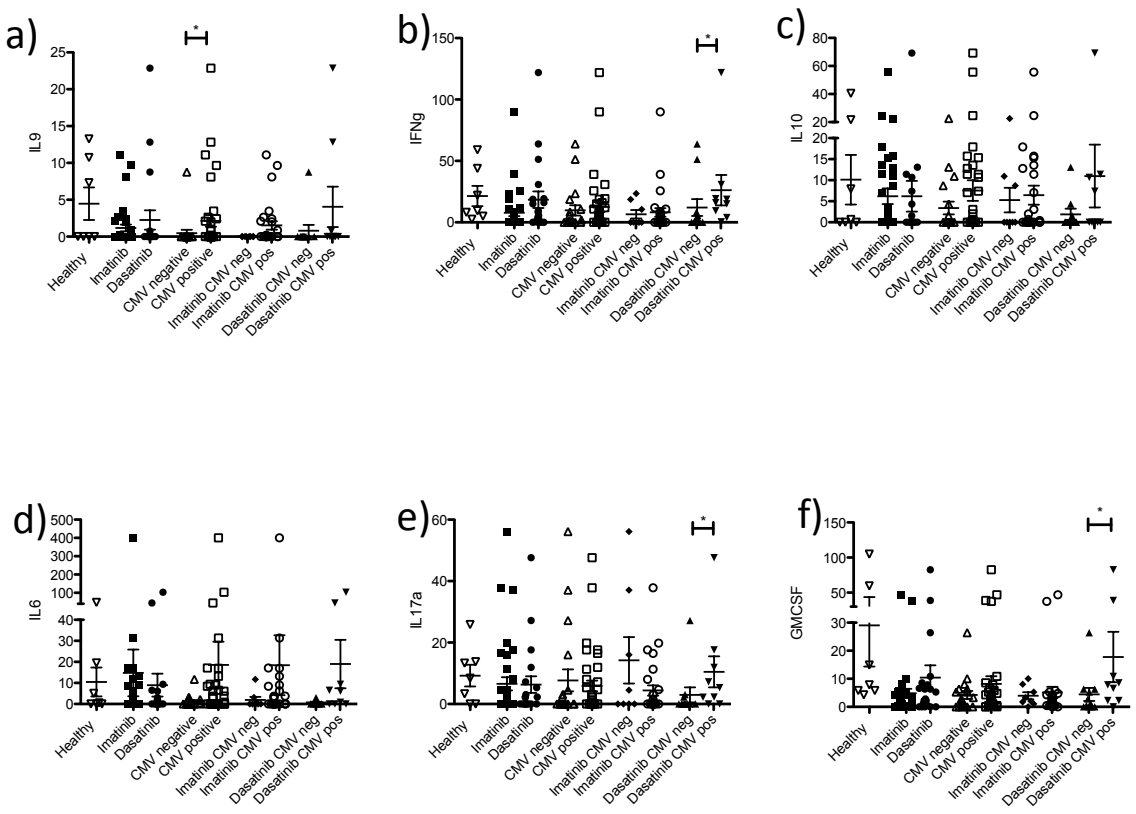


Figure 4.2: Changes in cytokine concentrations according to CMV serostatus and treatment

a) CMV seropositive patients have higher IL9 levels than CMV seronegative patients, and b) trend for higher IFNγ, c) IL10 and d) IL6 levels. Dasatinib-treated CMV seropositive patients have higher b) IFNγ, e) IL17a and f) GM-CSF levels than CMV seronegative patients

Changes in Lymphocyte Subsets in Patients taking Dasatinib or Nilotinib

Because the CMV IgG titre was noted to be higher in dasatinib treated patients, we went on to examine the effect of CMV seropositivity on peripheral blood lymphocyte subsets within this group.

Overall Lymphocyte Count is increased in Dasatinib compared to Imatinib or Nilotinib-Treated Patients

Dasatinib treated patients (n = 43) had significantly higher absolute lymphocyte counts compared to imatinib (n = 30) or nilotinib (n = 8)treated patients (p = <0.0001 versus imatinib treated patients, p = 0.0190 versus nilotinib treated patients, Table 4.1, Figure 4.3a).

	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	0.54	1.01	1.50	2.0	3.4
Nilotinib	1.08	1.21	1.93	2.27	2.27
Dasatinib	0.87	1.62	2.0	2.79	4.50

Table 4.1, absolute lymphocyte count (10^9/L) according to treatment*

The median lymphocyte count within the dasatinib treated group was 2.0 *10^9/L (range 0.87 *10^9/L to 4.5*10^9/L). The median lymphocyte count within imatinib treated patients was 1.50*10^9/L (range 0.54 *10^9/L to 2.5*10^9/L), and within nilotinib treated patients the median lymphocyte count was 1.93 *10^9/L (range 1.08 10^9/L to 2.27*10^9/L).

The Effect Of CMV Serostatus on Absolute Lymphocyte Count within the Different Treatment Groups

Because of the difference in absolute lymphocyte count on patients on dasatinib, we went on to see what effect CMV serostatus had on the lymphocyte count within each treatment group.

The median lymphocyte count for the CMV seropositive, dasatinib treated group was $2.28*10^9$/L ($1.0*10^9$/L - $4.5*10^9$/L) versus $1.67*10^9$/L for CMV seronegative, dasatinib treated patients ($0.87*10^9$/L - $3.06*10^9$/L). Within the imatinib treated group, the median lymphocyte count was $1.55*10^9$/L for CMV seropositive patients ($0.54*10^9$/L to $3.4*10^9$/L) versus $1.39*10^9$/L for CMV seronegative patients ($0.93*10^9$/L - $2.4*10^9$/L). For nilotinib treated patients, the median lymphocyte count was $1.93*10^9$/L for CMV seropositive patients ($1.08*10^9$/L -$2.27*10^9$/L), compared to $1.47*10^9$/L for CMV seronegative patients ($1.03*10^9$/L - $1.78*10^9$/L) (Figure 4.3b).

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.54	1.01	1.55	2.02	3.40
	-	0.93	1.14	1.39	1.80	2.40
Nilotinib	+	1.08	1.21	1.93	2.27	2.27
	-	1.03	1.22	1.47	1.65	1.78
Dasatinib	+	1.0	1.94	2.28	3.25	4.50
	-	0.87	1.50	1.67	2.07	3.06

Table 4.2 Lymphocyte counts (all $*10^9$/L) according to treatment and CMV serostatus

CMV seropositive dasatinib-treated patients had significantly higher overall lymphocyte counts compared to CMV seronegative patients ($p = 0.0175$, t-test). The median lymphocyte count for CMV seropositive, dasatinib treated patients was also significantly higher than that for the CMV seropositive, imatinib-treated group ($p = 0.0001$), and for

CMV seronegative, imatinib-treated patients (p = 0.0033). As previously stated, within the imatinib-treated group, CMV did not seem to have such a significant effect on the overall lymphocyte count (p = 0.65). There was a non-significant trend for a higher absolute lymphocyte count in CMV positive, dasatinib treated patients versus CMV positive, nilotinib-treated patients (p=0.14). Within the nilotinib treated patients, there was no significant differences between the median overall lymphocyte count in CMV seropositive versus CMV seronegative individuals, although a caveat to this is the low numbers of patients in the nilotinib treated group.

In a general linear model with CMV serostatus and treatment type as fixed factors and age as a covariable, both treatment type (p = 0.02) and CMV serostatus (p = 0.02) were found to be significantly associated with increases in lymphocyte count.

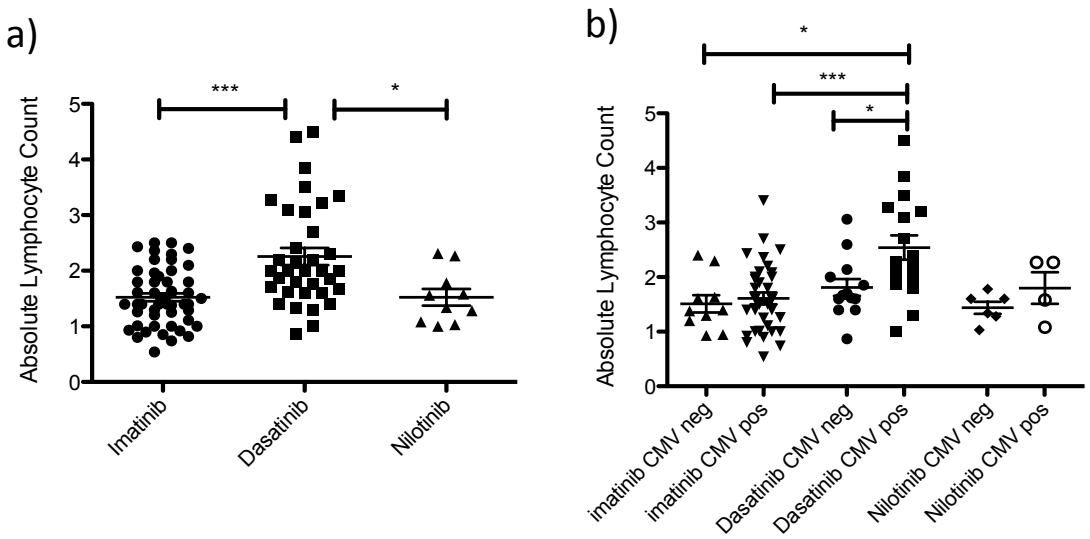


Figure 4.3: CMV seropositivity is associated with a higher absolute lymphocyte count in dasatinib-treated patients

a) Dasatinib-treated patients (n = 30) have higher total lymphocyte counts than imatinib (n = 43) or nilotinib-treated (n = 8) patients (p < 0.0001 versus imatinib-treated patients)

b) This effect is greatest amongst the CMV seropositive group of patients. Dasatinib-treated, CMV seropositive patients had significantly higher lymphocyte counts compared to dasatinib-treated, CMV seronegative patients (p = 0.0175), and imatinib-treated, CMV seronegative (p = 0.0033) amd CMV seropositive patients (p = 0.0001)

The magnitude of the individual Lymphocyte Subsets in relation to CMV serostatus and TKI treatment

We next went on to analyse lymphocyte subsets in different groups of patients according to TKI therapy and CMV serostatus to identify changes in the subsets associated with CMV serostatus.

As I had already demonstrated that CMV had a significant effect on the relative proportions and absolute numbers of different lymphocyte subsets within the imatinib-treated cohort, particularly CD8 T cells and Vδ1 γδ T cells, I investigated whether the same changes were observed within the dasatinib treated cohort.

The panel used for staining cells was the same panel as detailed for the imatinib-treated patients above. All samples were recovered from frozen lymphocytes which had been previously stored at -80 C as detailed in methods section. Samples were washed in PBS, and then resuspended in 100 μl PBS buffer. The vital dye was then added and samples left for 20 minutes, before being washed in MACS buffer and the rest of the antibodies added.

T cell count is increased in dasatinib treated patients, particularly if CMV seropositive

The median T cell count, defined by CD3 positivity, in dasatinib-treated patients was increased compared to both imatinib- ($p = 0.0007$) and nilotinib- ($p = 0.023$) treated patients (Figure 4.4a).

In imatinib-treated, CMV seropositive patients the median T cell count was $1.05*10^9$/L ($0.19*10^9$/L - $1.88*10^9$/L) compared to $1.07*10^9$/L ($0.64*10^9$/L - $1.91*10^9$/L) in CMV seronegative patients. Within nilotinib treated patients, the median CD3 count was

0.98*10^9/L for CMV seronegative patients versus 0.92*10^9/L for CMV seropositive patients. In patients treated with dasatinib, however the median T cell count for CMV seropositive patients was 150% that of CMV seronegative patients, at 1.78*10^9/L (0.63*10^9/L -2.76*10^9/L) compared to 1.17*10^9/L (0.40*10^9/L to 2.22*10^9/L); this difference was statistically significant (p = 0.049, t-test) (Table 4.3, Figure 4.4b).

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.19	0.70	1.05	1.44	1.89
	-	0.64	0.89	1.07	1.36	1.91
Nilotinib	+	0.90	0.90	0.92	1.13	1.13
	-	0.62	0.67	0.98	1.31	1.37
Dasatinib	+	0.63	1.37	1.78	2.03	2.76
	-	0.40	1.03	1.17	1.59	2.18

*Table 4.3 CD3 counts (all *10^9/L) according to CMV serostatus and treatment*

CMV seropositive, dasatinib-treated patients also had higher absolute CD3 counts than imatinib- or nilotinib-treated patients regardless of CMV serostatus (p = 0.0079 versus seronegative, imatinib treated patients p = 0.0001 versus CMV seropositive, imatinib-treated patients p = 0.027 versus nilotinib-treated CMV seronegative, p = 0.045 versus nilotinib-treated, CMV seropositive patients, all t-test). No other differences between the groups were statistically significant. These data show that dasatinib increases the T cell count in the blood of almost all patients but this effect is particularly pronounced in the CMV seropositive group.

Multivariate analysis with a general linear (ANOVA) model showed that dasatinib treatment had a highly significant effect on T cell count (p = 0.004). The effect of CMV serostatus was non-significant in this model (p = 0.27).

CD4:8 Ratios Within Different Treatment Groups

We previously showed that within the imatinib-treated cohort, CMV serostatus had a dramatic effect on the CD4:8 ratio. We went on to analyse the effect that CMV serostatus had within the different treatment groups.

Overall, comparing dasatinib-treated patients to the imatinib-treated group, there was a non-significant trend for a lower CD4:8 ratio within the dasatinib-treated cohort ($p = 0.15$, Mann-Whitney). The median CD4:8 ratio within the dasatinib-treated cohort was 1.69 (0.42 - 6.77) compared to 2.49 (0.60 - 6.64) within the imatinib-treated group (Figure 4.4c).

The median CD4:8 ratio in imatinib-treated, CMV seronegative patients was 3.67 (2.24 - 6.64), compared to 1.83 (0.60 - 5.88) in the imatinib-treated, CMV seropositive group. Within the dasatinib-treated cohort of patients, the median CD4:8 ratio was 3.22 within the CMV seronegative patients (0.69 - 6.77) compared to 1.24 (0.42 - 3.96) for CMV seropositive patients. For patients treated with nilotinib, the median CD4:8 ratio was 1.93 (1.17 - 3.71) for CMV seronegative patients compared to 1.83 (0.61 - 3.02) for CMV seropositive patients (Figure 4.4d).

CMV seropositivity was associated with significantly decreased CD4:8 ratios in both dasatinib-treated ($p = 0.037$, Mann-Whitney) and imatinib-treated ($p = 0.002$, Mann-Whitney) patients. Additionally, there were lower CD4:8 ratios in dasatinib treated, CMV seropositive patients compared to imatinib treated, CMV seropositive patients , but this was not significant ($p = 0.1016$, Mann-Whitney). In a general linear model (ANOVA) with CMV-serostatus and dasatinib versus imatinib-treatment as fixed factors, both treatment ($p = 0.021$) and CMV serostatus ($p < 0.001$) significantly altered the CD4:8 ratio.

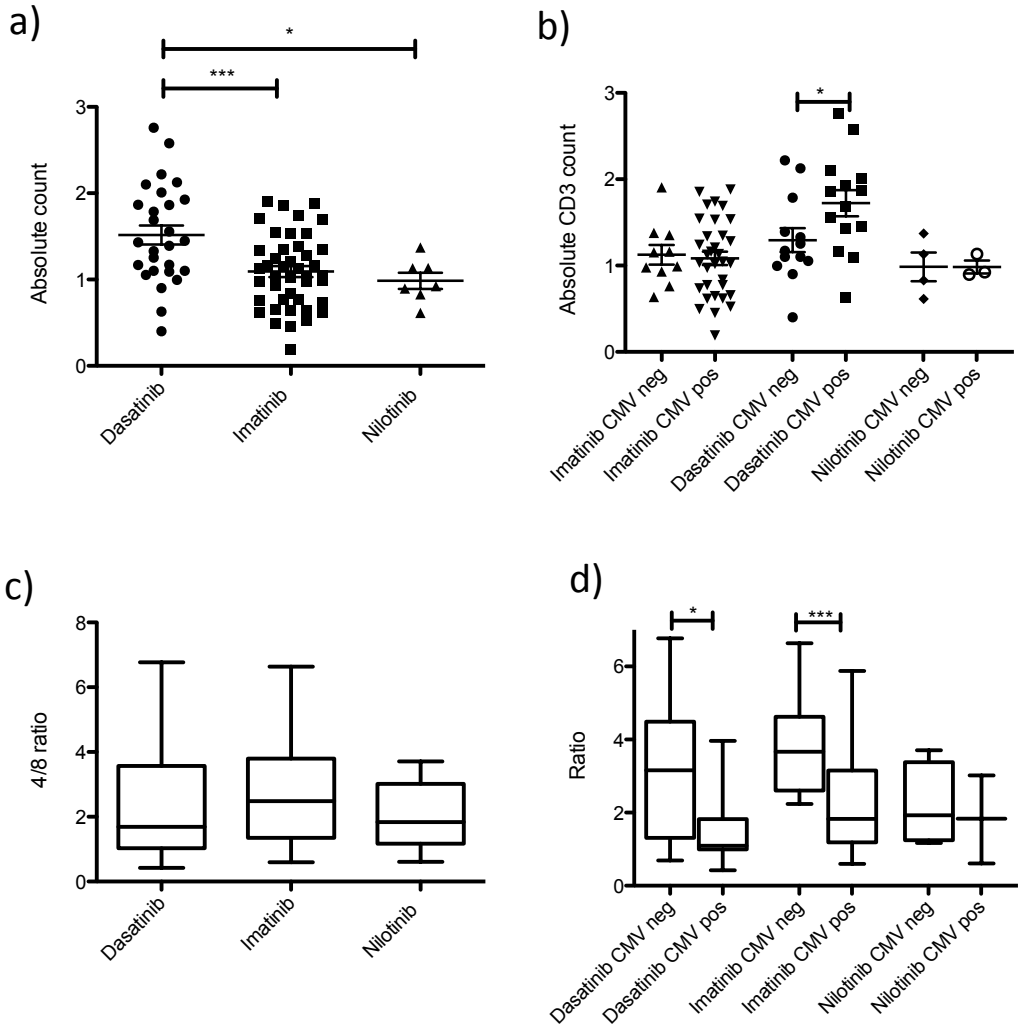


Figure 4.4: Relationship between CMV serostatus, TKI treatment and lymphoid profile

a) The CD3 count in dasatinib (n = 30) treated patients is increased compared to both imatinib (n = 43) (p = 0.0007) and nilotinib (n = 8) (p = 0.0228) treated patients.

b) This difference is most pronounced within the CMV seropositive, dasatinib-treated patients

c) A trend for a decreased CD4:8 ratio is seen in dasatinib-treated patients

d) CMV serostatus, rather than treatment, is the main factor altering in CD4:8 ratio

Relative and absolute CD8+ T cell numbers are increased in dasatinib versus imatinib treated patients

In order to determine if CMV's effects on the CD4:8 ratio were due to increases in CD8 T cells, decreases in CD4 counts, or both, we went on to examine relative and absolute numbers of CD8 and CD4 T cells in the different populations of patients.

Ignoring CMV serostatus, and examining the effect of TKI treatment on the absolute CD8 count, dasatinib-treated patients had median CD8 T cell counts 150% that of those on imatinib ($0.41*10^9$/L versus $0.26*10^9$/L, p = 0.0039, t-test, Figure 4.5a).
CMV seropositive patients had significantly more CD8 T cells compared to CMV seronegative patients (p = 0.023, t test). The median CD8 count in CMV seronegative patients was $0.24*10^9$/L ($0.060*10^9$/L - $0.92*10^9$/L) versus $0.41*10^9$/L ($0.036*10^9$/L - $1.03*10^9$/L) in CMV seropositive patients (Figure 4.5b). The proportion of CD8+ T cells relative to the lymphocyte population as a whole is shown in table 4.4, and Figure 4.5c

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.067	0.13	0.23	0.31	0.42
	-	0.038	0.10	0.11	0.17	0.24
Nilotinib	+	0.15	0.15	0.16	0.45	0.45
	-	0.086	0.096	0.14	0.30	0.34
Dasatinib	+	0.14	0.17	0.26	0.37	0.44
	-	0.069	0.11	0.19	0.28	0.42
Healthy	+	0.13	0.14	0.16	0.26	0.26
	-	0.13	0.13	0.16	0.17	0.17

Table 4.4 Proportions of CD8+ T cells according to treatment and CMV serostatus

The difference between the proportion of CD8 T cells in relation to CMV serostatus was statistically significant in dasatinib-treated patients (p=0.050, Mann-Whitney), as well as imatinib-treated patients (p = 0.0008, Mann-Whitney). There was a non-significant trend for higher relative proportions of CD8 T cells within the dasatinib treated, CMV

seropositive group versus the imatinib treated, CMV seropositive group (p=0.12). There was no difference of CD8 proportions according to CMV serostatus within the nilotinib treated patients. Multivariate analysis using a general linear model showed that CMV serostatus (p = 0.001) had a significant effect on the relative proportion of CD8 T cells. Type of TKI treatment had a non-significant effect (p = 0.065).

I next went on to study the absolute $CD8^+$ T cell count within the different groups. The absolute CD8 count was generated by multiplying the proportion of CD8 T cells by the absolute lymphocyte count (Table 4.5).

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.036	0.21	0.30	0.51	0.91
	-	0.084	0.15	0.17	0.29	0.40
Nilotinib	+	0.11	0.12	0.17	0.50	0.61
	-	0.24	0.24	0.25	0.48	0.48
Dasatinib	+	0.19	0.40	0.50	0.75	1.0
	-	0.06	0.23	0.32	0.48	0.92

Table 4.5 Absolute $CD8^+$ T cell counts ($*10^9/L$) according to CMV serostatus and treatment

Dasatinib-treated patients that were CMV seropositive had more CD8 T cells than those that were CMV seronegative, but this did not reach statistical significance. (p = 0.06, t-test). Dasatinib treated, CMV-seronegative patients had significantly higher CD8 T cell counts than imatinib-treated, CMV seronegative patients (p = 0.027, t –test). Dasatinib-treated, CMV seropositive patients had absolute CD8 counts 60% higher than imatinib-treated, CMV seropositive patients (p = 0.011, t test), see Figure 4.5d

Multivariate analysis showed that both CMV seropositivity (p < 0.001) and treatment with dasatinib (p = 0.001) were both associated with significant increases in CD8 T cell counts, suggesting that dasatinib treatment mobilises CD8 T cells to the peripheral circulation, irrespective of CMV serostatus.

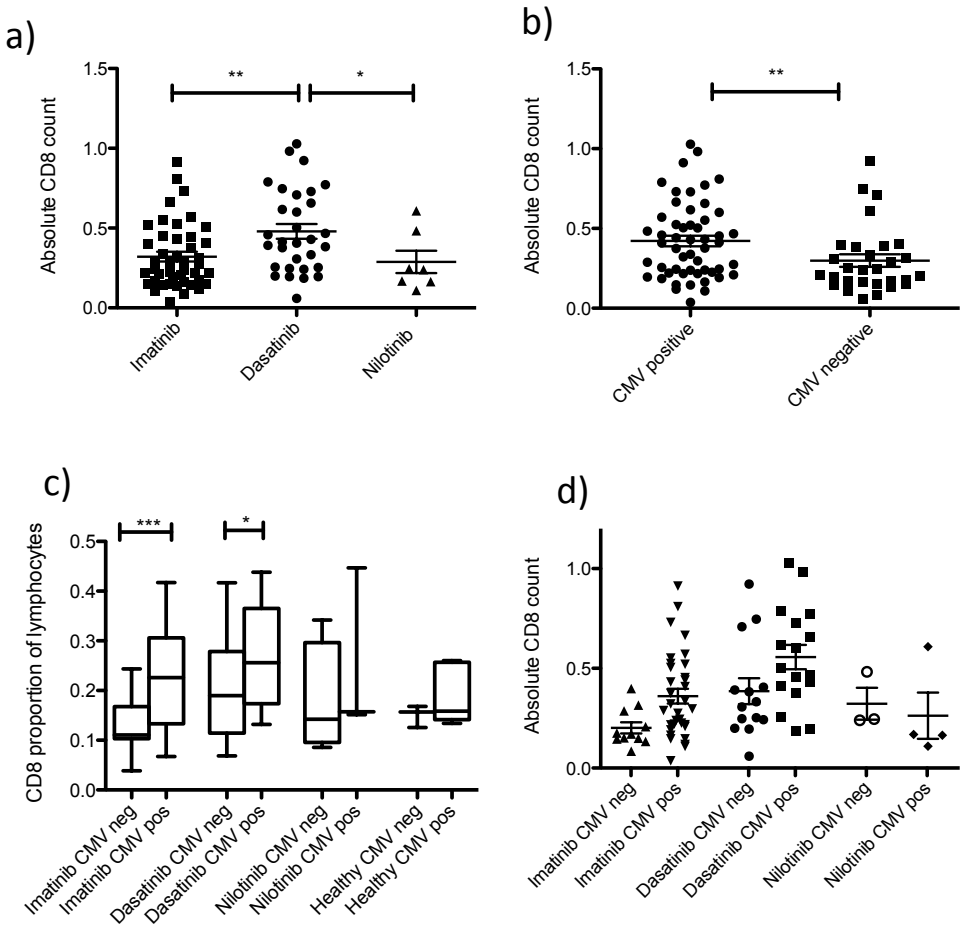


Figure 4.5: CD8 T cell numbers are expanded in dasatinib treated patients

a) The absolute CD8 count is higher in dasatinib (n= 30) treated patients compared to imatinib(n = 43) treated (p = 0.0044) or nilotinib treated (p = 0.0420) patients

b) Taking all patients together, CMV seropositivity is associated with a higher CD8 count (p = 0.0227)

c) CD8 T cells make up a greater proportion of lymphocytes in dasatinib treated patients versus imatinib treated patients, and CMV seropositivity further increases the proportion

d) Both CMV seropositivity (p < 0.001) and treatment with dasatinib (p =0.001) were associated with increases in CD8 T cell count.

Analysis of CD8 Memory Subsets in relation to CMV serostatus and TKI treatment

For the purposes of this analysis, the nilotinib treated patients were excluded due to the small size of the group.

There was no significant difference in the relative proportions of different memory subsets of CD8 T cells between different patient subsets according to treatment alone (Figure 4.6a). A similar pattern was observed within CMV seronegative patients (Figure 4.6b).

However, within CMV seropositive patients, the proportion of TEMRA CD45RA+ CD27- CD8 T cells was significantly increased in dasatinib-treated patients compared to those on imatinib (38.3% versus 42.9%, $p = 0.0385$, Mann-Whitney). There was also a significantly higher proportion of EM CD45RA- CD27- CD8 T cells (13.2% versus 22.3%, $p = 0.024$, Mann-Whitney) in dasatinib-treated patients. The proportion of naïve, CD45RA+ CD27+ CD8 T cells was correspondingly decreased in dasatinib-treated patients compared to imatinib-treated patients (26.8% versus 13.1%, $p = 0.0071$). There was no significant difference in the proportion of CM CD8 T cells between the two groups (Figure 4.6c).

Multivariate analysis showed that CMV serostatus significantly altered the proportions of all memory subsets with the exception of CD45RA- CD27– EM CD8 T cells. Treatment (dasatinib versus imatinib) did not significantly alter the proportion of CD8 memory subsets, although there was a trend towards decreased CD45RA+ CD27+ naïve CD8 T cells in dasatinib-treated patients.

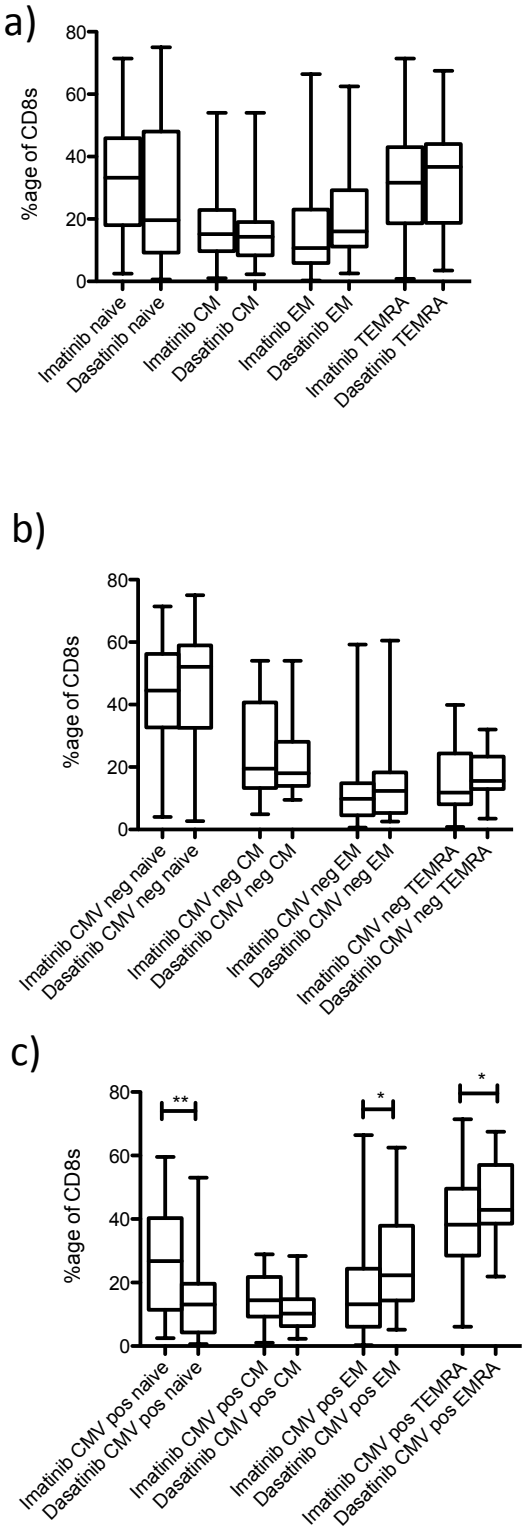


Figure 4.6: Dasatinib-treated CMV seropositive patients have an increase in terminally differentiated CD8 T cells

a) Ignoring CMV serostatus, no difference is seen between imatinib-treated and dasatinib treated patients in relation to CD8 memory subsets.

b) In CMV seronegative patients, there is also no difference in the proportion of different memory subsets of CD8 T cells

c) Within CMV seropositive patients, dasatinib-treated patients have lower proportions of CD45RA+ CD27+ naïve T cells (p = 0.0071), and increased CD45RA- CD27- EM (p = 0.024) and CD45RA+ CD27- TEMRA CD8 T cells (p = 0.0385)

I next went on to examine how CMV serostatus influenced absolute number of CD8 T cells and individual memory subsets.

Ignoring the effect of CMV serostatus, dasatinib-treated patients had significant increases in the numbers of CD45RA- CD27+ CM CD8 T cells (Figure 4.7a, p = 0.011), but no differences in other memory subsets.

Within CMV seronegative cohort, there were non-significant trends for increases in the numbers of all memory subsets with dasatinib treatment compared with imatinib, as illustrated in table 4.6 below and Figure 4.7b

Subset	Imatinib	Dasatinib	p
CD45RA+ CD27+, naïve	0.078	0.17	0.069
CD45RA- CD27+, CM	0.039	0.052	0.059
CD45RA- CD27-, EM	0.013	0.039	0.21
CD45RA+ CD27-, TEMRA	0.035	0.048	0.44

Table 4.6 Memory subsets in CMV seronegative patients, imatinib versus dasatinib (all $*10^9/L$)

However, within the CMV seropositive group, dasatinib treatment was associated with over double the number of CD45RA+ CD27- EMRA (p = 0.0096), and effector memory CD45RA- CD27 - CD8 T cells (p = 0.049) T cells compared to those treated with imatinib (Table 4.7, Figure 4.7c)

Subset	Imatinib	Dasatinib	p
CD45RA+ CD27+, naïve	0.076	0.069	0.80
CD45RA- CD27+, CM	0.050	0.048	0.13
CD45RA- CD27-, EM	0.034	0.090	0.049
CD45RA+ CD27-, TEMRA	0.11	0.24	0.0096

Table 4.7 Memory subsets in CMV seropositive patients, imatinib versus dasatinib (all $*10^9/L$)

Multivariate analysis with a general linear mode showed that treatment with dasatinib and CMV seropositivity were independently associated with significant increases in CD45RA- CD27- EM ($p = 0.020$ for treatment, $p = 0.003$ for CMV seropositivity), and CD45RA+ CD27- EMRA CD8 T cells ($p = 0.02$ for treatment, $p < 0.001$ for CMV seropositivity). Treatment with dasatinib was associated with increases in CD45RA- CD27+ CM cells ($p = 0.037$).

It is known that CMV seropostivity is associated with a more differentiated CD8 T cell phenotype. We have shown that this is true within our patient cohort and that furthermore, dasatinib treatment exaggerates this phenomenon. Compared with imatinib-treated patients, those CMV seropositive patients who take dasatinib had more differentiated CD8 T cells in both relative and absolute terms.

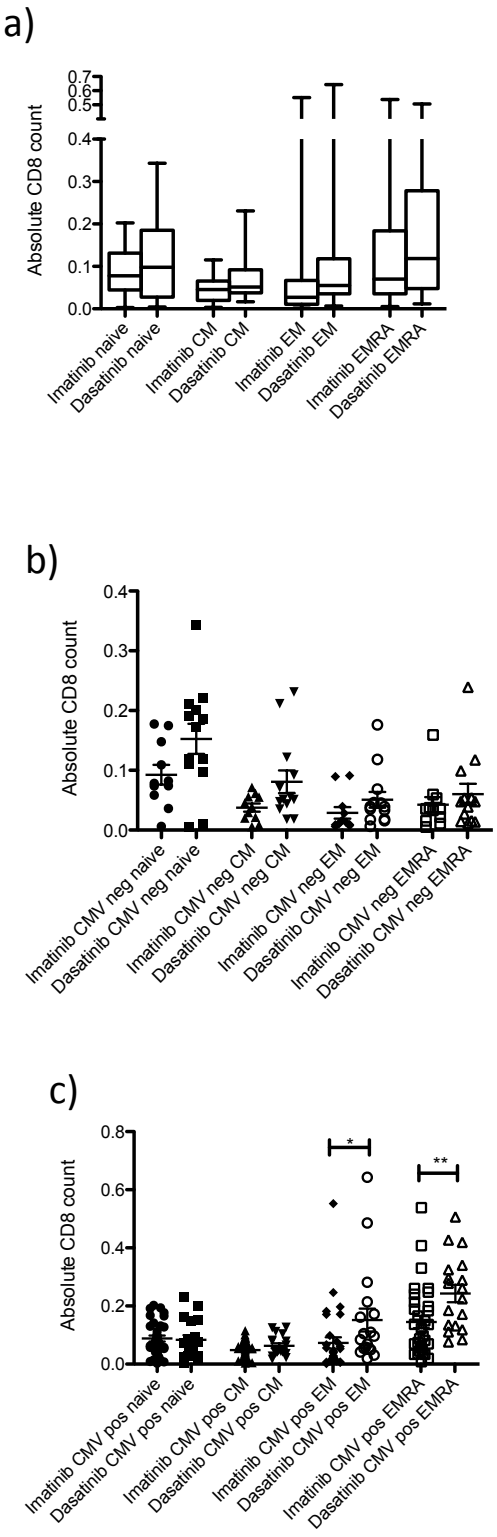


Figure 4.7: Absolute counts of total CD8 T cells and individual memory subgroups

a) Significantly higher numbers of CD45RA- CD27+ CM CD8 T cells are seen in dasatinib treated versus imatinib treated patients (p = 0.011)

b) Within the CMV seronegative group, no significant difference is seen in absolute numbers of CD8 T cells in any specific memory subset

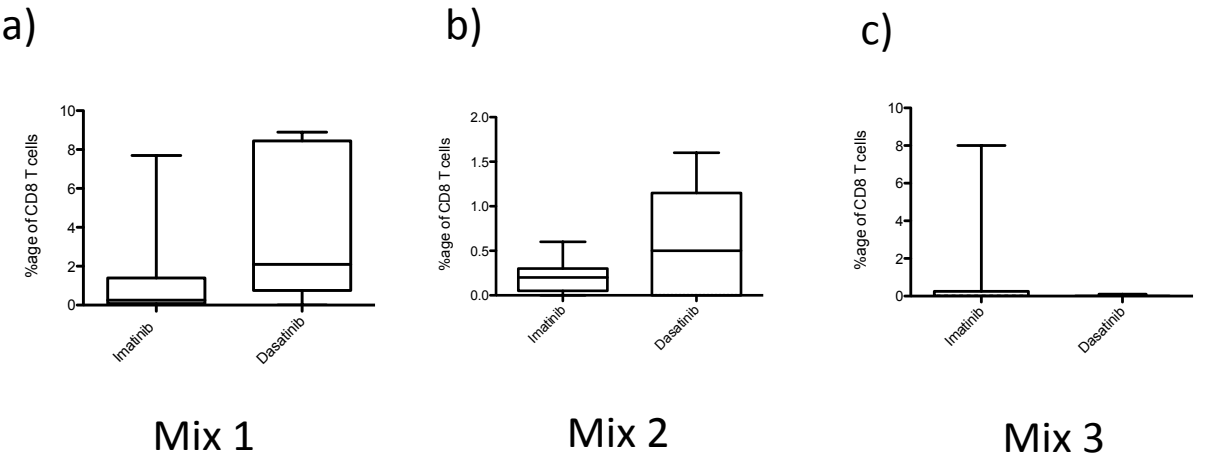
c) Within the group of patients that were CMV seropositive, there were a higher number of CD45RA- CD27- EM (p = 0.049) and CD45RA+ CD27- TEMRA (p = 0.0096) CD8 T cells in those patients taking dasatinib

CMV Specific CD8$^+$ T cell Responses Measured in Dasatinib versus Imatinib Treated Patients

In order to assess whether the increase in CD8 T cells seen in dasatinib-treated patients was due to a non-specific increase in total CD8 T cell subset, or a large number of CMV-specific T cells, I next went on to examine the magnitude of the CMV-specific T cell response by measuring responses to pools of immunogenic CMV peptides (Page 66). Cryogenically frozen PBMCs were defrosted, incubated at 37C with pools of immunogenic CMV peptides for 6 hours. At the end of the incubation period, the cells were washed, stained for surface CD3 and CD8, and then fixed, permeabilised and stained for intracellular IFNγ and TNFα. An unstimulated sample from the patient was run in parallel. The number of cytokine secreting CD8 T cells was measured by flow cytometric techniques, and the unstimulated cells background cytokine release subtracted from the stimulated cells' measured cytokine release. Patients whose blood was sent from external centres was not used in this analysis

Five CMV seropositive, dasatinib-treated patients were studied and contrasted with eight imatinib-treated patients' responses. Dasatinib treated patients had higher total measured CMV-specific CD8 T cells: the median percentage IFNγ producing CD8 T cells was 5.1% versus 1.4% in those treated with dasatinib ($p = 0.019$) There was a trend for dasatinib-treated patients to have higher CD8 T cell secretion of both IFNγ and TNFα to peptides contained in Mix 1 (mainly IE peptides) and Mix2 (mainly pp65 peptides), suggesting that dasatinib-treated patients may exhibit larger CMV-specific CD8 T cell responses (Figure 4.8).

IFNγ release



TNFα release

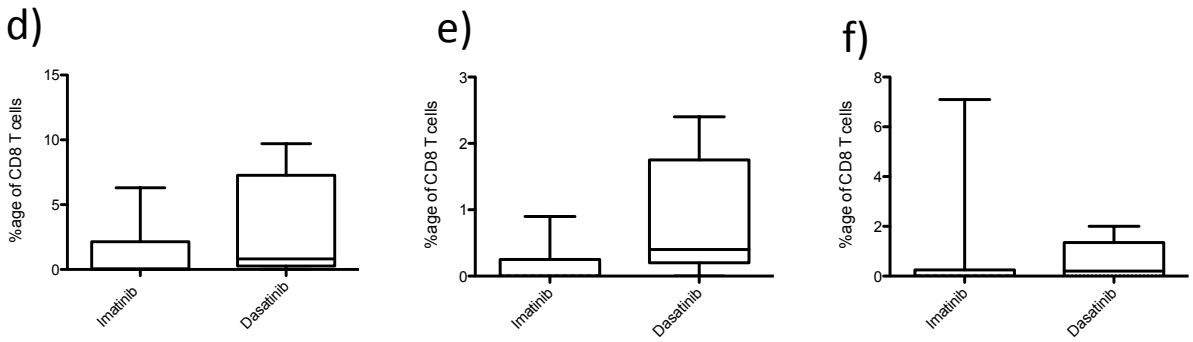


Figure 4.8: CMV-specific CD8⁺ T cell responses in dasatinib and imatinib treated patients measured by IFNγ release and TNFα release to CMV peptide pools

a) A trend for higher IFNγ release in response to peptide pools in Mix1, b) Mix 2 was observed from dasatinib-treated patients' (n = 5) CD8 T cells versus those on imatinib (n = 8) c) No difference in response to Mix3 peptide pools was seen. d) A trend for higher TNFα release from dasatinib-treated patients' CD8⁺ T cells to CMV peptide mixes was seen in Mix1 and e) Mix2, but there was f) no difference in response to mix 3

CD4 T cells in Imatinib versus Dasatinib treated Patients

I next went on to examine the influence of CMV serostatus and TKI treatment on the CD4 T cell populations. Treatment with dasatinib made no difference to the relative proportion or absolute number of CD4 T cells; the median proportion of $CD4^+$ T cells in imatinib treated patients was 43.5% (range 14.2% to 77.6%) versus 40.0% (range 18.6% to 72.0%) in dasatinib treated patients (ns, p = 0.323, Mann-Whitney). The median absolute CD4 was also similar in the two groups count; $0.69*10^9$/L (range $0.15*10^9$/L to $1.41*10^9$/L) versus $0.75*10^9$/L in dasatinib treated patients (range $0.27*10^9$/L to $2.20*10^9$/L), (ns, p = 0.14).

In both imatinib and dasatinib treated patients, CD4 T cells made up proportionally more of the lymphocyte population in CMV seronegative patients (p = 0.0423 and 0.0059, Mann Whitney) (Table 4.8 and Figure 4.9).

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.14	0.31	0.40	0.49	0.78
	-	0.40	0.42	0.53	0.61	0.69
Nilotinib	+	0.27	0.27	0.29	0.46	0.46
	-	0.13	0.19	0.39	0.45	0.47
Dasatinib	+	0.19	0.28	0.33	0.46	0.61
	-	0.23	0.36	0.46	0.58	0.72

Table 4.8 The proportions of $CD4^+$ T cells according to treatment and CMV serostatus

As I had already demonstrated that CMV seropositivity is associated with a relative and absolute increase in CD8 T cell numbers, I then went on to assess whether the difference in the relative proportion of CD4 counts in the CMV seropositive group was associated with an alteration in the absolute CD4 T cell number in the peripheral blood.

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.15	0.40	0.65	0.86	1.10
	-	0.41	0.70	0.79	1.13	1.41
Nilotinib	+	0.29	0.29	0.45	0.72	0.72
	-	0.16	0.22	0.51	0.69	0.71
Dasatinib	+	0.27	0.56	0.76	1.0	1.96
	-	0.31	0.45	0.68	0.96	2.20

Table 4.9 Absolute numbers of $CD4^+$ T cells ($*10^9$/L) according to treatment and CMV serostatus

Within the dasatinib treated group, there was no difference in the CD4 count between CMV seropositive and seronegative patients (p = 0.94, t test). As previously mentioned, the imatinib treated, CMV seropositive patients had lower absolute CD4 T cell counts compared to CMV seronegative patients (p = 0.0099, t test). Interestingly, the dasatinib treated, CMV seropositive patients had higher absolute CD4 counts than the imatinib treated, CMV seropositive patients (p = 0.043, t test).

However, neither CMV serostatus nor treatment with dasatinib compared to imatinib had a significant effect on CD4 count in a general linear model.

Distribution of CD4 T cells memory subsets in relation to CMV serostatus and TKI therapy

As in previous analyses I used CD45RA and CD27 expression to delineate the major memory subsets of CD4 T cells.

CMV serostatus had a marked influence on the distribution of individual CD4 memory subsets. CMV seropositivity is associated with lower proportions of naïve CD45RA+ CD27+ CD4 T cells (p = 0.0098, Mann Whitney), from a median of 43.2% (range 4.4% to 70.3%) to 27.6% (range 0.6% to 64.1%). There were lower proportions of CM CD45RA- CD27+ CD4 T cells (p = 0.019, Mann Whitney) in CMV seropositive patients (33.9%

(range 9.9% to 68.7%) versus 41.5% (range 25.3% to 81.3%)). The proportion of CD45-CD27- EM T cells was increased in CMV positive patients ($p < 0.0001$, Mann Whitney), from 6.5% of CD4 T cells (range 0.7% to 53.6%) to 17.3% (range 3.4% to 65.8%). The proportion of CD45+ CD27- TEMRA CD4 T cells was also increased ($p < 0.0001$, Man Whitney) from 2.90% in CMV seronegative patients (range 0.20% to 28.7%) to 10.7% (range 1.6% to 37.0%) in CMV seropositive patients.

Multivariate analysis with a general linear model showed that CMV serostatus had significant effects on the proportions of CD45RA+ CD27+ ($p = 0.017$), CD45RA- CD27+ ($p = 0.025$), CD45RA- CD27- ($p = 0.003$) and CD45RA+ CD27- ($p < 0.001$) subsets. Treatment with dasatinib versus imatinib did not affect any of them. This data shows that, while CMV serostatus alters the distribution of CD4 memory subsets, this is not modified by choice of TKI.

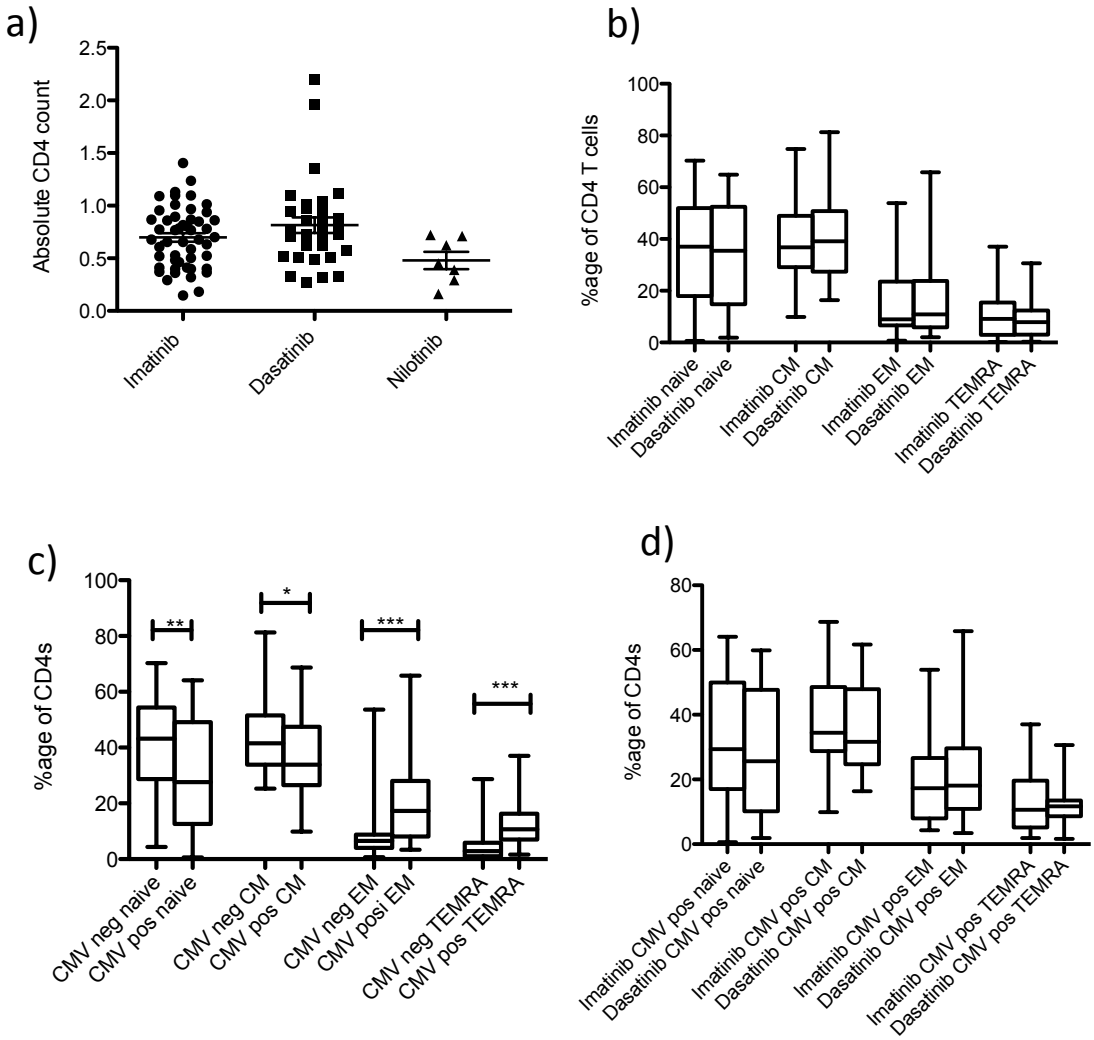


Figure 4.9: CMV seropositivity, but not treatment influences the memory phenotype of CD4 T cells

a) No significant difference was observed in absolute CD4 T cell count in dasatinib-treated patients compared to those on imatinib or nilotinib b) Treatment also makes no difference to the proportions of CD45RA + CD27+ naïve, CD45RA- CD27+CM, CD45RA- CD27- EM or CD45RA+ CD27- TEMRA CD4 T cells c) CMV seropositivity reduces the proportion of naïve ($p = 0.0098$) and central memory ($p = 0.019$) CD4 T cells, whilst increasing the proportion of EM ($p < 0.0001$) and TEMRA ($p < 0.0001$) CD4 T cells. d) Within the CMV seropositive group, there is no exaggeration of this effect in patients who take dasatinib, in contrast to the effect seen within the CD8 T cell group.

Analysis of γδ T cells within dasatinib-treated patients

Gamma-delta T cells are a subset of innate-like lymphoid cells that have been shown to be influenced by CMV serostatus (Lafarge et al., 2001).

Comparing dasatinib-treated (n = 30) patients to imatinib-treated patients (n = 43), γδ T cells made up a markedly greater proportion of the lymphocyte population within the dasatinib cohort; specifically they comprise 2.5% of the lymphoid pool compared to only 1.4% in the imatinib-treated patients (p = 0.0018, Mann-Whitney, Table 4.10, Figure 4.10a). There was no significant difference in the patients treated with nilotinib (n = 9) compared to those treated with imatinib.

	Minimum	**25th percentile**	**Median**	**75th Percentile**	**Maximum**
Imatinib	0.0015	0.0064	0.014	0.032	0.19
Nilotinib	0.0062	0.011	0.016	0.079	0.11
Dasatinib	0.0053	0.016	0.025	0.054	0.21

Table 4.10 γδ T cells as a proportion of the total lymphocyte population according to treatment

Dasatinib-treated patients had over twice as high absolute γδ T cell counts (p = 0.0051, Table 4.11, Figure 4.10b). There was no significant difference between nilotinib and imatinib-treated patients (p = 0.29), although a trend for increased γδ T cell numbers was seen.

	Minimum	**25th percentile**	**Median**	**75th Percentile**	**Maximum**
Imatinib	0.0019	0.012	0.020	0.047	0.37
Nilotinib	0.0083	0.016	0.047	0.13	0.17
Dasatinib	0.013	0.027	0.05	0.13	0.36

Table 4.11 Absolute γδ T cell count according to treatment (10^9/l)*

It then became of interest to see how CMV serostatus influenced this profile. Marked differences were seen between the different treatment groups. CMV seropositivity was associated with greatly increased total γδ T cell count within dasatinib treated patients. ($0.08*10^9$/L versus $0.03*10^9$/L, p = 0.0088, t-test). No such increase was seen in patients treated with imatinib (Table 4.12, Figure 4.10c). Because of small patient numbers, nilotinib-treated patients were not included in this analysis.

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.0019	0.011	0.019	0.068	0.37
	-	0.0030	0.013	0.028	0.042	0.069
Dasatinib	+	0.017	0.041	0.080	0.20	0.36
	-	0.013	0.019	0.031	0.050	0.14

Table 4.12 Absolute γδ T cell counts ($*10^9$/L) in imatinib and dasatinib treated patients according to CMV serostatus

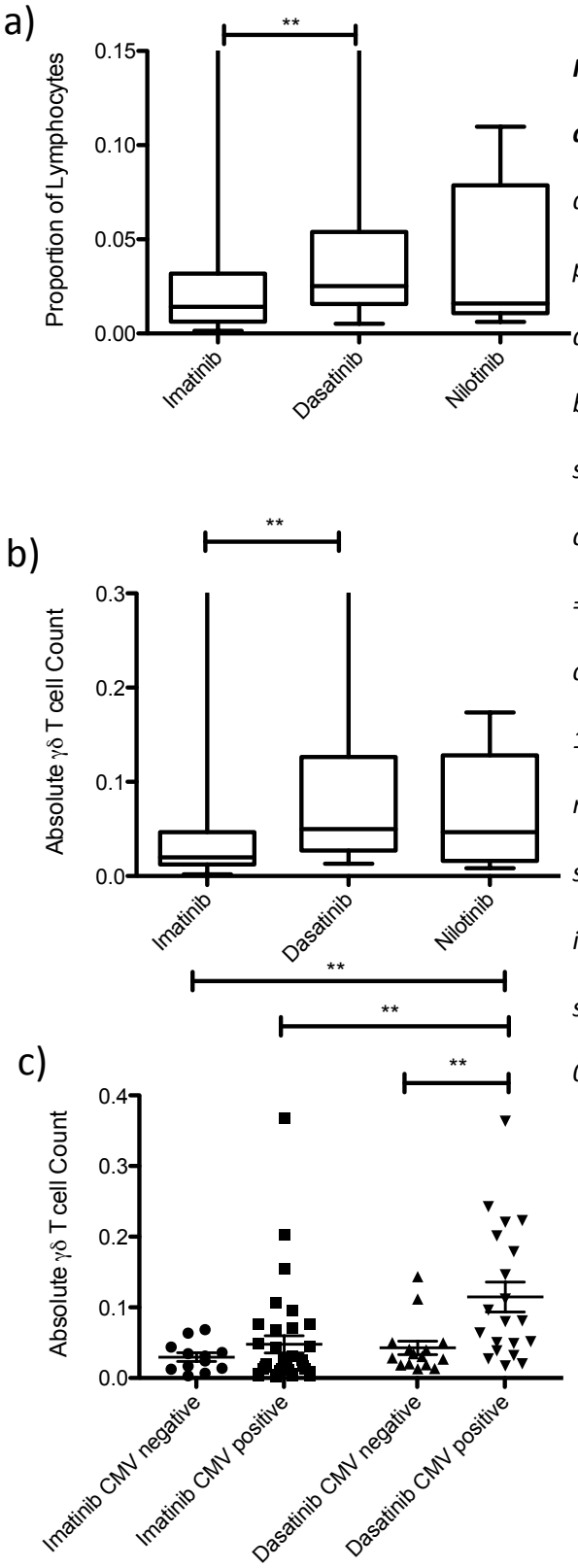


Figure 4.10: γδ T cell numbers are increased in dasatinib-treated CMV seropositive patients

a) γδ T cells make up a significantly greater proportion of the lymphocyte population in dasatinib-treated patients (p = 0.0018)

b) Dasatinib-treated patients (n = 30) have significantly increased absolute numbers of γδ T cells (p = 0.0051) compared to those on imatinib (n = 43)

c) Dasatinib treated CMV seropositive patients (n = 17) have significantly increased total γδ T cell numbers compared with dasatinib-treated CMV seronegative (n = 13) patients (p = 0.0088), and imatinib- treated patients, whether CMV seropositive (p = 0.0046) or CMV seronegative (p = 0.0048)

Analysis of individual γδ T cell subsets within patient groups

I had already demonstrated that CMV seropositive, imatinib-treated patients had higher proportions and absolute numbers of Vδ1 γδ T cells. I was interested to examine how this influenced the distribution of different γδ T cell subsets according to TKI treatment. As an initial assessment, I examined the relative proportion and number of Vδ1 γδ T cells.

As a proportion of CD3 T cells, there were significantly more γδ T cells of the Vδ1 subset in dasatinib-treated patients compared to the imatinib treated group ($p = 0.0070$, Mann Whitney, Figure 4.11a). The median proportion of Vδ1s within dasatinib treated patients was 1.0% (0.2% - 14.1%) compared to only 0.06% (0.1% - 31.5%) in imatinib treated patients. Within the imatinib treated group, there was one patient with a large percentage of 31.5%. This subject appeared to be an "outlier" but was included within the analysis. Within both the dasatinib- and imatinib-treated groups, CMV seropositivity was associated with a greater proportion of Vδ1 γδ T cells ($p = 0.003$ within dasatinib treated group, $p= 0.0018$ within imatinib group, t-test, Table 4.13 and Figure 4.11b). However, again, the dasatinib-treated, CMV seropositive group had a greater proportion of Vδ1 γδ T cells than the imatinib-treated CMV seropositive group ($p = 0.0041$). There was a non-significant trend for higher γδ T cells in the nilotinib-treated, CMV seropositive group compared to those that were CMV seronegative but this did not reach statistical significance ($p = 0.53$). However, there were low numbers of patients treated with this TKI (n=3 CMV seropositive, 4 CMV seronegative).

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.002	0.004	0.008	0.02	0.32
	-	0.001	0.002	0.003	0.005	0.012
Nilotinib	+	0.003	0.003	0.007	0.13	0.13
	-	0.009	0.011	0.016	0.044	0.053
Dasatinib	+	0.003	0.01	0.026	0.065	0.14
	-	0.002	0.005	0.006	0.008	0.014

Table 4.13 Vδ1 γδ T cells as a proportion of T cells

Analysis of the absolute numbers of Vδ1 γδ T cells within the treatment groups

I next went on to calculate the absolute number of Vδ1 γδ T cells within the groups by multiplying the proportion of Vδ1 γδ T cells determined by FACS by the T cell fraction of the total lymphocyte count, and then multiplying this by the absolute lymphocyte count.

There was a higher absolute Vδ1 count in dasatinib treated versus imatinib treated patients ($p = 0.0051$). The median count in imatinib treated patients was $0.0069*10^9$/L ($0.00057*10^9$/L - $0.37*10^9$/L) versus $0.014*10^9$/L ($0.000802*10^9$/L - $0.363*10^9$/L) in dasatinib treated patients (Figure 4.11c).

Dasatinib-treated, CMV seropositive patients had significantly higher absolute Vδ1 γδ T cell counts than dasatinib-treated, CMV-seronegative patients ($p = 0.011$, t test). Counts were also higher than imatinib-treated, CMV seropositive patients ($p = 0.019$, t test). Imatinib treated, CMV seropositive patients had a non-significant trend towards higher absolute Vδ1 counts versus imatinib treated, CMV seronegative patients ($p = 0.22$). Interestingly, dasatinib-treated, CMV seronegative groups also had a higher absolute δ1 count versus imatinib treated, CMV seronegative patients ($p = 0.028$); see Figure 4.11d and Table 4.14. Again, a trend for a similar pattern as that seen in patients treated with

dasatinib was seen amongst nilotinib-treated patients. Low numbers of patients in this group made meaningful comparisons impossible.

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.00057	0.0040	0.0097	0.026	0.37
	-	0.00064	0.0035	0.0038	0.0069	0.013
Nilotinib	+	0.0027	0.0027	0.0079	0.12	0.12
	-	0.0075	0.010	0.020	0.030	0.032
Dasatinib	+	0.0051	0.010	0.059	0.10	0.36
	-	0.00080	0.0057	0.0081	0.014	0.017

Table 4.14 Absolute numbers of Vδ1 γδ T cells according to treatment and CMV serostatus (10^9/L)*

A multivariate analysis with a general linear model demonstrated that both CMV seropositivity ($p = 0.002$) and treatment with dasatinib ($p = 0.013$) were associated with increased Vδ1 γδ T cells.

This suggests that dasatinib treatment serves to increase the number of these cells in the peripheral blood, independent of CMV serostatus. The combination of CMV seropositivity and treatment with dasatinib exaggerates the increase seen in CMV seropositive patients.

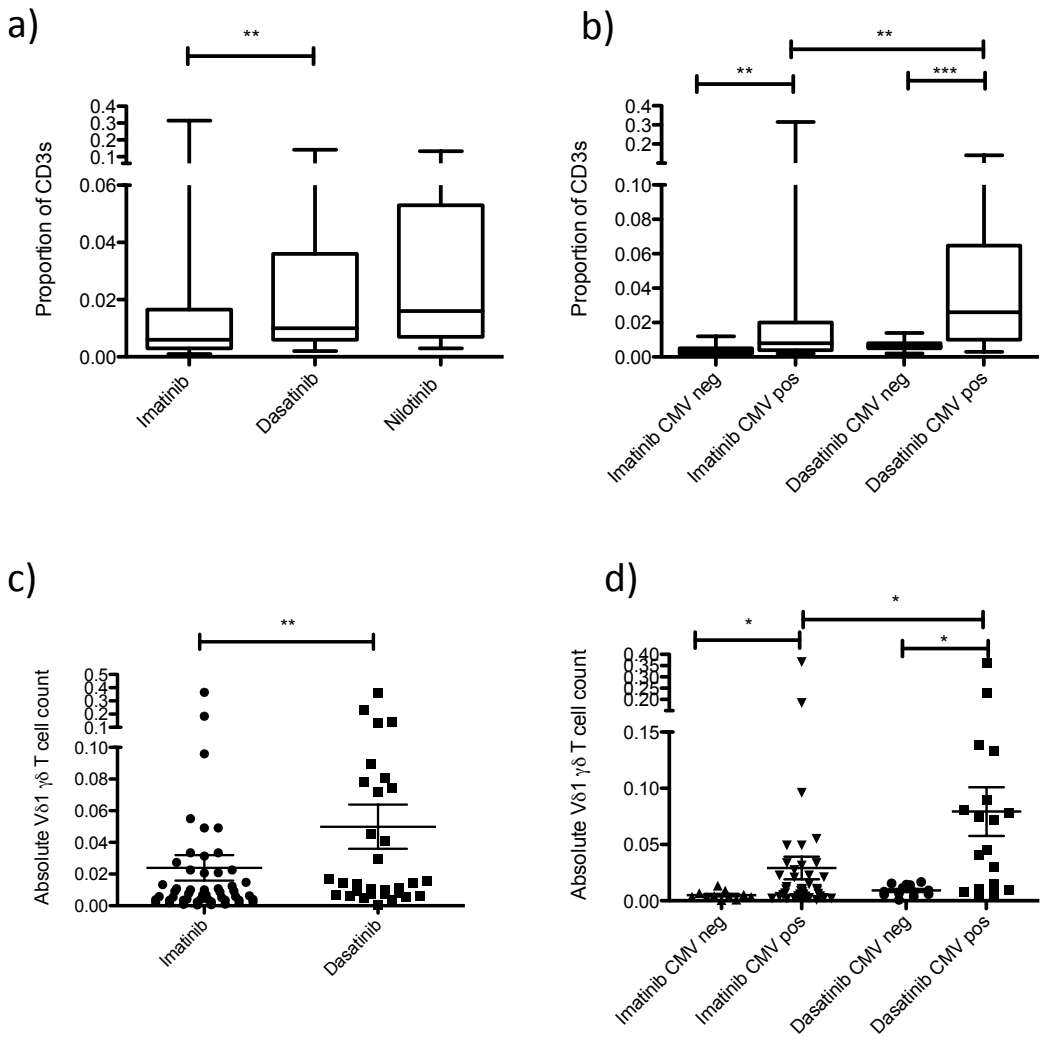


Figure 4.11: CMV seropositivity and dasatinib treatment are associated with increased Vδ1 γδ T cells

a) Dasatinib-treated patients exhibit a significantly increased proportion of Vδ1 γδ T cells within their total T cell population (p = 0.0070). b) CMV seropositivity is associated with more Vδ1 γδ T cells in both imatinib- (p = 0.0018) and dasatinib-treated (p = 0.003) patients. There are relatively more Vδ1 γδ T cells in dasatinib treated CMV seropositive patients versus imatinib treated CMV seropositive patients (p = 0.0041). c) Dasatinib treatment is associated with significantly increased absolute Vδ1 γδ T cells compared with imatinib-treated patients (p = 0.0051) d) dasatinib-treated, CMV seropositive patients have higher absolute Vδ1 γδ T cell counts than imatinib-treated, CMV seropositive patients (p = 0.019)

Memory Subsets of Vδ1 γδ T cells

I have previously demonstrated that CMV seropositivity causes an increase in the proportion and absolute number of CD45RA+ CD27- Vδ1 γδ T cells in imatinib-treated patients, and I went on to investigate whether this was influenced by dasatinib treatment.

I initially examined patients according to the type of treatment that they were on (dasatinib versus imatinib) regardless of CMV serostatus. There was no significant difference in proportions of any if the memory subsets in imatinib versus dasatinib treated patients (Figure 4.12a).

Within the whole group of patients, CMV seropositivity was associated with a significant decrease in the proportion of naïve CD45RA+ CD27+ Vδ1 T cells ($p = 0.004$, Mann-Whitney), and central memory CD45RA- CD27+ Vδ1 T cells ($p < 0.0001$, Mann-Whitney), accompanied by an increase in the proportion of EMRA δ1 T cells ($p = 0.0003$, Mann Whitney) (Figure 4.12b).

Next I compared the effect of treatment on CMV seropositive and CMV seronegative patients separately, to assess if the effects of CMV seropositivity were exaggerated within the dasatinib-treated group of patients. There was no significant difference in the proportions of different memory subsets of Vδ1 γδ T cells between imatinib and dasatinib treated patients regardless of CMV serostatus.

Although dasatinib treatment makes no difference to the proportions of different memory subsets of Vδ1 γδ T cells, we hypothesized that it may make a difference to the absolute numbers of different memory subsets of Vδ1 γδ T cells in the peripheral blood, as the absolute lymphocyte count is increased in patients taking dasatinib.

Within the CMV seronegative patients, treatment with imatinib versus dasatinib made no significant difference to the absolute numbers of different memory subsets of Vδ1 γδ T cells (Figure 4.12c). However, within CMV seropositive patients, dasatinib treatment was associated with a higher absolute number of CM CD45RA- CD27+ Vδ1 γδ T cells (p = 0.0075, t test), CD45RA- CD27- EM Vδ1 γδ T cells (p = 0.040, t test) and CD27- CD45RA+ TEMRA Vδ1 γδ T cells (p = 0.0045, Mann-Whitney), as illustrated in Figure 4.12d and Table 4.15

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.00007	0.0011	0.0044	0.014	0.36
	-	0.00012	0.00026	0.0017	0.0027	0.0052
Dasatinib	+	0.011	0.0044	0.032	0.077	0.22
	-	0.00033	0.00062	0.0012	0.0069	0.012

*Table 4.15 Accumulation of CD45RA+, CD27- Vδ1 γd T cells (all *10^9/L) in dasatinib-treated CMV seropositive patients*

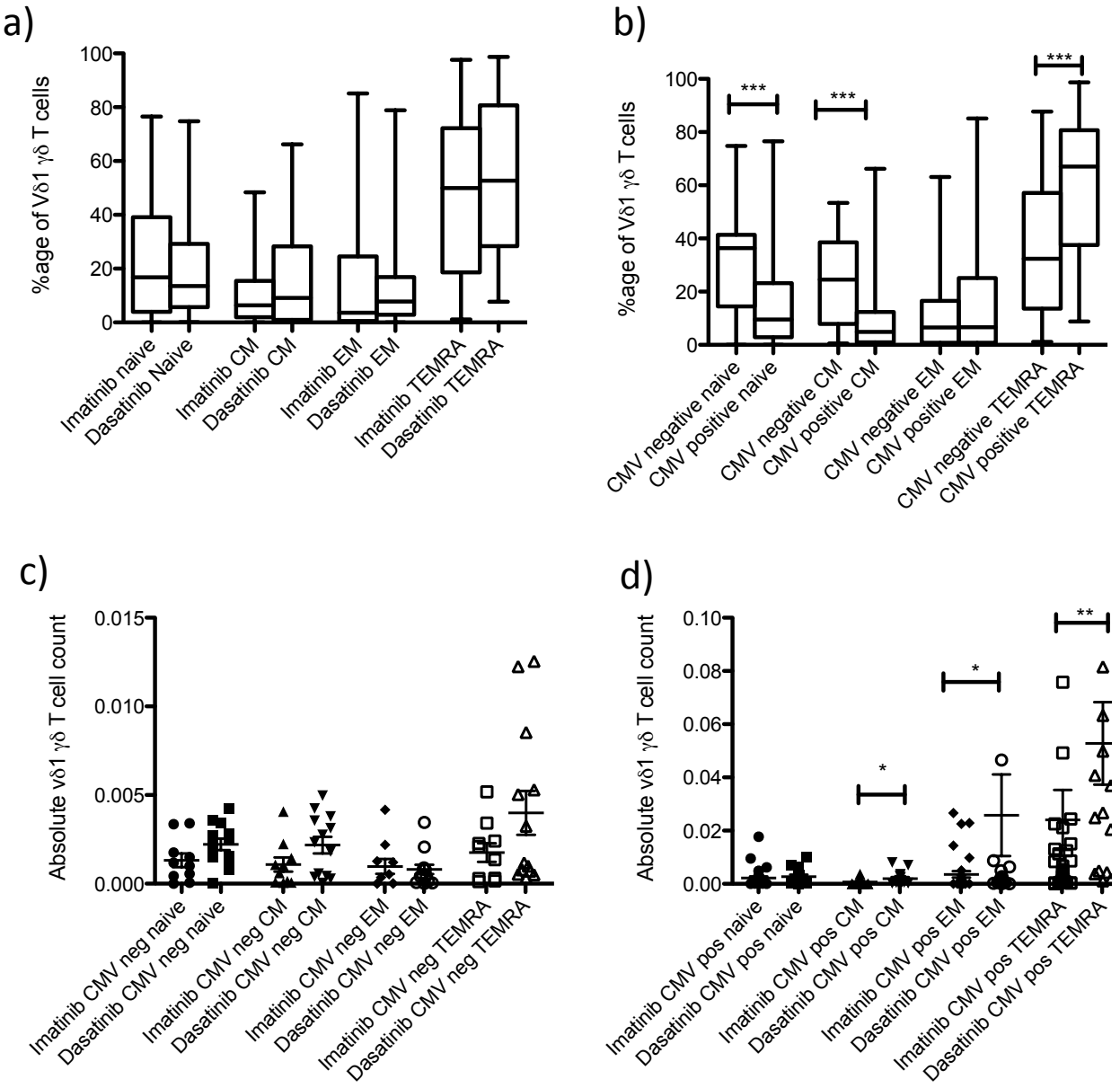


Figure 4.12: CMV serostatus and TKI treatment in relation to memory subsets of Vδ1 γδ T cells

a) Treatment does not alter the proportions of different memory subsets of Vδ1 γδ T cells b) CMV seropositivity is associated with a significant decrease in the proportion of CD45RA+ CD27+ naïve (p = 0.004) and CD45RA- CD27+ central memory (p < 0.0001) Vδ1 γδ T cells, with an increase in CD45RA+ CD27- TEMRA Vδ1 γδ T cells (p = 0.0003) c) Within the CMV seronegative group, treatment with dasatinib makes no difference to the absolute numbers of different memory subsets of Vδ1 γδ T cells. Within the CMV seropositive group, dasatinib treatment is associated with an increase in the absolute numbers of CM (p = 0.0075), EM (p = 0.040) and TEMRA (p = 0.0045, Mann-Whitney) vδ1 γδ T cells

Effect of Dasatinib and CMV Serostatus on Vδ2 γδ T cells

In the next section I went on to perform a similar analysis on the Vδ2 γδ T cell subset. No significant difference was seen in the proportion of Vδ2 γδ T cells (expressed as a proportion of CD3 T cells) between imatinib and dasatinib treated patients (Figure 4.13a). The median proportion of Vδ2 γδ T cells was 1.20% (range 0 to 7.6%) in imatinib treated patients versus 1.85% (range 0% to 24.2%) in dasatinib treated patients (p = 0.17, Mann-Whitney). Furthermore, CMV serostatus within the patient treatment subsets made no difference to the proportions of Vδ2 γδ T cells (Figure 4.13b and Table 4.16).

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0	0.004	0.010	0.024	0.076
	-	0.001	0.008	0.016	0.029	0.056
Nilotinib	+	0	0.0013	0.0075	0.11	0.14
	-	0.001	0.0013	0.002	0.018	0.023
Dasatinib	+	0	0.005	0.0075	0.029	0.12
	-	0.01	0.012	0.020	0.035	0.24

Table 4.16 Vδ2 γδ T cells as a proportion of $CD3^+$ T cells

CMV Serostatus Makes No Difference to Absolute numbers of Vδ2 γδ T cells

Looking next at absolute numbers of Vδ2 γδ T cells, calculated by multiplying the proportion of Vδ2 γδ T cells (of total lymphocytes) by the absolute lymphocyte count, there was a non-significant trend for higher numbers of Vδ2 γδ T cells in dasatinib treated patients (p = 0.1, t test), as illustrated in Figure 4.13c.

	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	0.00050	0.0050	0.010	0.032	0.11
Nilotinib	0.00083	0.0019	0.0058	0.052	0.17
Dasatinib	0	0.0097	0.020	0.037	0.20

Table 4.17 Absolute numbers of Vδ2 γδ T cells according to treatment (all $*10^9/l$)

However, there was no significant difference between CMV seropositive or seronegative patients in dasatinib, imatinib or nilotinib treated patients (p = 0.94, 0.40 and 0.16 respectively, all t-test). Multivariate analysis with a general linear model demonstrated that neither CMV seropositivity (p = 0.52) nor treatment with dasatinib (p = 0.31) was associated with changes in the Vδ2 γδ T cell count (Figure 4.13d and Table 4.18).

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.00050	0.0042	0.0085	0.022	0.11
	-	0.0059	0.0084	0.026	0.044	0.057
Nilotinib	+	0.0090	0.0090	0.087	0.17	0.17
	-	0.00083	0.0012	0.0025	0.011	0.014
Dasatinib	+	0	0.0058	0.015	0.040	0.20
	-	0.0016	0.013	0.025	0.036	0.13

Table 4.18 Absolute numbers of Vδ2γ9 γδ T cells according to treatment and CMV serostatus (all $*10^9$/L)

I looked at CD45RA and CD27 expression on Vδ2 γδ T cells to assess changes in the distributions of memory subsets in the different groups. Neither type of treatment nor CMV serostatus made any difference to the proportions of memory subsets.

In summary, CMV seropositivity, and dasatinib treatment, are associated with an increase in the relative proportion and absolute number of Vδ1 γδ T cells. CMV seropositivity is associated with a skewing of the memory phenotype towards a terminally differentiated (CD45RA+, CD27-) phenotype. CMV serostatus has no effect on the phenotype or numbers of Vδ2 γδ T cells.

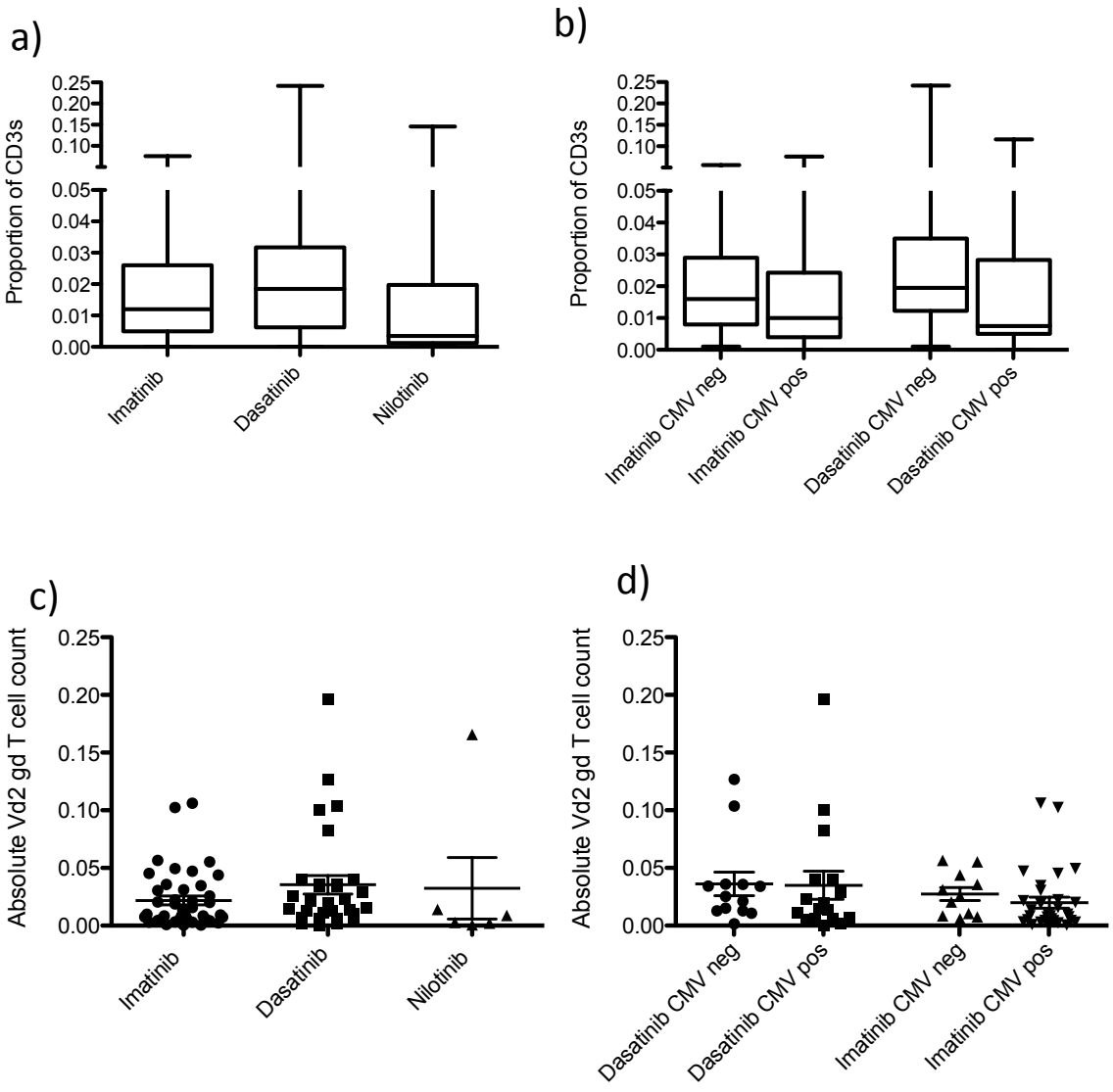


Figure 4.13: The proportion and number of Vδ2 γδ T cells is not influenced by TKI treatment or CMV serostatus *a) No significant difference is seen in Vδ2 γδ T cells as a proportion of T cells in dasatinib versus imatinib treated patients b) CMV serostatus has no impact on the proportion of Vδ2 γδ T cells c) There is no difference in absolute Vδ2 γδ T cell counts in dasatinib treated patients d) CMV serostatus makes no difference to the absolute Vδ2 γδ T cell count in any treatment group*

Natural Killer Cells

Natural killer cells are the major effector cells of the innate immune system and it was felt to be important to study how their numbers were influenced by TKI treatment and CMV serostatus. $CD56^{dim}$ NK cells have been shown to expand in dasatinib treated patients (Mustjoki et al., 2009).

There were no differences in median $CD56^{dim}$ NK cell count between different groups according to treatment subtype or CMV serostatus, in either relative or absolute terms (Figure 4.14). There were, however four patients in the dasatinib-treated, CMV seropositive group with significant $CD56^{dim}$ NK cell expansions; the maximum number of $CD56^{dim}$ NK cells in a dasatinib-treated patient was $2.01*10^9$/ L, which was almost 4 times as high as the maximum count seen in an imatinib-treated patient (Figure 4.14d, Table 4.19)

	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
Imatinib	+	0.026	0.084	0.27	0.38	0.53
	-	0.021	0.058	0.15	0.31	0.79
Nilotinib	+	0.27	0.27	0.40	0.45	0.45
	-	0.039	0.079	0.22	0.28	0.29
Dasatinib	+	0.043	0.085	0.19	0.46	2.01
	-	0.054	0.12	0.16	0.26	0.35

Table 4.19 The absolute $CD56^{dim}$ NK cell count according to treatment and CMV serostatus ($*10^9$/l)

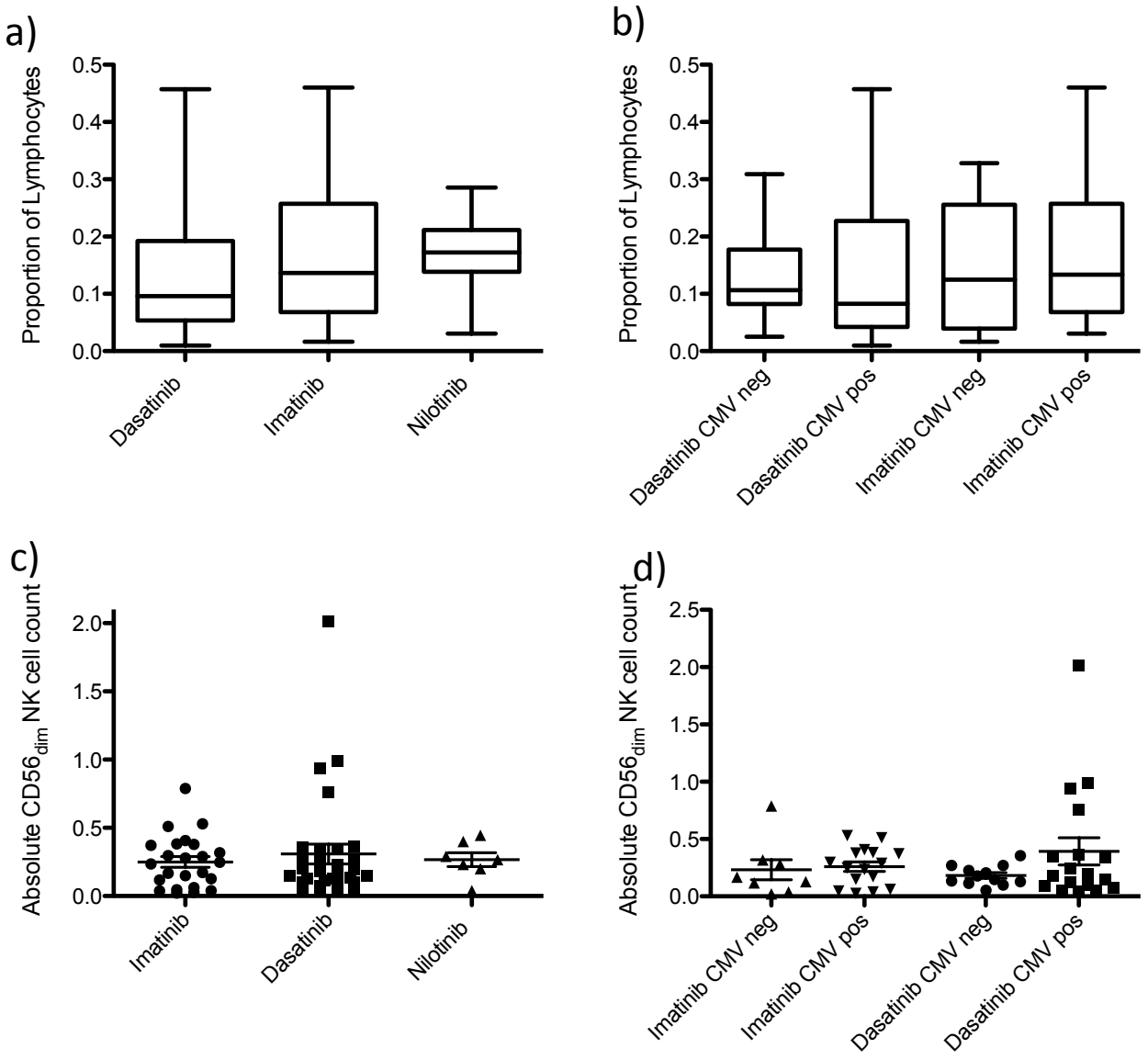


Figure 4.14: CD56^{dim} NK cell count and treatment

a) Type of TKI treatment made no significant difference to the proportion of CD56^{dim} NK cells, b) CMV serostatus made no significant difference to the proportion of CD56^{dim} NK cells c) Some patients in the dasatinib treated cohort had NK cell expansions, but the median NK cell count was not significantly different between the different treatment groups d) The patients with NK cell expansions within the dasatinib treated group were all CMV seropositive. The median NK count did not differ between different groups of patients according to CMV serostatus or treatment

CMV reactivation in dasatinib treated patients

My data had indicated that several lymphoid subsets that are increased in CMV seropositive patients are further amplified during treatment with dasatinib. This raises the possibility that the drug may lead to subclinical reactivation of the virus, leading to a "boost" in cellular immunity. In order to examine this hypothesis I went on to look at the antibody response to CMV in different groups. I started by measuring serum CMV specific IgM within patients. This might be expected to reflect recent reactivation, and has been shown to have a similar sensitivity to PCR methods (Jahan et al., 2008). This revealed that a number of patients had strong IgM responses, which may reflect recent CMV reactivation. All of the patients with detectable CMV IgM were also positive for CMV IgG, indicating that this was not a primary infection.

Of the patients with CMV IgM positivity, four (QE10, QE18, QE02 and Glasgow12) were undergoing with dasatinib. One patient with CMV IgM seropositivity was on imatinib. One nilotinib treated patient was also weakly positive for CMV IgM.

I went on to detect for the presence of CMV DNA from DNA extracted from PBMCs, on 20 CMV IgG seropositive patients. CMV DNA was not detected in any patients, including, interestingly, all of the patients who were positive for CMV IgM.

Patients with CMV IgM Seropositivity have higher numbers of Vδ1 γδ T cells

4 out of the 6 patients with detectable CMV-specific IgM antibody were undergoing treatment with dasatinib. We therefore compared the proportions of CD8, CD4 and Vδ1 γδ T cells in these patients relative to CMV seropositive, dasatinib-treated, patients who did not have a detectable CMV-specific IgM response.

IgM seropositivity was not associated with any difference in the absolute $CD8^+$ T cell count (p = 0.53, t test). The median CD8 count in patients with CMV IgM positivity was $0.72*10^9$/L ($0.19*10^9$/L - $1.05*10^9$/L) versus $0.60*10^9$/L ($0.20*10^9$/L - $0.98*10^9$/L) in patients that were CMV IgM negative (Figure 4.15a). CMV IgM seropositivity was not associated with any changes in the proportions of individual memory subsets.

A non-significant trend for a lower absolute CD4 count was observed in patients with CMV IgM compared to those without (p = 0.26). Specifically, the median CD4 count in patients that were positive for IgM was $0.51*10^9$/L (range $0.44*10^9$/L to $1.01*10^9$/L) versus 0.77 $*10^9$/L (range $0.27*10^9$/L to $1.96*10^9$/L) in patients that were IgM negative. There was also no effect of IgM positivity on the NK count in this cohort (p = 0.93).

Patients who were positive for anti-CMV IgM had significantly higher numbers of circulating Vδ1 γδ T cells (p = 0.014) compared to the IgM negative group. Specifically, the median Vδ1 T cell count in dasatinib treated patients with detectable IgM was threefold higher at $0.16*10^9$/L($0.041*10^9$/L - $0.36*10^9$/L) versus 0.046 $*10^9$/L ($0.0051*10^9$/L - $0.14*10^9$/L) in patients that were CMV IgG positive but IgM seronegative (Figure 4.15b).

The memory phenotype of the Vδ1 γδ T cells was also compared between the two groups (Figure 4.15 c). There was a significant increase in the absolute count of CD45RA+ CD27- EMRA Vδ1 γδ T cells (p = 0.0024, t test) The median Vδ1 CD27- CD45RA+ EMRA count in the IgM seropositive patients was four times that of IgM seronegative patients at 0.10 $*10^9$/L (0.037 $*10^9$/L - $0.22*10^9$/L) versus $0.025*10^9$/L (0.0017 $*10^9$/L - $0.063*10^9$/L).

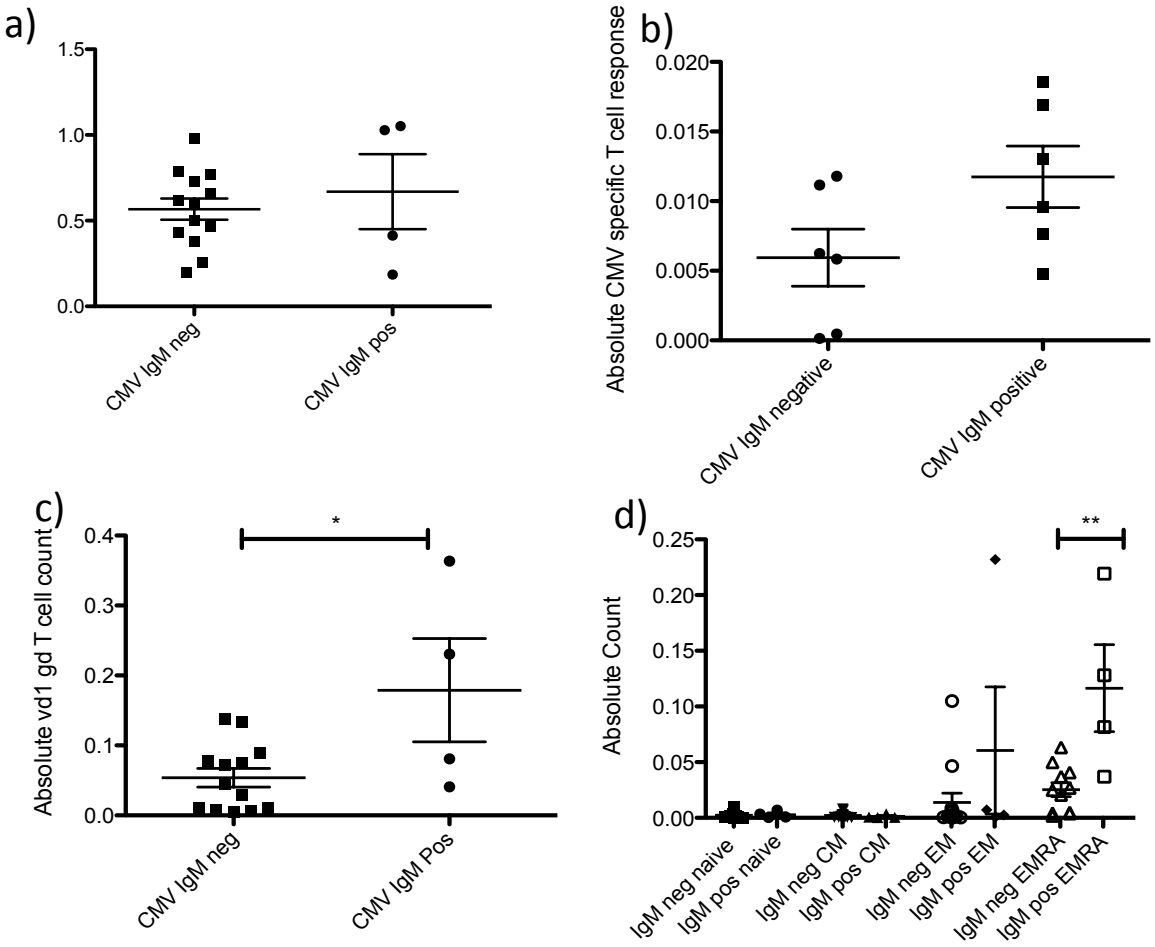


Figure 4.15: Patients seropositive for both CMV IgG and IgM have higher absolute Vδ1 γδ T cell counts

a) There is no difference in the absolute CD8 T cell count between patients positive for CMV IgM/ IgG (n = 4) and those positive for CMV IgG only (n = 13) b) A non-significant trend for a higher total CMV-specific CD8 T cell response in patients dual positive for CMV IgG/IgM compared to those seropositive for IgG only c) significantly higher absolute Vδ1 γδ T cell count in patients positive for CMV IgM/IgG (p = 0.014) d) The largest expansion of Vδ1 γδ T cells are in the CD45RA +, CD27- EMRA compartment (p = 0.0024).

Further Characterisation of Vδ1 γδ T cells in Patients with CMV IgM Seropositivity

In order to further characterize the Vδ1 γδ T cells in patients with expansions, we went on to study Vγ chain usage (Vγ9 positive versus negative) as well as expression of CD69, CD4, CD8, CCR7 and CD45RA. This panel was used to sort Vδ1Vγ9 T cells versus Vδ1nonVγ9 T cells and these cells were used for TCR repertoire analysis.

γ Chain usage in Vδ1 γδ T cells

A diversified TCR dependent mechanism of action has been hypothesized for non Vδ2 γδ T cells.(Willcox et al., 2012). The chain usage of paired Vγ chains is likely to be important in determining target specificity (O'Brien and Born, 2010). In all of the patients that had significant expansions of Vδ1 γδ T cells, the majority of the Vδ1 γδ T cells were coupled with nonVγ9 chains. The median percentage of Vδ1Vγ9 T cells was 33.4% (range 5% to 39.9%) versus 66.6% (range 60.1% to 95%) Vδ1Vnonγ9 T cells (Figure 4.16a).

CD8αα expression

CD8αα is a homodimer whose precise function is uncertain, but may facilitate expansions of certain virus-specific αβ T cells in vivo (Zhong and Reinherz, 2005). It is expressed on a subset of Vδ1 γδ Tcells which are usually associated with tissue epithelia (Davey et al., 2011), and which have been shown to have anti-leukaemia properties (Scheper et al., 2013). There was variable expression of CD8αα on the Vδ1 γδ T cells. Of the patients with the expansion, vδ1 γδ T cells made up between 5.11% and 21.41% (median 13.42%) of CD8 T cells.

CD69 Expression on Vδ1 γδ T cells

CD69 has been used as a marker of T cell activation (Halary et al., 2005) and is expressed on T cells transiently after encountering antigen. Measuring the median

fluorescence intensity of the CD69 antibody revealed significant differences between the different subsets. Specifically, the median fluorescence intensity of CD69 expression on the CD8 T cell population was 101.0 (range 70 to 142), versus 231 (range 151 to 390) in the Vδ1 T cells population (Figure 4.16b). This difference was statistically significant ($p = 0.029$, Mann-Whitney).

There was a non significant trend for the median MFI of CD69 in the Vδ1nonVγ9 γδ T cells, at 281 (258 - 368), to be higher than that of the Vδ1Vγ9 γδ T cells, at 38 (0 - 375) versus ($p = 0.3$, Mann-Whitney) (Figure 4.16c).

Thus, in the patients with the Vδ1 γδ T cell expansion, the majority of the γδ T cells are Vδ1nonVγ9 T cells, and these cells express higher levels of the activation marker CD69 compared with the Vδ1Vγ9 γδ T cells. Furthermore, these cells make up a significant proportion of the CD8 T cells in these patients. The majority of the Vδ1 γδ T cells in both CMV IgM seropositive and negative patients were CD45RA+ CD27- TEMRA cells. The proportions of these did not change between the two groups (Figure 4.16d).

These data suggest that patients with serological evidence of recent CMV reactivation have expanded CD45RA+ CD27- Vδ1 γδ T cell. The majority are coupled with a nonVγ9 chain. A large proportion of them express CD8, to the extent that they make up significant numbers of the CD8 T cell population in these patients. They have evidence of recent activation, as shown by their increased CD69 expression compared to the background CD8 T cell population.

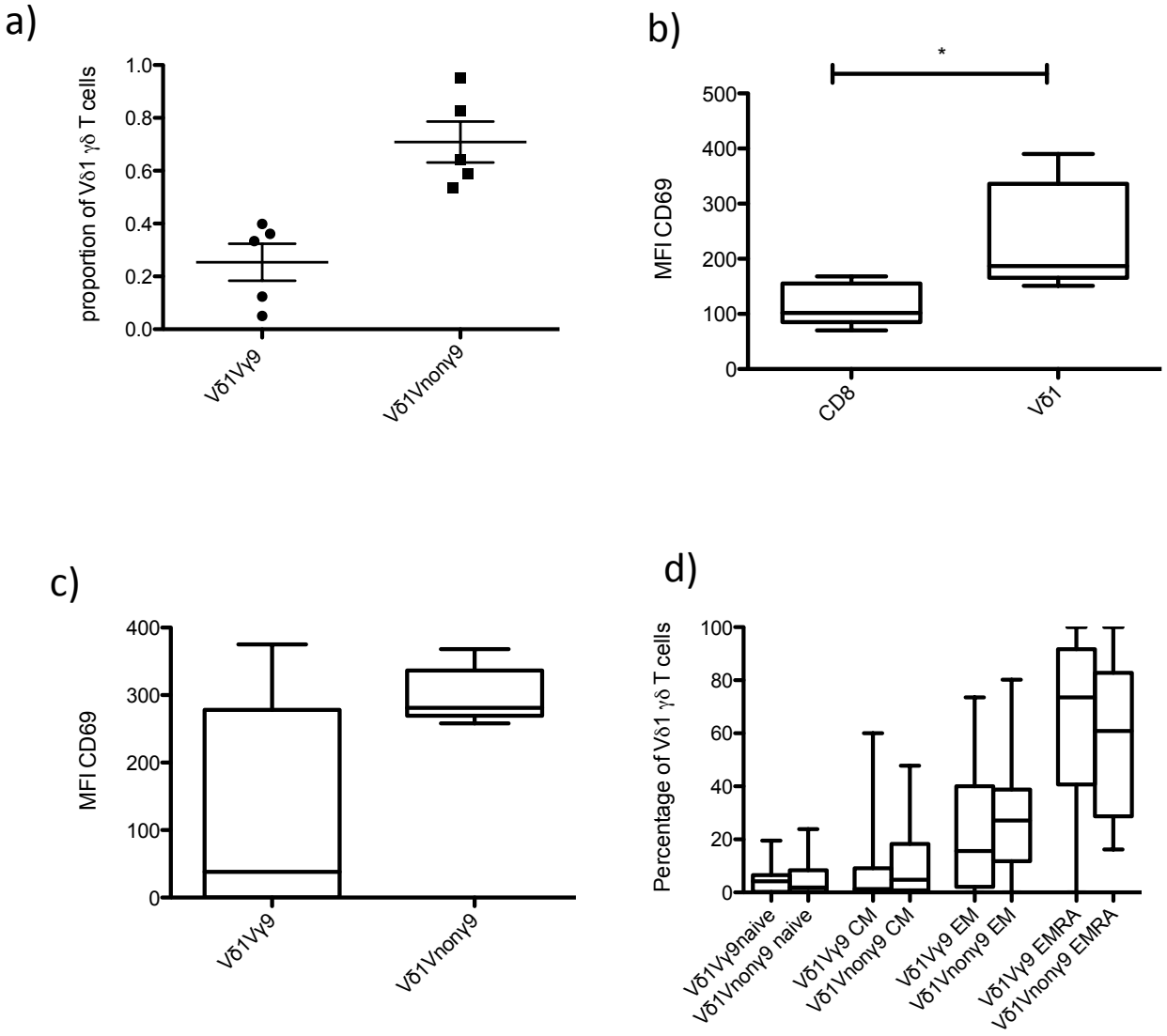


Figure 4.16: Vδ1 γδ T cells in Patients with CMV IgM Seropositivity

a) Most Vδ1 γδ T cells in patients with Vδ1 expansions are composed of Vδ1Vnonγ9 T cells, with a small population of Vδ1Vγ9 γδ T cells.

b) CD69 expression is higher on Vδ1 γδ T cells compared to the background CD8 population (p = 0.0426)

c) Non significant trend for increased CD69 expression on the Vδ1Vnonγ9 population compared to the Vδ1γ9 population

d) No significant difference is seen in the proportions of different memory subsets of Vδ1γ9 or Vδ1nonγ9 T cells, based on CD45RA and CD27 expression

CMV Specific CD8 T cell Responses in CMV IgM Positive Patients

In order to assess if the presence of CMV-specific IgM antibody was a reflection of subclinical viral reactivation, I then went on to study the magnitude of the CMV-specific T cell responses within this group. HLA peptide CMV protein tetramers consisting of peptide-MHC complexes expressing immunodominant peptides from CMV peptides pp65, pp50 and IE1 were used to assess CMV specific T cell responses, as described in the methods section. The panel used for assessing tetramer responses comprised anti-CD3, anti-CD8, anti-CD27, and CD45RA, anti-CD45RO, anti CCR7, anti CD28, anti CD4. We compared the tetramer responses of patients that were CMV IgG positive, IgM negative, with patients that were dual positive for CMV IgG and IgM.

There was an increased proportion of CMV-specific CD8 T cells in anti-CMV IgM positive patients (Figure 4.17a). The median proportion of CMV-specific CD8 T cells within the group with evidence of CMV reactivation was 1.13% (0.48% - 1.86%) compared to 0.57% (0.015% - 1.40%) in patients that were negative for CMV IgM ($p = 0.044$, Mann Whitney).

As most of the patients that reactivated CMV were in the dasatinib-treated group, I also compared CMV IgM positive patients with CMV IgG positive, IgM negative patients that were treated with dasatinib. The total tetramer response was added together, and compared. All responses greater than 1% of the CD8 T cell population were anti-pp65 responses. The patients with the higher overall tetramer response were largely within the IgM positive group (Figure 4.17b), suggesting that it was the presence of CMV IgM, presumed secondary to CMV reactivation that was driving the tetramer response rather than the treatment itself. There was a non-specific trend for a higher percentage of effector memory CD45RA- CD27- tetramer positive CD8 T cells in the CMV IgM seropositive group. (Figure 4.17c).

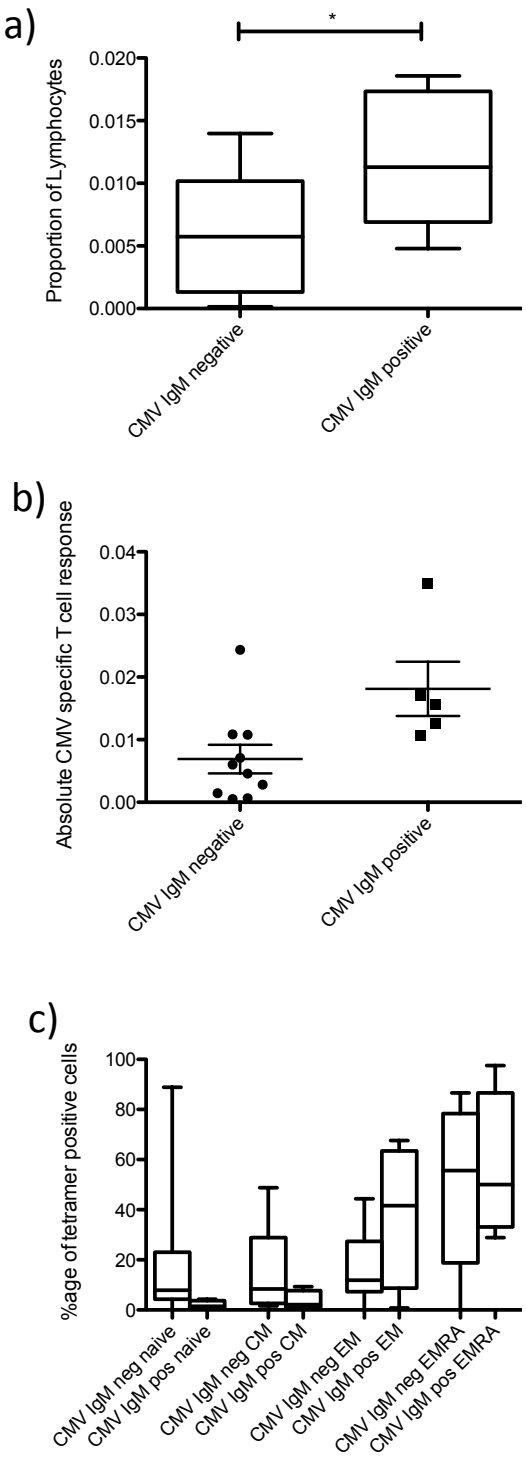


Figure 4.17: CMV-Specific CD8 T cell response measured by HLA-peptide CMV tetramers

a) In CMV IgM/IgG dual positive patients, the CMV tetramer response makes up proportionately more of the lymphocyte population compared to patients that are CMV IgG positive

b) The absolute CMV specific CD8 T cell response as measured by the tetramer response shows a trend towards higher values in CMV IgM positive patients

c) A non-significant trend for higher CD45RA- CD27- effector memory and CD45RA+ CD27- terminally differentiated CMV specific CD8 T cells in was also seen in CMV IgM positive patients

Quantitative EBV PCR and TKI treatment

In order to assess whether CMV was the main factor causing the immune changes seen in these patients, it was important to rule out other causes. One potential cause was poor control of EBV, a gamma herpes virus which is known to be under immune surveillance and which can reactivate in immunosuppressed individuals (Fox et al., 2013, Parvaneh et al., 2013).

To this end, quantitative EBV PCRs were carried out on a number of patients on dasatinib, imatinib and nilotinib. Because the range of PCRs was wide, they were logarithmically transformed and a t-test used to compare the populations.
This showed that dasatinib-treated patients had significantly higher EBV viral loads as measured by quantitative PCR compared to imatinib treated patients ($p = 0.038$, Figure 4.18a).

Patients that were seropositive for CMV IgM did not have Raised EBV PCRs

In order to assess if patients who had indirect evidence of CMV reactivation as measured by CMV specific IgM response had worse control of herpes viruses in general, the EBV PCRS of patients with CMV IgM and IgG dual positivity were compared with patients with CMV IgG positivity. There was a non-significant trend for a lower EBV PCR in CMV IgM seropositive patients (Figure 4.18b). The median EBV PCR for the patients that were CMV IgG and IgM positive was 126 copies/10^5 cells(range 0 to 262). The median EBV PCR for patients that were CMV IgG positive, but IgM negative was 678.0 copies/10^5 cells (range 0 to 8620). As above, the log of the PCRs was calculated and these were compared. The difference was not statistically significant ($p = 0.3313$, Figure 4.18b).

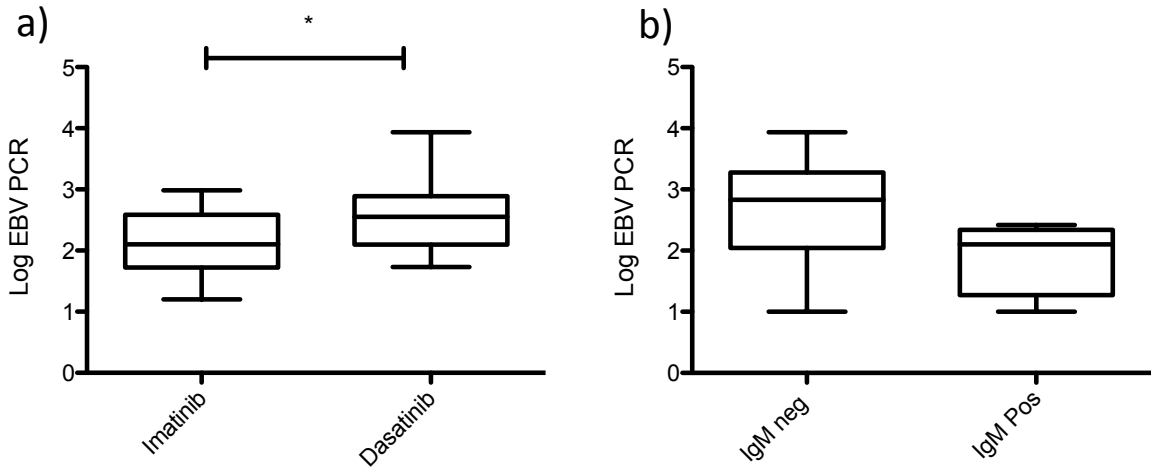


Figure 4.18: EBV PCR level is higher in dasatinib-treated patients, but not in patients positive for CMV IgM

a) Of patients with detectible EBV PCR, the median log EBV PCR was higher in dasatinib-treated patients compared to imatinib-treated patients (p = 0.038)

b) The patients with the highest detectible EBV PCRs were in the CMV IgM negative group.

CMV serostatus and TKI treatment are associated with significant increases in Vδ1 γδ T cells and CD8 T cells, whereas EBV viral load is associated with increased Vδ2 γδ T cells

Because of the complex nature of the dataset, and to ensure the absence of confounding factors, we analysed the data a general linear model. CMV serostatus and treatment type were treated as fixed factors with EBV PCR as a covariate.

CMV seropositivity was associated with significantly higher numbers of Vδ1 γδ T cells and CD8 T cells, whereas increased EBV PCRs were associated with higher numbers of Vδ2 γδ T cells. Treatment with dasatinib was associated with increased numbers of total lymphocytes, CD8 T cells and Vδ1 γδ T cells. EBV PCR did not have any significant relationship with the levels of Vδ1 γδ T cells, CD8 T cells or on the memory phenotypes of the different T cell subsets.

Lymphocyte subset	CMV serostatus	Treatment Type	EBV PCR
Absolute Count	0.084	0.001	0.17
CD8 T cells	0.001	<0.001	0.60
CD4 T cells	0.21	0.144	0.10
Vδ1 γδ Tcells	0.002	0.014	0.47
Vδ2 γδ T cells	0.34	0.052	0.03

Table 4.20 General linear model of lymphocyte subsets including CMV serostatus, Treatment type and EBV PCR (all numbers are p values)

Functional analysis of γδ T cells from TKI treated patients

As my previous work had shown that TKI treatment had a direct influence on the number and phenotype of γδ T cell subsets, I then went on to assess their functional capacity. Functional analysis was performed on γδ T cells from 6 patients treated with TKIs. Peripheral blood mononuclear cells were prepared as documented in the methods

section, and then enriched for γδ T cells using a MACS negative selection kit (pp 54). The sorted lymphocytes were then rested overnight in 500 μl of RPMI plus human serum media. Enriched γδ T cells were then stimulated with PMA and ionomycin in order to assess their pattern of cytokine stimulation (page 55). It has been demonstrated previously that Vδ1 γδ T cells are variously able to secrete IL17 (Wakita et al., 2010), TNFα and IFNγ (Devaud et al., 2009).

I demonstrated that following PMA and ionomycin stimulation, the majority of the Vδ1 γδ T cells from TKI treated patients are able to secrete IFNγ. I did not see any evidence of IL17 production, although there was some production of TNFα. Specifically, the median percentage of Vδ1 γδ T cells secreting IFNγ in response to PMA was 56.1% (0.7% - 96.9%). 1.7% of Vδ1 γδ T cells secreted TNFα (0.2 - 5.8%). There was no secretion of IL2 or IL17 in any experiments (Figure 4.19a).

In some functional assays, CD107a was added to assess if Vδ1 γδ T cells had capacity to degranulate and have direct cytotoxic activity. There was variable success with these assays, as PMA frequently caused cell death. However, up to 23.5% of Vδ1 T cells were able to secrete CD107a with PMA stimulation (median 1%, range 0.1% to 23.5%).

The pattern of cytokine release for Vδ2 γδ T cells was similar to that seen in Vδ1 γδ T cells. The majority of Vδ2 T cells secreted IFNγ with PMA stimulation. The median proportion of IFN secreting Vδ2 γδ T cells was 60% (range 26.6% to 93.4%). The median proportion of TNFα secreting cells was 21% (range 0 to 34%). No Vδ2 γδ T cells secreted IL17 or IL2 in response to PMA (Figure 4.19b).

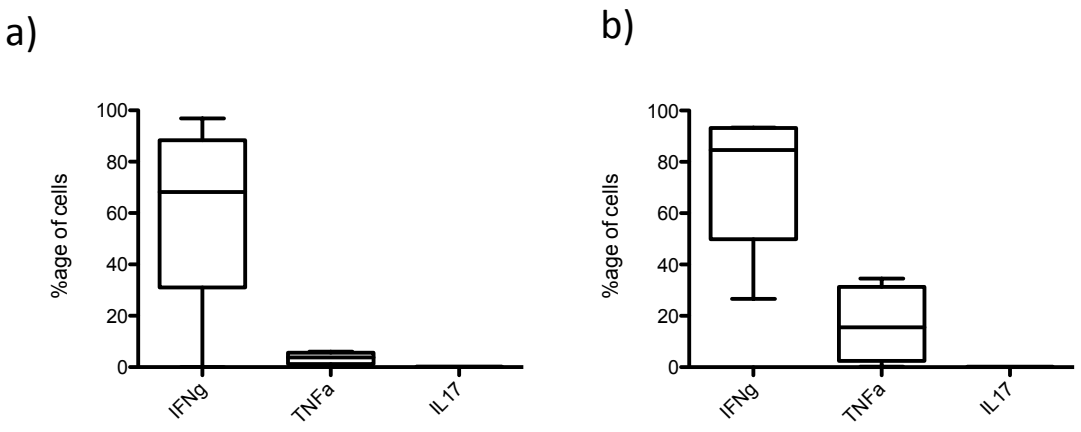


Figure 4.19: Pattern of cytokine production by γδ T cells

a) Vδ1 γδ T cells demonstrate predominant expression of IFNγ in response to PMA/ionomycin. There is less TNFα production compared to vδ2 γδ T cells. No IL17 production was elicited

b) Vδ2 γδ T cells are capable of secreting IFNγ in response to PMA/ionomycin stimulation

γδ T cell Recognition of CMV Infected Fibroblasts

In order to assess whether γδ T cells could recognize CMV infected fibroblasts, we first created a model of CMV infection by infecting cultured human fibroblasts as described in the methods section, page 46. Briefly, human fibroblast cell lines were seeded into a 24-well plate at $6*10^4$ cells/well. Two wells per sample were left uninfected as controls. Sorted patient γδ T cells were added to the fibroblasts, incubated for 6 hours at 37C, and subsequently stained for IFNγ production as described elsewhere.

Vδ1 γδ T cells were able to produce IFNγ after incubation with CMV infected fibroblasts (Figure 4.20a). Of six patients, the median number of IFNγ secreting nonVδ2 γδ T cells was 1.7% (minimum 0, maximum 9.1%). There was no significant secretion of IFNγ to CMV-infected fibroblasts in any experiment performed by Vδ2γ9 γδ T cells (Figure 4.20b).

γδ T cell recognition of K562 cell Lines

In order to investigate whether γδ T cells may be polyfunctional, and have anti-tumour as well as anti-infective properties, we decided to use an *in vitro* model to assess cytokine release by γδ T cells when used against K562 cell lines. Sorted γδ T cells from patients were incubated with target cells for 6 hours. After this period, the cells were washed, resuspended in MACS buffer, surface antibodies added. The cells were then fixed and permeabilised and intracellular antibodies added. The IFNγ release from cells incubated with K562 cell lines was compared with that from γδ T cells left unstimulated for 6 hours, but otherwise treated identically. There was no significant difference between the unstimulated cells and the cells stimulated with K562s. The median IFNγ release from the unstimulated cells was 1.35% (0 - 4.4%) versus 2.25% (0.1 - 20.1%) in the cells stimulated with K562s ($p = 0.504$, Mann-Whitney) (Figure 4.20).

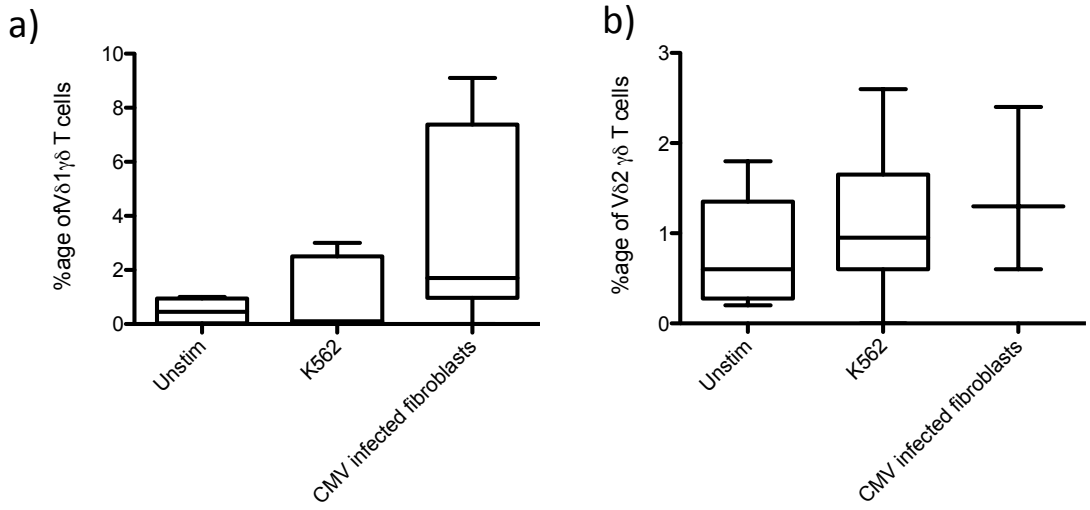


Figure 4.20: Activity of sorted Vδ1 and Vδ2 γδ T cells against targets

a) Vδ1 γδ T cells secrete IFNγ when incubated with CMV infected fibroblasts. No significant activity against K562 cells

b) Vδ2 γδ T cells do not show significant activity against CMV infected fibroblasts or K562 cells

High Throughput Vδ1 γδ T cell TCR Repertoire Analysis

It has been shown that Vδ2 -ve γδ T cells recognize targets in a TCR dependent manner (Willcox et al., 2012). We noted heterogenous expansions of Vδ1 γδ T cells, as demonstrated by the variation in the Vγ chain usage. Because of the variation in Vγ chain usage amongst the Vδ1 population, we FACS sorted (see page 58) lymphocytes based on Vδ1, Vδ2 and Vγ (Vγ9 versus Vnonγ9) chain usage and went on to sequence the TCRs in the different populations.

There are two currently available high throughput methods for obtaining data about TCR usage in these populations, and we considered both. Immunoseq offer a multiplex PCR DNA based method, which offers great sensitivity, and generates TCR usage data on the populations examined at a very high resolution. However, immunoseq currently only offer primers based around the rearranged gamma chain of the Vγδ TCR, so there would be no information on the delta chains obtained via this method; there may also be cells with rearrangements that are not actually expressed (Personal communication, Prof Ben Willcox). Alternatively, mRNA can be amplified, to give information about TCR expression on the examined population. Furthermore the use of both Vγ and Vδ specific primers allows interrogation of both chains, and may allow the gamma/delta chain pairings to be inferred based upon the relative frequencies of each present.

We decided to use the RACE-PCR method (see page 58) because, although it is not as high resolution, it will give TCR repertoire data on the most prevalent of the TCR clones present within the population and will also give data on both the gamma and delta chains of the TCR, as well as ensuring that the TCRs obtained are expressed.

The minimum requirement for sorting cells for the RACE-PCR method was 5,000 cells per sorted population. Only patients with these numbers of cells could be included in the analysis.

Sorting was performed on a FACS/ARIA flow sorter, as detailed in methods. The lymphocytes were sorted into Vδ1/Vγ9, Vδ1/nonVγ9, Vδ2 γ9 and Vδ2 nonγ9 populations. The delta and gamma chains of these cells were then PCRed separately according to the method described in the methods section.

TCR repertoire of Vδ1 γδ T cells is highly Oligoclonal

RACE-PCR method was used to generate the sequences for the Vδ1 γδ T cells. TCR sequences were successfully amplified from patient QE02, glasgow12 (two separate populations – a Vδ1Vγ9 and a Vδ1Vnonγ9 population, although no Vγ chains were successfully amplified from the Vnonγ9 sequences), QE10 (Vδ1nonγ9) and QE23. All of the sequenced patients were CMV seropositive; there were no CMV seronegative patients with sufficient Vδ1 γδ T cells to sequence.

Both the sequenced Vδ and Vγ chains from each patient demonstrated remarkable oligoclonality (Figure 4.21). The full sequences obtained are detailed in table 4.21

Patient QE23 was a CMV seropositive, imatinib-treated patient. The most common Vδ1 sequence made up 54.4 % of all sequences, with the second most common making up 32.2%. Of the corresponding Vγ9 sequences, 53.8% of sequences were made up of one sequence and 40.4% of the second most common sequence.

Patient Glasgow12 was a dasatinib-treated, CMV IgG and IgM seropositive patient. There were two populations sequenced: a Vδ1γ9 population that made up 35% of the Vδ1

population, and a Vδ1nonγ9 population that made up 65% of the Vδ1 population. Within the Vδ1γ9 population, 95.7% of the sequenced Vδ1 chains were made up of one clone, and all of the other sequence clones were only different from the most common clone by 2-3 amino acids in the CDR3 region. Similarly, of the sequenced Vγ9 population, 97.3% of the sequences obtained were of one clone, with the second most common clone having only one amino acid difference in the CDR3 region.

Patient QE02 was a dasatinib-treated patient that had been switched to dasatinib due to low-level PCR positivity for BCR-ABL transcripts. They were strongly CMV IgG positive and also positive for CMV IgM. The sequenced population was the Vδ1nonγ9 population, as this made up 97% of the total Vδ1 population. Within the sequenced Vδ1 population, the most common TCR sequence comprised 92.7% of the total sequences obtained. Two other sequences comprising a further 3.6% of the sequences were only different by one amino acid in the CDR3 region. The sequences obtained from the Vγ population were oligoclonal, but more heterogenous than the Vδ sequences obtained. 66% of the Vγ sequences were comprised of a Vγ2*02 chain. The next most common Vγ chain was a Vγ3 chain. Strikingly, the CDR3 regions of these two chains were different by only four amino acids. The remaining sequences were composed of a Vγ2, a Vγ8 and a Vγ2 chain.

Finally, patient QE10 was a dasatinib-treated patient with both CMV IgG and IgM seropositivity. A Vδ1nonγ9 population was sequenced. The most common Vδ1 sequence made up 54.2% of the sequenced population, with the next most common making up 25.4%. The CDR3 regions of these sequences differed by seven amino acids. Two sequences; a Vγ8 population and a Vγ3 population, with similar CDR3 sequences, dominated the Vγ population.

TCR Sequences Obtained from Vδ and Vγ sequencing of patients Vδ1 γδ T cells

TRDV	CDR3	TRDJ	Frequency (%)	Count
1	CALGDHVGVLGDAYTDKLIF	1	54.4	49
1	CALGELYGWGLYGSDKLIF	1	32.2	29
1	CALGEASLLYWGTRGTDKLIF	1	5.6	5
1	CALGDPVGVLGDAYTDKLIF	1	4.4	4
1	CALGEASLLYWGTRGTDELIF	1	1.1	1
1	CALGEASLLYWGTRGTDKLIL	1	1.1	1
1	CALGELYGRGLYGSDKLIL	1	1.1	1

Vδ1 sequences from patient QE23

TRGV	CDR3	TRGJ	Freq (%)	Count
9*01	CALWEVLPKLF	1*02 or 2*01	53.8	28
9*01	CALWEVTIWELGKKIKVF	P*01	40.4	21
9*01	CALWEVLGKKLF	1*02	3.8	2
9*01	CALREVTIWELGKKIKVF	P*01	1.9	1

Vγ9 sequences from QE23

TRDV	CDR3	TRDJ	Freq (%)	Count
1	CALGELRSWGILSRLTDKLIF	1	95.7	88
1	CALGELRSWGMLSRLTDKLIF	1	2.2	2
1	CALGELRSWEILSRLTDKLIF	1	1.1	1
1	CALGELRSWGIPSRLTDKLIF	1	1.1	1

Vδ1 sequences from Glasgow12 Vd1Vγ9 population

TRGV	CDR3	TRGJ	Freq (%)	Count
9*01	CALWEISHKKLF	2*01 or 1*02	97.3	73
9*01	CALWEIPHKKLF	2*01 or 1*02	2.7	2

Vγ9 sequences from Glasgow12Vd1Vγ9 population

TRDV	CDR3	TRDJ	Freq (%)	Count
1	CALGDVWDEPYTKGVRETDKLIF	1	98.7	75
1	CALGDVWDEPYTKGVRETDKLTF	1	1.3	1

Vδ1 sequences from Glasgow12Vδ1Vnonγ9 population

TRDV	CDR3	TRDJ	Freq (%)	Count
1	CALGEKNPVRNWGYNNDKLIF	1	92.7	51
1	CALGDCRRPSYLYWGIGETDKLIF	1	3.6	2
1	CALGEKNPARNWGYNNDKLIF	1	1.8	1
1	CALGEKNPVCNWGYNNDKLIF**	1	1.8	1

Vδ1 sequences from QE02 Vδ1 γδ T cell population

TRGV	CDR3	TRGJ	Freq (%)	Count
2*02	CATWDGPLNYKKLF	1*02	66.1	37
3*01	CATWRMGYYKKLF	1*02	21.4	12
2*02	CAAWDGPLNYKKLF	1*02	5.4	3
8*01	CATWDRRYWIKTF	P2*01	5.4	3
2*02	SATWDGPINYKKLF	1*01	1.8	1

Vγ sequences from QE02, Vδ1Vnonγ9 population

TRDV	CDR3	TRDJ	Freq (%)	Count
1	CALGELVAPRVLGELDKLIF	1	54.2	32
1	CALGELEDGTAGVSDKLIF	1	25.4	15
1	CALGAPPFLPLLGVPRPEADKLIF	1	8.5	5
1	CALGELVAPRVLGEPDKLIF	1	6.8	4
1	CALGEVVAPRVVGELDKLIF	1	1.7	1
1	CALGEVEDGTAGESDKLIF	1	1.7	1
1	CALGELGLPIEYWGDKLIF	1	1.7	1

Vδ1 sequences from QE10 Vδ1Vnonγ9 population

TRGV	CDR3	TRGJ	Freq (%)	Count
8*01	CATWDRPNYYKKLF	2*01	50.0	26
3*01	CATWDRYHYYKKLF	1*02	46.2	24
4*02	CATWDGFYYYKKLF	1*02	3.8	2

Vγ sequences from QE10 Vδ1Vnonγ9 population

Table 4.21 TCR Sequences Obtained from Sorted Vδ1 γδ T cells. TRD/GV = Variable (V) region employed by relevant δ/γ sequence (NB all δ sequences use δ1 as selected for this population). CDR3 = sequence of CDR3 loop. TRD/GJ = Joining (J) region employed by each δ/γ sequence. Freq = frequency of sequence.

These data demonstrate remarkable oligoclonality within the Vδ1 γδ T cell subset of each patient. There was, however, no similarity in the CDR3 region from patient to patient. No public TCR was identified, in contrast to responses seen to foetal CMV infection (Vermijlen et al., 2010). Whilst the oligoclonality suggests antigen-driven proliferation, and therefore an adaptive response, the antigen driving the expansions may be different from patient to patient. All of the Vδ1 sequences utilized a Jδ1 segment; there are four Jδ gene segments in VDJ recombination in the human genome (Davis and Bjorkman, 1988). It is unclear whether this is a fundamental requirement for these cells' TCRs, or whether there may have been some selection bias introduced – for example, the anti-Vδ1 antibody used may have been specific for Jδ1.

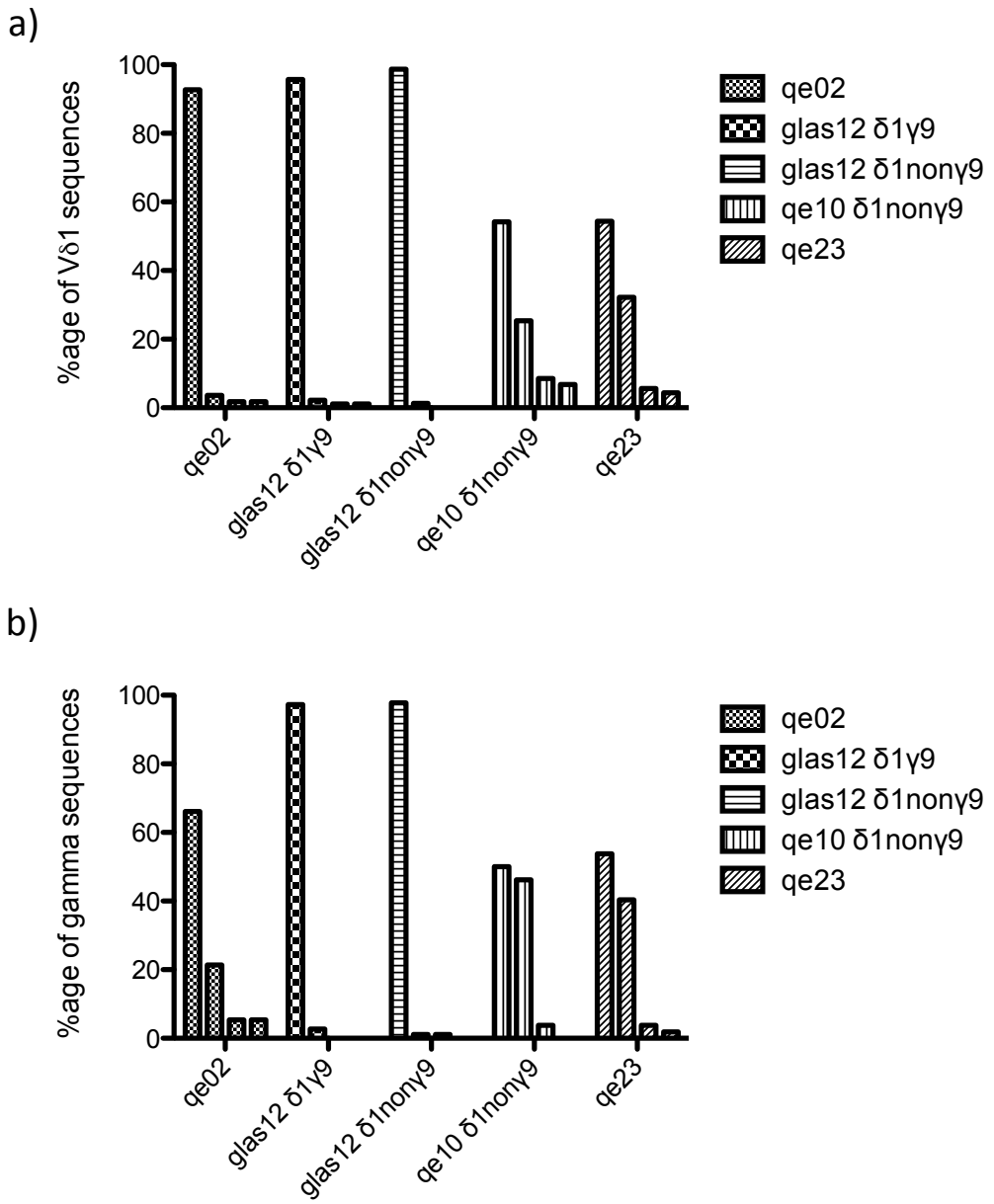


Figure 4.21: Oligoclonal Vδ and Vγ Chain usage in Vδ1 γδ T cells of patients with expansions of Vδ1 γδ T cells

a) The Vδ chains of patients with Vδ1 γδ T cell expansions are remarkably oligoclonal

b) The Vγ chains of sorted and sequenced Vδ1 γδ T cells are oligoclonal. Where indicated, they are sorted subpopulations of Vδ1Vγ9/ Vδ1Vnonγ9 T cells.

Discussion

Dasatinib has profound effects on T cell activation

Dasatinib has activity against a wider range of kinase targets, including *Src* family kinases, which are important mediators of TCR receptor signaling (Giles et al., 2009). It has been found to have profound inhibitory effects on T cell activation *in vitro* on CD8 T cells (Seggewiss et al., 2005, Cwynarski et al., 2004), and on NK cells (Salih et al., 2010), more so than imatinib. It is therefore perhaps unsurprising that dasatinib-treated patients have significant changes in their lymphoid subsets compared with similar patients treated with other TKIs. (Pawelec et al., 2009)

CMV Reactivation Drives CD8 T cell Expansions

Primary CMV infection is thought to occur at about 0.5-1% per year of life, possibly peaking in the 4th decade (Hecker et al., 2004, Pawelec et al., 2009). It is usually asymptomatic but leads to persistent infection, and its control becomes a priority for the immune system.

Increased levels of antibodies against CMV and EBV have been detected in elderly populations, and it has been suggested that this is due to impaired cellular responses and subsequent upregulation of viral expression (Stowe et al., 2007, Khan et al., 2004). Of note, overt reactivation manifested by CMV PCR positivity was not detectible in these populations, suggesting that a pattern of subclinical reactivation may be occurring.

Increased serum levels of certain cytokines, particularly IFNγ, have been shown to be associated with active herpes virus infection (Schuster et al., 1993). Dasatinib-treated CMV seropositive patients within our cohort had increased serum IFNγ levels compared with seronegative patients, suggestive of ongoing or recurrent herpes virus infection.

Large clonal expansions of T cells have been noted in response to CMV (Schwab et al., 1997, Lang et al., 2002), and can comprise up to 10% of the CD8 T cell response (Gillespie et al., 2000). In mouse models, these CMV-specific T cells accumulate as time goes on (Snyder et al., 2008). The CMV-specific CD8 T cells generally express a phenotype suggestive of extensive antigen-driven differentiation; specifically they express low amounts of co-stimulatory CD27 and CD28 (Appay et al., 2002).

The term "memory inflation" has subsequently been used to describe the pattern of changes seen in ageing CMV seropositive populations (Beswick et al., 2013, Pawelec et al., 2009).

Dasatinib is thought to be associated with rare CMV reactivation (Tanaka et al., 2012). We demonstrate that subclinical CMV reactivation may be more common than previously thought. Dasatinib treatment in CMV seropositive patients causes changes similar to those described in the phenomenon of "memory inflation". In particular, a dramatic increase in the terminally differentiated, CD45RA+ve CD27-ve CD8 T cell population is seen in dasatinib treated patients compared to similar patients treated with imatinib.

We have demonstrated that CMV seropositive dasatinib-treated patients have evidence of repeated subclinical CMV reactivation, as evidenced by increased CMV IgG titres compared to imatinib-treated patients, and an increase in the anti-CMV IgG titre over time. They also show an increase in CMV specific CD8 T cell responses by IFNγ release assays compared with imatinib-treated patients, and have increased levels of IFNγ detectible in their serum. Furthermore, some patients treated with dasatinib have evidence of recent CMV reactivation evidenced by anti-CMV IgM seropositivity. Patients

with anti-CMV IgM tend to have the most profound changes in their lymphocyte subsets, and higher numbers of CMV-specific CD8 T cells.

I also saw increased quantitative EBV PCRs in dasatinib-treated patients versus imatinib-treated patients, but increases in EBV PCR were not associated with significant increases in CD8 T cells. This is consistent with other studies, which have demonstrated that CMV, and not other herpes viruses, is important in driving CD8 T cell expansions (Derhovanessian et al., 2011). Indeed, the increase in CMV-specific T cell responses has been shown to impair responses to EBV (Khan et al., 2004), and EBV viral loads are increased in elderly populations (Stowe et al., 2007).

Lymphocytosis on Dasatinib Therapy

A clonal expansion of NK or T cells has been demonstrated in some patients on dasatinib therapy, at a median time of onset at 3 months of treatment (Mustjoki et al., 2009). The predominant phenotype in 15 patients was an αβ CD8+, CD3+ cytotoxic T cell. 7/22 patients had a CD3-, CD16+, and CD56+ NK cell lymphocytosis. All patients with a T-LGL phenotype had mono or oligoclonal expansions. The appearance of the lymphocytosis was occasionally associated with other side effects of dasatinib therapy. Importantly, in this study, patients that developed lymphocytosis had significantly better outcomes when compared with matched controls.

Similarly in a series of eight patients on dasatinib treatment, 8 of 18 patients commenced on dasatinib developed a LGL lymphocytosis. The majority of these patients showed NK cell expansions. Furthermore, the NK cells from dasatinib treated patients had greater cytolytic activity against K562 cell lines compared to healthy controls (Kim et al., 2009).

In another study, four of fifteen patients treated with dasatinib developed an LGL lymphocytosis, with lymphocytes >3.8*10^9/L. They developed this lymphocytosis between one and nine months after commencement of therapy. In three of the four it was a CD3+, CD8+, CD56+, CD57+, CD16+ve CD8 T cell. In the fourth patient, it was CD3-, CD56+, CD57+, CD16+, CD8+ve. NK cell. The four patients with a peripheral blood LGL lymphocytosis all had a good response to therapy, with undetectable RT-PCR transcripts for Bcr/Abl (Valent and Schiffer, 2011).

In another study, clonal lymphocyte proliferations were found in 15 of 18 (83%) of patients with chronic phase CML, compared to only one of twelve healthy donors. Most patients developed more than one clonal TCR rearrangement. From 15 of the CML patients, a follow up sample was available. Of the three on dasatinib, all had persistence of the clonal lymphocytosis, as did 10 of the 12 patients taking imatinib that had a follow up sample available. In 9 of 13 cases, the follow up clone was identical to the clone found at diagnosis. Although clones were present in patients taking imatinib, none of the imatinib treated patients had increased absolute lymphocyte counts during therapy. In the 3 patients without a clonal lymphocyte population detectable at diagnosis, no clonal products were found during treatment with TKI therapy (Kreutzman et al., 2010). The lymphocytes were BCR/ABL negative by FISH analysis. It has previously been shown (Takahashi et al., 1998) that lymphocytes in chronic phase CML patients are negative for the BCR/ABL translocation. Of patients with clonal TCR rearrangements detectable and who had CMV serology available, 21 (91%) were positive for CMV IgG. Only two of six patients without a clonal lymphocytosis were CMV positive. The greater prevalence of clonal proliferations in CML patients compared to healthy controls was not due to differences in the age, as the median age of the patient group was 51 compared to 46 in the healthy controls.

High frequencies of CMV-specific CD8 T cells have been observed, concurrent with CMV PCR positivity in a group of patients treated with dasatinib. The majority of patient with CMV PCR positivity were treated for ALL or CML in blast crisis, however. T cells from dasatinib-treated patients with lymphocytosis were more sensitive to apoptosis after OKT3 stimulation. CMV-specific T cell responses were found to be less sensitive to the inhibitory effect of dasatinib on proliferation compared to other CD8 T cell responses, and in vitro dasatinib prevented downregulation of TCR and CD8 in vitro. They found increased frequencies of CD8 T cells and decreased CD4 T cells in patients with the LGL expansion. The CD8 T cells were mainly CD45RO+, CD27-ve, as were the CMV specific T cells, consistent with late differentiated EM T cells. Clonal expansions appeared 12 weeks after the initiation of dasatinib (Kreutzman et al., 2011a).

Dasatinib has been shown to induce rapid increases in lymphocyte counts shortly after administration, proportional to the concentration of dasatinib in the patients' plasma. Natural killer cells, B cells and cytotoxic effector CD8 T cells increased. CMV seropositive patients had higher lymphocyte counts than CMV seronegative patients. Six of seven patients with high lymphocyte counts achieved complete molecular responses, compared to only one of thirty that did not achieve high counts (Mustjoki et al., 2013).

This is the first study to systematically examine lymphocyte subsets in patients on dasatinib and compare them to similar patients treated with other TKIs. I demonstrated increases in median overall lymphocyte counts, particularly of differentiated CD8 T cells and Vδ1 γδ T cells in patients taking dasatinib. The influence of CMV serostatus, as documented above, is exaggerated in patients taking dasatinib.

Overall lymphocyte counts were not as high as reported in some of the studies described above. This could be for two reasons; firstly because the standard dose of dasatinib used in the studies above was 140mg/day, whereas in patients in this study the dose was 100mg/day. Secondly, it may be that the lymphocyte count increases occur shortly after administration of the drug and sampling time may be important. It was not possible to control the time of sample collection after administration of the drug. The combined effect of CMV seropositivity and dasatinib treatment in our cohort of patients was sufficient to significantly raise the absolute lymphocyte count.

Whilst I did not see a statistically significant expansion of CD56+ CD16+ CD3- NK cells, there were 4 patients in the dasatinib-treated cohort with NK expansions greater than $0.5*10^9$/L, all of whom were CMV IgG positive.

CMV Drives Expansions of Vδ1 γδ T cells in dasatinib-treated patients

Vδ2Vγ9 γδ T cells, the most common subset in the peripheral blood of humans, are known to be very polyclonal (Allison et al., 2001). CDR3 regions of γδ T cells studied elsewhere have been seen to be very diverse (Hayday, 2000b); up to 3 D gene segments with N-addition can be used to generate diversity. The Vδ1 γδ T cell subset has been shown to expand in some immunosuppressed states, for example in patients with AIDS (De Paoli et al., 1991). It has recently been shown that highly differentiated Vδ2 negative γδ T cells accumulate in CMV seropositive individuals as they age (Roux et al., 2013). There was no associated expansion of Vδ2 positive γδ T cells with time in the CMV positive patients.

Vd1 γδ T cells are often associated with intestinal epithelia (Chowers et al., 1994) Expanded Vδ2-ve γδ T cells from renal transplant patients with CMV viraemia have been

shown to produce TNFα and IFNγ in response to colon carcinoma cell lines (Halary et al., 2005), in a NKG2D independent, TCR dependant manner. Effector functions of the cloned Vδ2 negative γδ T cells included cytotoxicity and perforin production. One such Vδ2 negative γδ T cell was shown to express the Vδ5γ4 TCR, and the TCR was subsequently found to recognise the endothelial protein C receptor (EPCR) (Willcox et al., 2012).

We demonstrated significant expansions of Vδ1 γδ T cells in patients treated with dasatinib that had evidence of CMV reactivation. These expansions were associated expansions of terminally differentiated CD8 T cells.

It is possible that dasatinib-treatment leads to preferential expansion of γδ T cells; Lck-/-, Fyn -/- mice do not develop αβ T cells, but do develop some γδ T cells (van Oers et al., 1996). Furthermore, it has been shown that some γδ T cells use different kinases, notably the Blk kinase, to initiate T cell signalling (Laird et al., 2010).

Vδ1 γδ Tcells from the dasatinib-treated CMV seropositive patient cohort were strikingly skewed towards a well-differentiated CD45RA+ CD27- subset. This is similar to the phenotype seen in renal-transplant patients following CMV reactivation. Whereas CMV negative donors' Vδ2 -ve γδ cells predominantly expressed a CD45RA+, CD27+, CD62L+ naive phenotype, Vδ2-ve γδ cells from CMV positive donors expressed a CD45RA+, CD27-, CD62L- TEMRA phenotype. In most of the CMV positive individuals, the Vδ2 –ve cells were predominantly Vδ1 γδ cells but some patients showed expansions of Vδ3 cells. The TEMRA Vδ2 –ve γδ T cells expressed high levels of perforin and granzyme B in CMV positive individuals, compared to the naïve δ2-ve γδ T cells in CMV negative individuals.

Furthermore, the Vδ2 –ve γδ T cells were oligoclonal in CMV positive individuals (Pitard et al., 2008).

Vδ1 γδ T cells from dasatinib-treated CMV seropositive patients were functional, and able to secrete IFNγ , and small amount of TNFα and in response to stimulation with PMA and ionomycin. This pattern of cytokine secretion is similar to that seen with other nonVδ2 γδ T cell populations, which have been shown to have anti-tumour properties in a TCR dependent manner (Devaud et al., 2009). Other studies have demonstrated an IL17 producing subset of γδ T cells, which have anti-tumour properties in mouse models (Schmolka et al., 2013). We did not find any evidence of IL17 producing γδ T cells, either Vδ1 or Vδ2, in any of our patients.

We also demonstrated activity of sorted Vδ1 γδ T cells from dasatinib treated patients against CMV-infected fibroblasts. Furthermore, the pattern of cytokine secretion was similar to that described from Vδ2-ve γδ T cells which have anti-tumour activity (Devaud et al., 2009). We did not demonstrate any in-vitro anti-tumour activity against K562 cell lines. I used fresh sorted γδ T cells whereas in other studies cloned, expanded γδ T cells were used. It is possible that the environment in which γδ T cells are expanded ex vivo may influence the in-vitro activity.

Vδ2Vγ9 T cells have been shown to be markedly polyclonal , but there are conserved hydrophobic residues amongst all Vδ2Vγ9 γδ T cells capable of recognising phosphoantigens, which may be antigen selected (Davodeau et al., 1993).

Sequencing the TCR repertoire from sorted Vδ1 γδ T cells of dasatinib-treated, CMV seropositive patients revealed a striking oligoclonality. Furthermore, the CDR3 regions

of the subclones only differed by a handful of amino acids in the CDR3 region within each individual. However, the TCRs were different from patient to patient. Whilst the CDR3 regions differed from individual to individual, there was significant conservation of regions of CDR3. The marked oligoclonality of the expansion suggests an antigen driven proliferation and is similar to the oligoclonality of CD8 T cells seen secondary to expansion due to chronic stimulation by herpes virus infections such as CMV (Wedderburn et al., 2001). The oligoclonality and the fact that the Vδ1 γδ T cells express a highly differentiated phenotype suggests chronic antigen-driven proliferation.
A "public" Vδ1 γδ T cell receptor has also been described in relation to foetal CMV infection, using a Vγ8Vδ1 pairing (Vermijlen et al., 2010). Whilst one patient within our cohort analysed did have evidence of this pair, the CDR3 region was not the same as the sequence found in this "public" TCR sequence. All of our patients used for TCR analysis were CMV IgG positive, and none had evidence of this TCR.

The targets of the Vδ1 γδ TCRs are not fully understood. Recent studies have suggested that upregulated self-antigens, such as EPCR may serve as stress antigens (Willcox et al., 2012) in infected or malignant cells. Others have noted CD1d, an MHC-like molecule, recognition by Vδ1 γδ TCRs (Bai et al., 2012, Luoma et al., 2013). However, in these studies γδ T cells have been expanded in the presence of PBMCs, which express CD1 and may bias the TCR selection. It is possible therefore, that TCRs with self-reactivity were positively selected using this culture method. In contrast, we used a high throughput method from patients' blood with no ex-vivo manipulation, and it is possible that the TCRs may recognise CMV peptides directly; in the mouse, for example, a Vg2Vd8 clone which recognises HSV glycoprotein 1 has been described (Sciammas et al., 1994).

Others have shown that in vitro treatment of γδ T cells with dasatinib potentiates their proliferation and may alter their ability to degranulate in a dose-dependent manner (Wu et al., 2013).

Interestingly, whilst there was no association between CMV and Vδ2Vγ9 γδ T cells, there was a positive correlation with quantitative EBV PCR. Others have reported increased numbers of this subset in EBV infection (De Paoli et al., 1990).

The immune control of CML

It has been shown that CML is a particularly good target for immune control. Studies using DLI after HSCT are particularly effective in CML. Natural killer and cytotoxic CD8 T cells are thought to be the main mediators of anti-CML immune effects (Rezvani et al., 2003, Savani et al., 2007a).

In patients previously treated with IFN-α therapy, there are an increased number of NK cells in patients that have successfully discontinued IFN-α therapy. Previous treatment with IFN-α was associated with higher numbers of antigen experienced CD45RO positive T cells. Clonal TCR Vγ and Vδ rearrangements were found in the γδ T cell population, with increased Vγ9+ T cells in patients that had finished IFN-α therapy. They suggest that a major factor in the response to IFN-α is due to the expansion of these cytotoxic cells (Kreutzman et al., 2011b).

It has been demonstrated that relative increases in lymphocytes predict improved responses to dasatinib treatment; patients with lymphocyte counts which increased on dasatinib to 150% of baseline at 1 month or 200% at 3 months have deeper molecular responses to dasatinib compared with those that do not (Kumagai et al., 2014).

Changes in the killer-cell immunoglobulin-like receptor (KIR) gene profile has been demonstrated to correlate with response to dasatinib therapy; absence of the inhibitory KIR2DL5A, 2DL5B and 2DL5all genes have been shown to correlate with improved molecular responses to dasatinib (Kreutzman et al., 2012).

Oligoclonal expansions of LGLs have been demonstrated after primary CMV infection (Rossi et al., 2007). After HSCT, LGL lymphocytosis associated with CMV reactivation was associated with long term complete remissions in 5/6 patients. (Mohty et al., 2002).

All of these studies suggest that innate or innate-like subsets of lymphoid cells may contribute to the immune control of CML. This is an increasingly important area of study, with the observation that a number of patients are maintained in a complete molecular response after stopping TKI therapy. The ability to predict which patients are likely to remain in remission after stopping TKI therapy will have a tremendous impact on the clinical course of these patients, as well as having significant economic benefits.

Conclusions

Dasatinib has a broad activity against tyrosine kinase targets, including Src family kinases vital for the initiation of TCR mediated activation of T cells.

I have demonstrated that patients taking dasatinib have evidence of repeated subclinical CMV reactivation; some have detectible anti-CMV IgM, and the titre of the anti-CMV IgG increases as time on dasatinib treatment goes on.

CMV infection drives the expansion of CMV-specific CD8 T cells. It also drives the expansion of atypical Vδ1 γδ T cells. These cells have a terminally differentiated phenotype, and express high levels of activation markers. They have activity against CMV-infected fibroblasts in vitro.

Dasatinib itself has been shown to rapidly mobilise lymphocytes into the peripheral blood shortly after administration. CMV serostatus and treatment with dasatinib were independent factors in causing increased numbers in the blood of CD8 and Vδ1 γδ T cells. The numbers of these cells was increased to such an extent that it caused a dramatic increase in the overall lymphocyte count, which was not seen with relation to CMV serostatus in the imatinib-treated patients.

High-throughput sequencing of the TCRs of sorted Vδ1 γδ T cells from CMV seropositive patients with expansions of this subset revealed remarkable oligoclonality; in every patient, the dominant TCR sequence made up greater than 50% of the total sequences obtained, with only minor variations in individual patients from TCR to TCR sequence. Between individuals, however, there was little similarity in the CDR3 regions; we did not find evidence of a public TCR, suggesting that the antigen that drives the expansions may differ from individual to individual.

Others have shown anti-leukaemic properties of Vδ1 γδ T cells, and it is possible that their increase may be of some benefit in this group of patients

Chapter 5: The influence of CMV Seropositivity on Immune Reconstitution following allogeneic stem cell transplantation for myeloid malignancies

Introduction

Allogeneic haematopoietic stem cell transplantation (allo HSCT) is a standard therapy for high-risk acute myeloid leukaemia (AML). However, traditional myeloablative alloHSCT is associated with considerable toxicity in populations older than 40 years old. It has proved important to develop alternative strategies, as the median age of onset of AML is 66 years. Reduced intensity conditioned (RIC) transplants, which utilise fludarabine, melphalan and alemtuzumab (Campath) have are far less toxic than myeloablative regimens and have been shown to be highly effective in the treatment of high-risk AML (Tauro et al., 2005). It is known that the use of alemtuzumab for T-cell depletion reduces transplant-related mortality (TRM) by reducing the incidence of GvHD (Chakraverty et al., 2002). Reported incidences of grade II-IV acute GvHD range from 2-21%, with rates of 0-13% for chronic extensive GvHD. It is known that alemtuzumab delays immune reconstitution (Chakraverty et al., 2002), in particular a high rate of CMV reactivation is seen in recipients of alemtuzumab containing regimens (Chakrabarti et al., 2002) and delayed CD4 T cell recovery following T cell depletion with alemtuzumab.

Evidence of manipulation of a potential graft versus tumour effect may affect relapse risk and outcome is evidenced by the impact that ciclosporin levels have on outcome following RIC allo HSCT for AML (Craddock et al., 2010).

CMV is traditionally associated with a significant morbidity post alloHSCT (Osarogiagbon et al., 2000, Ljungman, 2008, Craddock et al., 2001). For this reason, CMV seronegative donors are usually matched with CMV seronegative patients wherever possible (Ljungman et al., 2004). Recipient CMV seropositivity has been identified as the most important predictor for CMV reactivation following allo HSCT. CMV seropositivity has been linked with higher rates of TRM, often due to infection (Broers et al., 2000),

and it has also been suggested that it may increase the rate of GvHD, particularly in the setting of T-cell depletion. Little is currently known about the effect of CMV seropositivity on immune reconstitution in the context of immune reconstitution following allo HSCT with T cell depletion.

As yet, there has been no study examining the relationship between CMV serostatus and immune reconstitution in T-depleted, RIC alloHSCT.

The aims of this study were to assess the effect of CMV serostatus of patient and/or donor on immune reconstitution following alloHSCT, and the effect of T cell depletion, in a range of haematological malignancies. We went on to examine what impact CMV serostatus had on the outcome of patients transplanted for myeloid malignancies transplanted with RIC alloHSCT.

Methods

Patients were classified according to their donor type (matched unrelated versus sibling donor), and CMV serostatus. The age of the patients was also included. Kaplan Meier plots are shown, and Cox multivariate analysis was performed to identify significant factors in affecting outcome. CMV serostatus was defined by recipient/donor pairs e.g. +/- means recipient CMV seropositive, Donor CMV seronegative. Patients were classified as "CMV-at-risk" if the donor and/or the recipient were CMV seropositive.

Conditioning Regimens

Within the cohort of patients analysed for immune reconstitution, a number of different conditioning regimens were used. Myeloablative regimens included Cyclophosphamide and total body irradiation (TBI), or Busulfan and Cyclophosphamide. All myeloablative-conditioned allo HSCTs from unrelated donors were T-cell depleted with alemtuzumab

in vivo at 10mg/day for 5 days. Reduced intensity conditioned allografts were conditioned with BEAM (BCNU, Etoposide, Cytarabine and Melphalan) /alemtuzumab or fludarabine/melphalan and alemtuzumab, as described below. Patients in the cohort analysed for outcome were conditioned with fludarabine 30mg/m^2 on days -6 to -2, melphalan 140mg/m^2 on day -1. Alemtuzumab was given at a dose of 10mg/day for 5 days on D-7 to -3. Ciclosporin was given for GvHD prophylaxis. The CMV serostatus of all patients and donors had been measured by ELISA prior to transplant. CMV PCRs were performed weekly on CMV-at-risk patients. Prophylaxis was given in the form of aciclovir, and a policy of pre-emptive therapy for CMV reactivation was used according to local protocol based on CMV PCR positivity. Graft-versus-host disease was diagnosed and treated according to local protocols. All patients were followed up at their respective centres, and events were calculated from follow up data. NRM was defined as death in the absence of confirmed relapse. Patients' blood was collected with informed consent, according to ethics Q5/Q2707/175. Patient characteristics for outcome data in RICs for AML are shown below. CMV-at-risk patients were all patients with either donor or recipient CMV seropositivity. Patient characteristics of the group undergoing lymphocyte subset analysis are shown in table 5.1.

Statisical Analysis

Overall survival and event-free survival were estimated using the Kaplan-Meier method. Probabilities of NRM and relapse were summarized using cumulative incidence estimates. NRM was defined as death without relapse, and was considered a competing risk for relapse. Relapse was considered a competing risk for NRM. Univariate analyses were compared using Log-Rank tests, and multivariate analyses were carried out with Cox regression. All analyses were carried out in SPSS version 21 for Mac. Non-relapse mortality and relapse rates were calculated using a competing risk analysis (Pintilie, 2007).

Patient Characteristics

CMV Serostatus (Patient/Donor)	+/+	-/-	+/-
Number	18	18	14
Median patient age	42.5	42.5	45
Range of patient ages	26-67	23-70	32-61
Patient gender, M/F	11/7	13/5	6/8
Mean weeks post transplant	11.9	11.5	11.8
Diagnosis:			
Acute Myeloid Leukaemia	7	9	7
Chronic Myeloid Leukaemia	3	1	3
Hodgkin's Lymphoma	1	2	0
Non-Hodgkin's Lymphoma	3	2	2
Burkitt's Lymphoma	1	0	0
Acute Lymphoblastic Leukaemia	1	2	2
Myelofibrosis	1	1	0
Mantle Cell Lymphoma	0	1	0
Myelodysplastic Syndrome	1	0	0
Type of Donor:			
Sibling	12	12	8
Unrelated	6	6	6
Conditioning:			
Cy/TBI	7	7	7
Bu/Cy	2	2	0
Flu/Mel/Alemtuzumab	7	7	5
Beam/Alemtuzumab	2	2	2
CMV reactivation	9	-	9

Table 5.1 Patient characteristics of cohort used for lymphocyte subset analysis NB Cy=cyclophosphamide, TBI=Total Body Irradiation, Bu=Busulfan, Flu=Fludarabine, Mel=Melphalan. CMV serostatus is represented as recipient/donor CMV serostatus

Lymphocyte Subsets at 3 months post transplant

Patients treated with Alemtuzumab have significantly lower lymphocyte counts after transplant than patients undergoing T-replete allografts

I first compared lymphocyte subsets in patients conditioned with regimens employing T-cell depletion with alemtuzumab with those that did not, at 3 months post transplant. Lymphocyte depletion with alemtuzumab was shown to have profound effects on immune reconstitution at 3 months post allo HSCT. Patients given alemtuzumab had significantly lower T cell counts ($p < 0.0001$, Figure 5.1a), CD4 counts ($p < 0.0001$, Figure 5.1b), CD8 counts ($p = 0.0079$, Figure 5.1c), and Vδ1 γδ Tcell counts ($p = 0.0001$, Figure 5.1d) compared to patients given T-replete transplants. Indeed, total T cell counts were 5 times higher in the recipients of T-replete grafts compared to those that were conditioned with alemtuzumab (Table 5.2).

Subset	Alemtuzumab	Minimum	25th percentile	Median	75th Percentile	Maximum
CD3	N	0.19	0.38	0.66	0.90	2.10
	Y	0	0.029	0.13	0.24	1.54
CD8	N	0.069	0.12	0.25	0.54	1.66
	Y	0	0.020	0.046	0.14	1.18
CD4	N	0.036	0.21	0.27	0.40	0.62
	Y	0	0.016	0.049	0.081	0.33
Vδ1 γδ	N	0.00094	0.0013	0.0027	0.008	0.023
	Y	0	0.00008	0.00023	0.0011	0.0091

Table 5.2 Total T cell, CD8, CD4 and Vδ1 γδ T cell counts (10^9/L) at 3 months post allo HSCT. Patients are grouped according to the use of alemtuzumab (Y) or not (N) in the transplant regimen.*

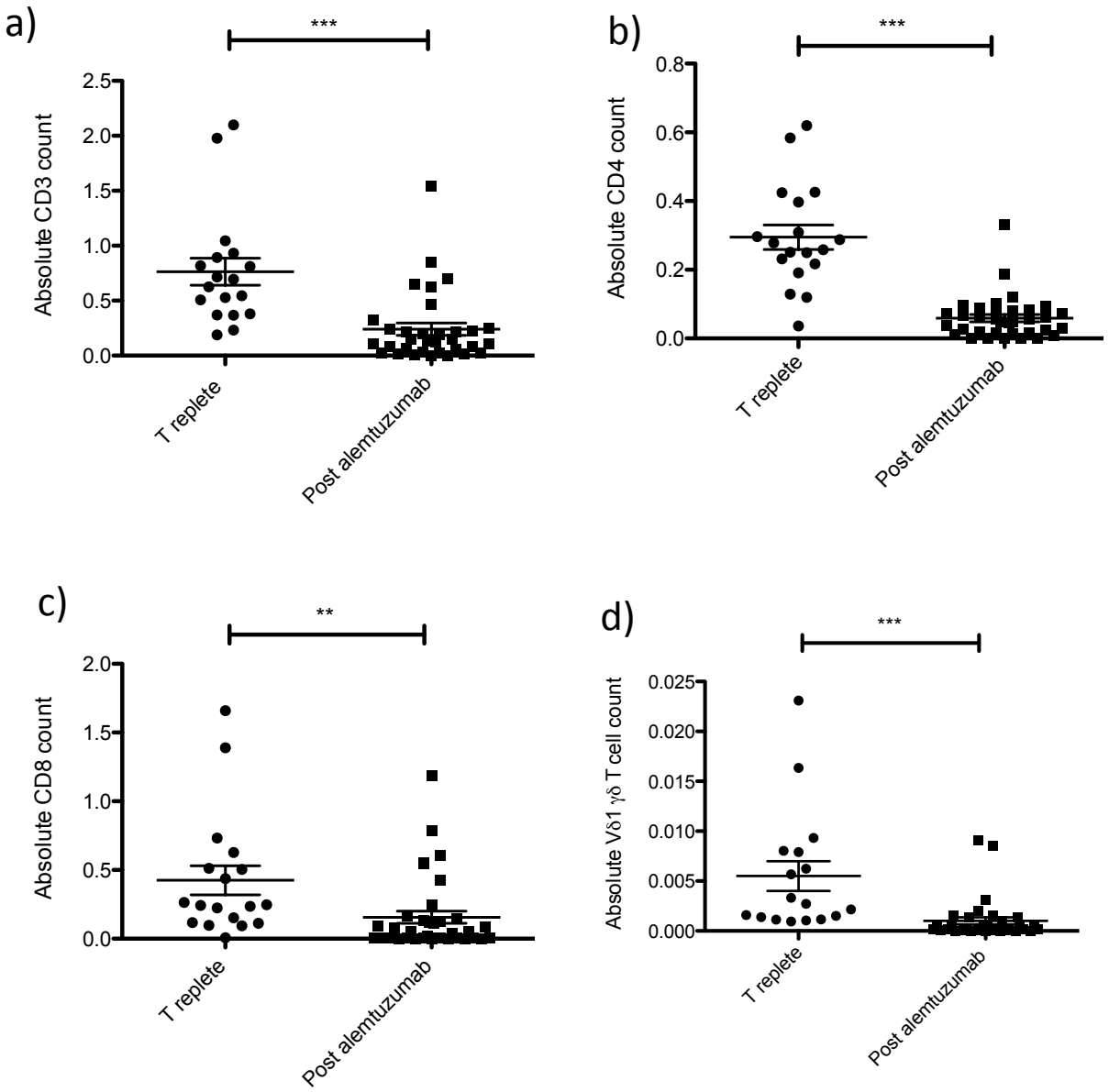


Figure 5.1: Comparison of CD3, CD8, CD4 and Vδ1 γδ T cell counts in alemtuzumab-conditioned versus T-replete allo HSCT recipients

a) Patients that receive alemtuzumab (n = 36) have significantly lower total CD3 counts at 3 months post allograft ($p < 0.0001$) b) lower CD4 T cell counts ($p < 0.0001$), c) CD8 T cell counts ($p = 0.0079$) and d) Vδ1 γδ T cell counts ($p < 0.0001$) than those give T-replete regimens (n = 18)

The Effect of CMV serostatus on Lymphocyte Subsets at 3 months

Recipient CMV seropositivity is associated with a higher T cell count in Recipients of T-replete Grafts

I next went on to examine the effect of CMV serostatus on immune reconstitution; CMV-at-risk status was defined as either recipient or donor CMV seropositivity. Within the T-replete transplants, median CD3 count was higher in CMV-at-risk patients, but this did not reach statistical significance ($0.81*10^9$/L versus $0.54*10^9$/L, p = 0.13). CD8 T cell counts in this group were also greater ($0.50*10^9$/L versus $0.22*10^9$/L, p = 0.069). The median Vδ1γδ T cell count was 3 times higher in CMV-at-risk patients, but this difference did not reach statistical significance (Table 5.3). There was no difference in median CD4 T cell count.

Subset	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
CD3	-/-	0.23	0.37	0.54	0.77	0.89
	At risk	0.19	0.44	0.81	1.51	2.10
CD8	-/-	0.095	0.11	0.22	0.35	0.51
	At-risk	0.0068	0.19	0.50	1.06	1.66
CD4	-/-	0.13	0.20	0.28	0.41	0.43
	At-risk	0.0037	0.18	0.25	0.44	0.62
Vδ1 γδ	-/-	0.0011	0.0012	0.0021	0.0057	0.016
	At-risk	0.00094	0.0012	0.0060	0.0090	0.023

Table 5.3 The effects of CMV serostatus on lymphocyte subsets at 3 months post allo HSCT in recipients of T-replete allografts

Patients who have undergone T cell depletion with alemtuzumab demonstrate a marked increase in T cell numbers at 3 months in association with CMV seropositivity

CMV-at-risk patients treated with alemtuzumab had higher total lymphocyte count (median $0.8*10^9$/L versus $0.4*10^9$/L, p = 0.0039) at 3 months after transplant, compared to CMV -/- patients. The total T cell count of CMV-at-risk patients was 7 times that of CMV -/- patients. There was a marked increase in CD8 T cells (median

0.092*10^9/L versus 0.0027*10^9/L, p =0.039) associated with CMV seropositivity. The median CD4 T cell count in CMV-at-risk patients was twice as high as in CMV -/- patients (median 0.027*10^9/L versus 0.059*10^9/L, figure 5.2b), although this difference was not statistically significant. Similarly, the median number of Vδ1 γδ T cells was 3 times higher in CMV-at-risk patients compared to -/- transplants (0.00043*10^9/L versus 0.00012*10^9/L, figure 5.2d), although this did not reach statistical significance (Table 5.4).

Subset	CMV	Minimum	25th percentile	Median	75th Percentile	Maximum
CD3	-/-	0	0.0192	0.031	0.086	0.20
	At risk	0.00012	0.11	0.22	0.50	1.54
CD8	-/-	0	0.0017	0.0027	0.021	0.12
	At-risk	0	0.0059	0.092	0.34	1.19
CD4	-/-	0	0.013	0.027	0.074	0.097
	At-risk	0	0.018	0.059	0.086	0.33
Vδ1 γδ	-/-	0	0.00007	0.00013	0.00024	0.00042
	At-risk	0	0.00011	0.00043	0.0014	0.0091

Table 5.4 Absolute numbers of lymphocyte subsets in CMV-at-risk versus CMV -/- patients transplanted with alemtuzumab-containing regimens at 3 months post transplant

Comparing CMV -/-, T-replete graft recipients with CMV -/- patients treated with alemtuzumab, there were very markedly higher CD3 (median 0.031*10^9/L versus 0.54*10^9/L $p < 0.0001$), CD8 (0.22*10^9/L versus 0.0027*10^9/L, $p = 0.0002$) and CD4 counts (0.28*10^9/L versus 0.027*10^9/L) $p < 0.0001$.

These data demonstrate that, regardless of lymphodepletion, CMV seropositivity is associated with significantly higher T cell counts at 3 months post transplant, and this is particularly seen within the CD8 T cell subset. Within the cohort treated with alemtuzumab, this difference is particularly marked. Whereas CMV seropositivity is associated with 1.5 times higher T cell counts at 3 months in recipients of T-replete grafts, the difference is 7 times higher in the T-deplete setting.

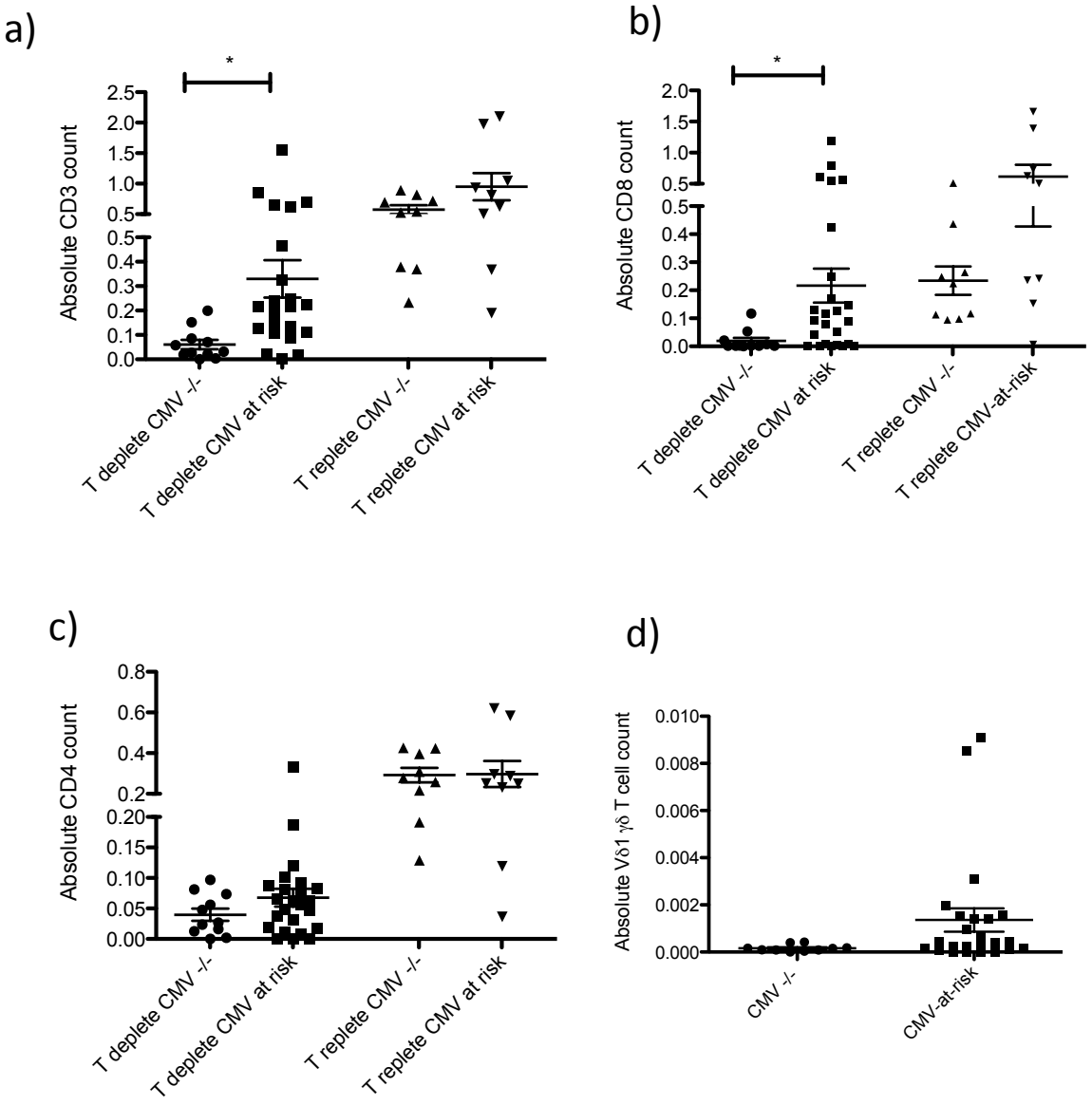


Figure 5.2: The influence of CMV serostatus and T-cell depletion with alemtuzumab on CD3, CD8, CD4 and Vδ1 γδ T cell counts at 3 months post transplant

a) Within the recipients of T-depleted grafts, patient CMV seropositivity is associated with significantly increased CD3 T cells at 3 months (p = 0.0208). b) This is mainly accounted for by an increase in CD8 T cells, which are significantly increased in CMV seropositive recipients of SCT (p = 0.0134). c) CD4 T cell numbers are not significantly increased in the CMV seropositive group d) Non-significant trend for increased Vδ1 γδ T cell numbers in CMV at risk recipients of SCT vs -/- (p = 0.13)

CMV seropositive recipients of Sibling alloHSCT have improved T cell reconstitution following T-deplete stem cell transplant

As CMV serostatus has a dramatic effect on the lymphocyte repertoire at 3 months, I next went on to study how these values were affected by sibling or unrelated donors. Within CMV-at-risk alemtuzumab-treated patients, there was a non-significant trend for higher CD3, and CD8 T cell count in recipients of sibling compared to unrelated donor recipients.

Subset	Donor	Minimum	25th percentile	Median	75th Percentile	Maximum
CD3	Sib	0.00012	0.15	0.32	0.64	1.54
	MUD	0.0052	0.070	0.14	0.24	0.85
CD8	Sib	0.00011	0.10	0.25	0.55	1.19
	MUD	0.0011	0.0068	0.065	0.13	0.79
CD4	Sib	0	0.027	0.056	0.088	0.33
	MUD	0.0025	0.017	0.062	0.089	0.19
Vδ1 γδ	Sib	0.0000002	0.0002	0.0010	0.0023	0.0091
	MUD	0	0.00012	0.00032	0.0014	0.0085

Table 5.5 CD3, CD4 and CD8 absolute counts (10^9/L) in recipients of CMV-at-risk sibling and unrelated donor transplants*

The largest differences between recipients of sibling and unrelated donor grafts was in the CD8 T cell subset; specifically the median total T cell count is 2.3 times higher in recipients of sibling donor, T-depleted grafts compared to that from T-depleted, unrelated donors (p = 0.13, Figure 5.3a). The median CD8 T cell count is 3.8 times higher in sibling transplants (p = 0.092, Figure 5.3b). The median CD4 count is unchanged, at 1.1 times as high (Figure 5.3c). The highest absolute Vδ1 γδ T cell counts were seen in recipients of sibling allo HSCT that were CMV at risk (median 0.0010*10^9/L versus 0.00032*10^9/L, siblings versus unrelated, p=0.30, Figure 5.3d), see Table 5.5.

There were insufficient patient numbers in the CMV -/- sibling transplant group (n=3) to make meaningful comparisons between the CMV -/- group according to donor source.

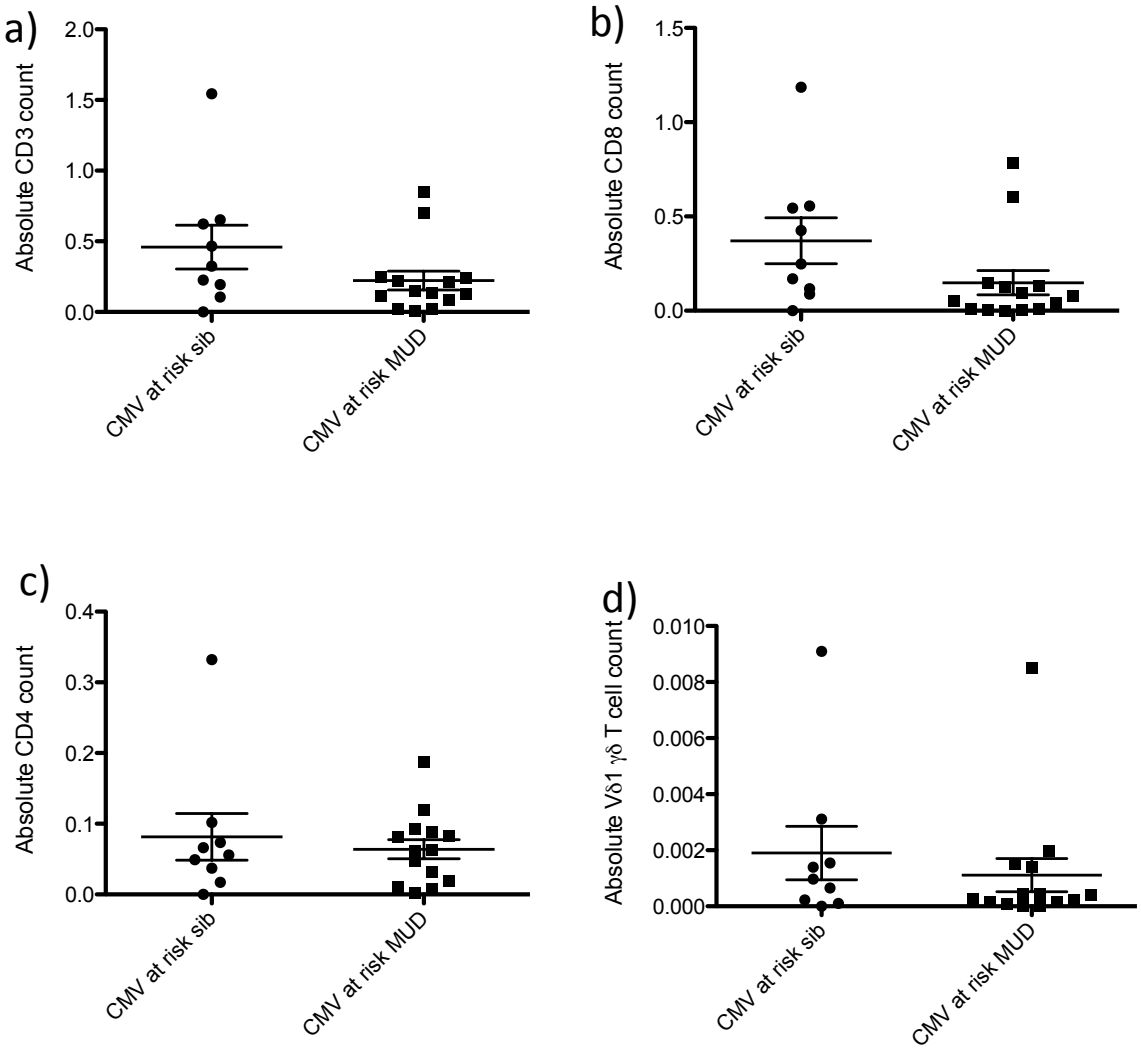


Figure 5.3: The effect of sibling versus unrelated donor on lymphocyte reconstitution at 3 months in CMV-at-risk patients

a) There was a non-significant trend for higher total T cell count in recipients of sibling alloHSCT (n = 9)

b) The trend was also for a higher CD8 T cell count in recipients of a sibling donor (n = 9) compared to an unrelated donor (n = 14)

c) Median CD4 T cell count was not increased in recipients of sibling allo HSCT

d) There was a trend for the median Vδ1 γδ T cell count to be higher in recipients of sibling allogeneic stem cell transplants versus unrelated donors

Memory Phenotypes of CD8 T cells in T-depleted transplants

Because CMV seropositivity is known to be associated with a higher number of more differentiated CD8 T cells, I went on to examine the frequencies of CD8 T cell memory subsets defined by CD45RA and CD27 expression in patients treated with alemtuzumab-containing regimens at 3 months post transplant.

There were significantly greater numbers of more differentiated CD8 T-cells in CMV-at-risk patients compared with CMV -/- patients (Figure 5.4a). CD45RA- CD27– EM cells were over twenty times as high ($0.0017*10^9$/L compared with $0.037*10^9$/L, p = 0.0083), and CD45RA+ CD27- EMRA CD8 T cells were almost 100-fold greater ($0.00035*10^9$/L versus $0.032*10^9$/L, p = 0.0057). The numbers of naïve and CM CD8 T cells were not significantly different between the two groups.

The effect of CMV on CD8 T cell differentiation was most marked amongst recipients of sibling allo HSCTs (Figure 5.4b); the median number of EMRA CD8 T cells in CMV-at-risk sibling recipients was 25 times higher, at $0.131*10^9$/L compared with $0.0049*10^9$/L in recipients of unrelated grafts (p = 0.0074). There was a non-significant trend for increased EM CD8 T cells in recipients of sibling allo HSCT ($0.11*10^9$/L versus $0.033*10^9$/L, p = 0.14).

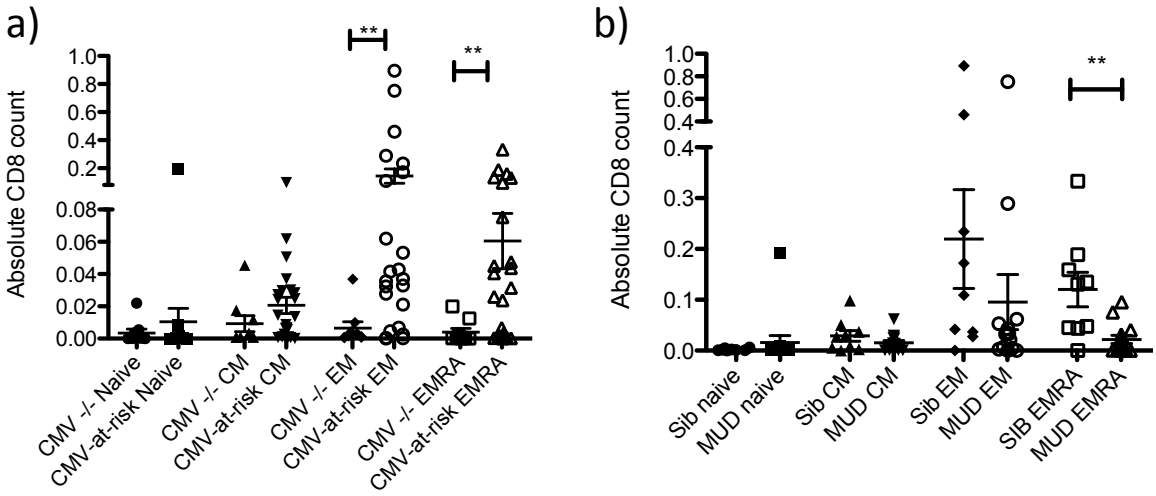


Figure 5.4: CD8 Memory Subsets in Alemtuzumab-treated patients

a) There are more frequent CD45RA- CD27- EM (p = 0.0083) and CD45RA+ CD27- EMRA (p = 0.0057) CD8 T cells in CMV-at-risk recipients of allogeneic stem cell transplant compared to CMV -/- patients.

b) Within the CMV-at-risk group, there were more CD45RA+ CD27- EMRA CD8 T cells in recipients of sibling allo HSCT compared with recipients of unrelated donors (p = 0.0074)

Improved Immune Reconstitution at 3 months correlates with reduced relapse risk

The efficacy of RIC alloHSCT is thought to be very dependent on an alloimmune responses to reduce relapse risk (Soiffer et al., 2011). I therefore went on to identify relapse rates within the cohort in which I had measured lymphocyte reconstitution. Due to the heterogeneity of the patient cohort and differences in definitions of relapse and response of different diseases, I elected to examine only the patients transplanted for myeloid malignancies transplanted using RIC regimens.

There were non-significant trends for a number of lymphocyte subsets to be increased in patients that remained in remission. Specifically, patients transplanted for myeloid malignancies that went on to relapse (n = 4), compared to those that did not (n = 16) had lower T cell counts (median $0.024*10^9$/L versus $0.12*10^9$/L, ns, Figure 5.5a), CD8 T cell counts (median $0.0055*10^9$/L versus $0.066*10^9$/L, ns, Figure 5.5b). The median CD4 count in patients that went on to relapse was only one third of the number ($0.020*10^9$/L versus $0.059*10^9$/L, Figure 5.5c) in patients that did not, but this was not statistically significant. The Vδ1 γδ T cell number also correlated with protection from relapse ($0.00010*10^9$/L versus $0.00022*10^9$/L, Figure 5.5d), but the difference did not reach statistical significance.

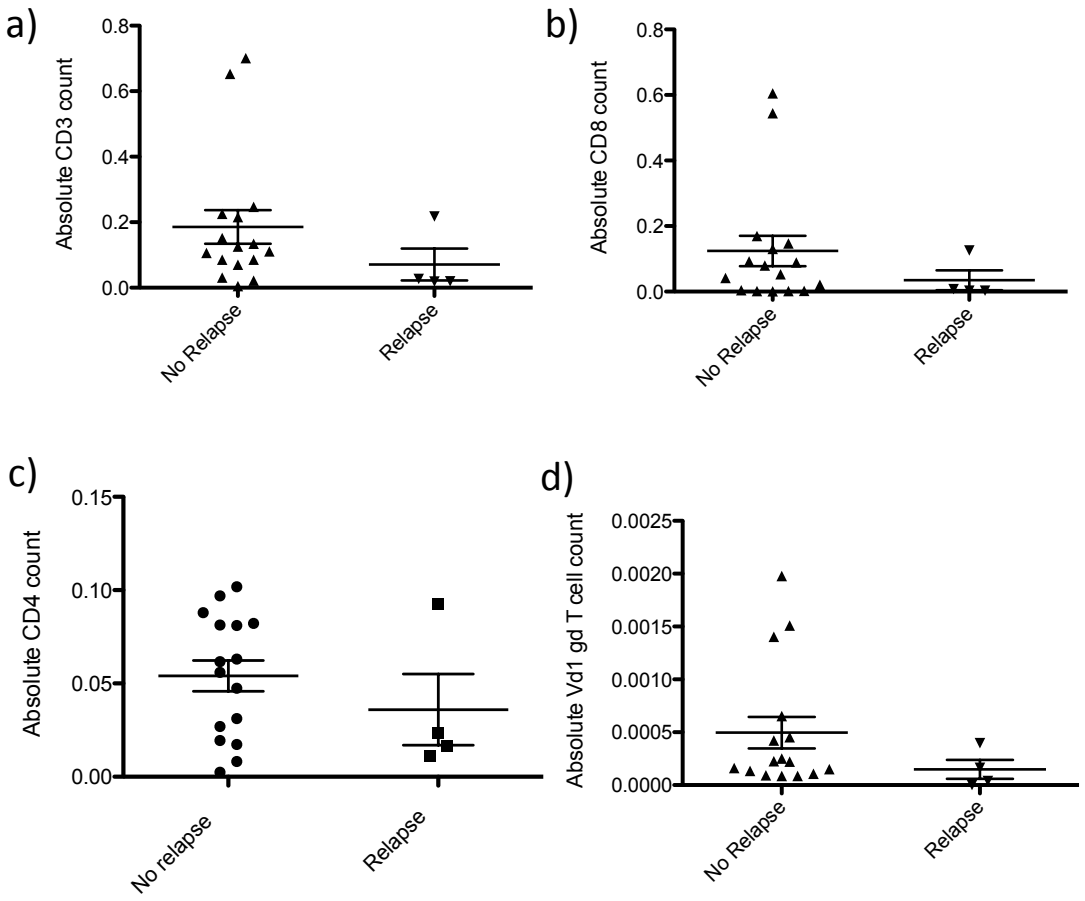


Figure 5.5: Comparison of Lymphocyte subsets at 3 months post transplant of patients that went on to relapse versus those that did not

a) There was a non-significant trend for higher CD3 counts in patients that remained in remission (n = 16) versus those that relapsed (n = 4) b) Median CD8 T cell count in those patients that remained in remission was higher than in those that relapsed, although this did not reach statistical significance. c) There was a non-significant trend for higher CD4 count in patients that remained in remission versus those that relapsed, and d) a non-significant trend for higher Vδ1 γδ T cell count in those that remained in remission.

Outcome of Fludarabine, melphalan and alemtuzumab-conditioned AlloHSCT for AML and MDS

Because of the marked differences in immune reconstitution at 3 months post alloHSCT according to donor source and CMV serostatus, I next went on to examine the outcome of patients transplanted with fludarabine/melphalan and alemtuzumab conditioned HSCTs for AML and MDS. Patients were recruited from University Hospital Birmingham (n = 146), Birmingham Heartlands Hospital (n = 39) and University College Hospital, London (n = 37) from 1998-2011.

Patient characteristics are detailed in tables 5.6 and 5.7.

		CMV-at-risk	CMV -/-
Disease	AML	113 (77%)	64 (84%)
	MDS	33 (23%)	12 (16%)
Donor Type	Sibling	63 (43%)	25 (37%)
	Unrelated	81 (57%)	48 (63%)
Median Age		56.7	54.4
CR1		110 (75%)	52 (68%)
CR>=2		34 (23%)	12 (16%)

Table 5.6 Patient Characteristics according to CMV-at-risk status. NB CR1 = 1st complete response.

CMV serostatus	**-/-**	**-/+**	**+/-**	**+/+**
Total	76 (33%)	16 (7%)	48 (21%)	82 (36%)
Sibs	25 (33%)	7 (44%)	20 (42%)	36 (44%)
Unrelated	48 (67%)	9 (56%)	26 (58%)	46 (56%)
CR1	52 (68%)	13 (81%)	34 (71%)	63 (77%)
CR >=2	12 (16%)	3 (19%)	14 (29%)	13 (16%)
Unk	12 (16%)	0	0	6 (7%)

Table 5.7 Patient Characteristics according to Patient/Donor CMV serostatus

The median follow up of the group was 3.53 years (2.99 to 4.97 95% CI, Kaplan-Meier estimate). The median follow up of sibling transplants was 3.53 (2.86 to 4.20) years and for unrelated donors it was 3.31 (2.48 to 4.14) years. Median survival was 2.04 years (Figure 5.6a, and Table 5.8).

Time from transplant	6 month	12 month	2 year	5 year
Percentage alive	73%	61%	51%	43%

Table 5.8 Kaplan-Meier estimates of overall survival for Fludarabine/Melphalan/Alemtuzumab Conditioned allo HSCTs for AML and MDS

Most deaths occurred within the first 6 months of transplant. Relapse rates at 6 months were 14%, with a NRM of 18%. At 1-year post transplant, the relapse rate was 23%, and the NRM estimated at 23%. At 2 years, 29% of patients had relapsed, and 24% had died of NRM.

Patients transplanted from sibling donors had significantly better overall survival than unrelated donor transplants

Firstly, I examined the effect of donor source on outcome. The median overall survival for patients transplanted from sibling donors was not reached, with a 5 year estimated survival of 58% years, versus 1.1 years from unrelated donors, 5 year estimated survival 33% ($p < 0.0001$, Log-Rank), see Figure 5.6b and Table 5.9

Donor	6 month	12 month	2 year	5 year
Sibling	83%	72%	64%	58%
Unrelated	67%	54%	42%	33%

Table 5.9 Kaplan-Meier estimates of overall survival of recipients of sibling versus unrelated donors

In order to calculate whether the difference in outcome was due to increase relapse rates or due to other factors, a competing risk analysis was used to calculate relapse rates (with NRM as a competing risk) and NRM (with relapse as a competing risk). The difference in survival between recipients of unrelated donors and sibling donors was due to a significantly higher rate of NRM ($p<0.001$, Log rank) in the recipients of unrelated donors compared to the recipients of sibling donors, largely during the first 6 months post transplant (Table 5.10).

Donor	**6 month**	**12 month**	**2 year**	**5 year**
Sibling	7%	11%	12%	12%
Unrelated	25%	31%	33%	36%

Table 5.10 Competing risk estimates of NRM according to donor source

There was no significant difference in the relapse rates between recipients of unrelated and sibling donors (log rank $p = 0.41$, table 5.11).

Donor	**6 month**	**12 month**	**2 year**	**5 year**
Sibling	12%	25%	28%	32%
Unrelated	14%	21%	29%	29%

Table 5.11 Competing risk estimates of cumulative relapse incidence according to donor source

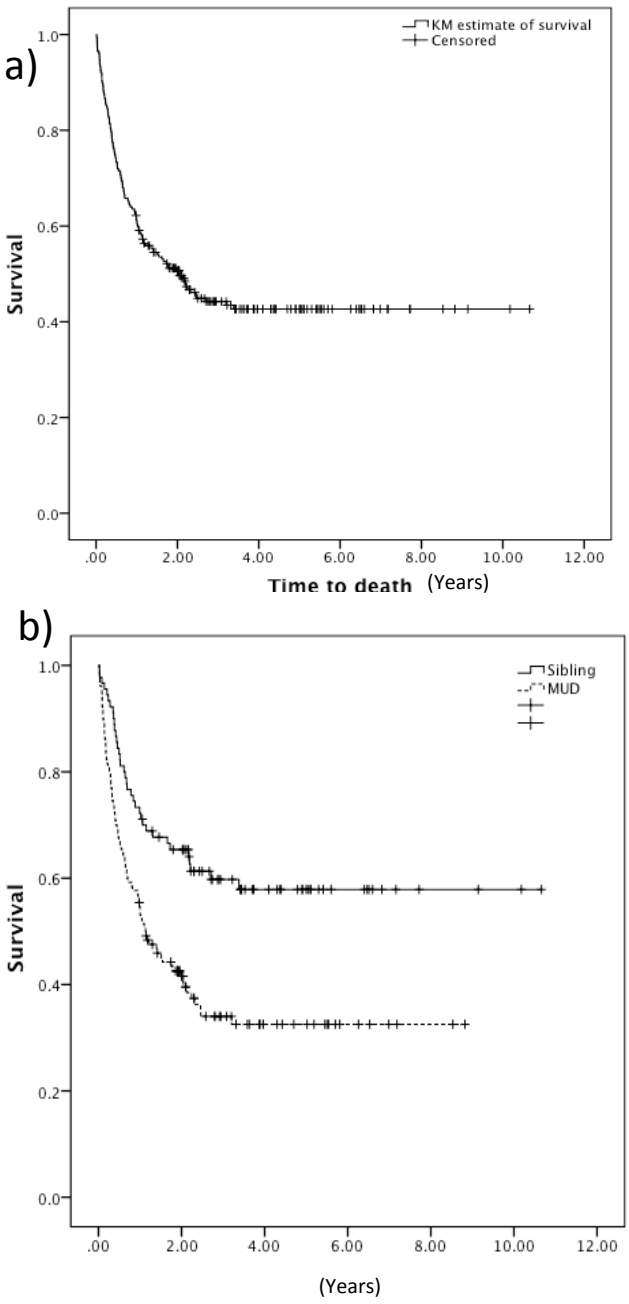


Figure 5.6: There was significantly improved survival in recipients of sibling compared with unrelated alloHSCT with this regimen

a) The overall survival curve for recipients of fludarabine/melphalan/alemtuzumab conditioned alloHSCT for AML and MDS

b) Recipients of sibling alloHSCT had significantly improved survival compared to patients transplanted from unrelated donors

"CMV-at-risk status" patients exhibit significantly better overall survival following transplant with reduced intensity conditioning

Because of the dramatic difference in immune reconstitution of difference lymphocyte subsets according to CMV serostatus, I went on to examine the relationship between CMV serostatus and outcome in this group of patients.

CMV-at-risk patients had a significantly improved overall survival compared to CMV -/- patients in this cohort (Figure 5.7a and Table 5.12). Within the CMV-at-risk patients, the median survival was 2.31 years versus 1.00 for the CMV -/- patients (p = 0.037 by Log-Rank).

CMV serostatus	**n**	**6 month**	**12 month**	**2 year**	**5 year**
At-risk	144	77%	66%	54%	47%
-/-	73	67%	51%	43%	33%

Table 5.12 Kaplan-Meier estimates of overall survival based on CMV-at-risk status

This survival difference was evident early post-transplant; by six-months post transplant CMV -/- patients had almost a 1.5 risk of death compared to the CMV-at-risk patients.

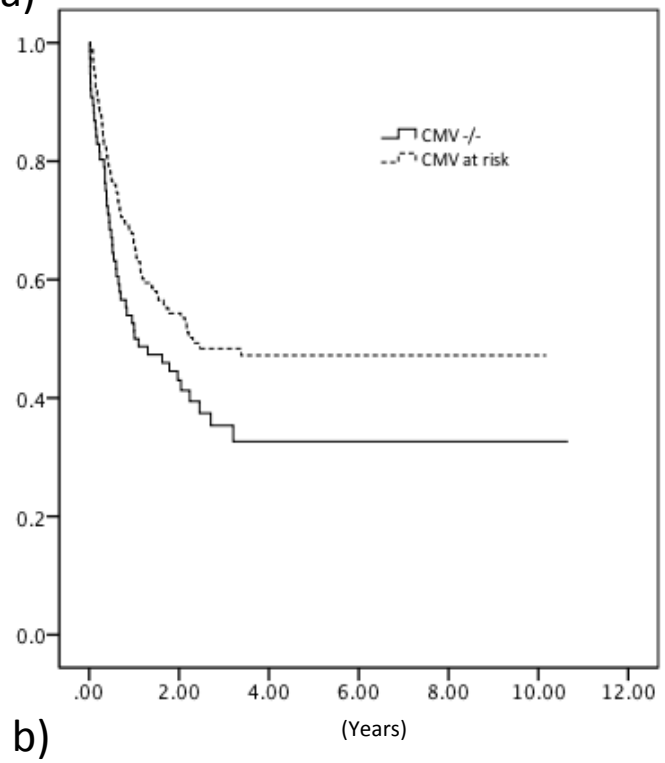
Within patients transplanted from unrelated donors, there was no difference in overall survival according to CMV-at-risk status

Because there were differences seen in immune reconstitution in recipients of sibling donors and unrelated donors, we went on to examine the effect of CMV serostatus in these two groups separately. Within the cohort of patients transplanted from unrelated donors, the median survival for CMV-at-risk patients was 1.18 years versus 1.00 years for CMV -/- patients (ns, Figure 5.7b, Table 5.13).

CMV serostatus	*n*	6 month	12 month	2 year	5 year
-/-	48	67%	52%	43%	27%
At-risk	81	67%	54%	40%	35%
(-/+)	9	56%	44%	22%	22%
(+/-)	26	69%	62%	45%	36%
(+/+)	46	70%	54%	43%	37%

Table 5.13 Kaplan-Meier estimates for overall survival based on CMV serostatus of recipient/donor pairs in patients transplanted from MUD donors

a)



b)

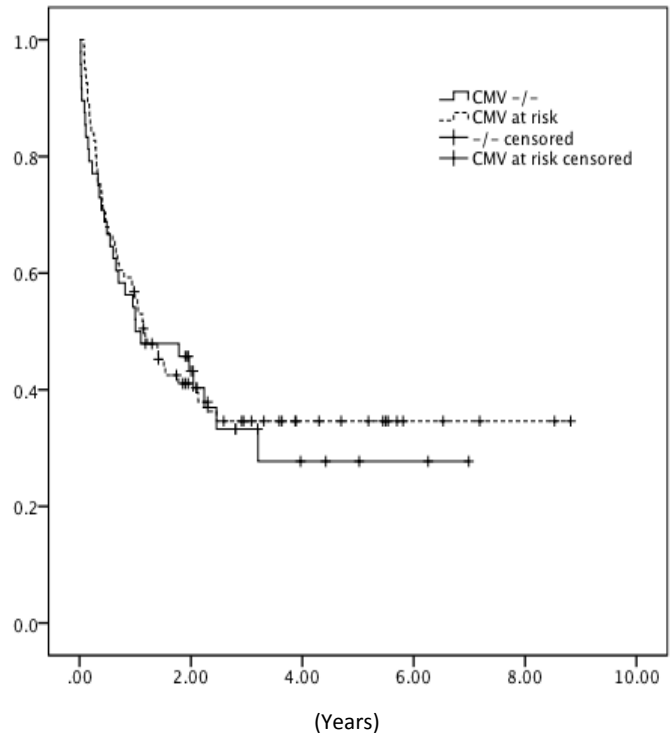


Figure 5.7: Kaplan Meier Plots for Overall Survival in RIC transplants for AML and MDS

a) CMV-at-risk patients (n = 144) have improved overall survival compared to CMV -/- patients (n = 73) post transplant for AML and MDS (p = 0.037)

b) Within the group of patients transplanted from an unrelated donor, CMV-at-risk status did not make a difference to overall survival

The survival difference is confined to recipients of sibling donors

For recipients of transplants from sibling donors, the median survival for CMV-at-risk patients was not met, with 2-year survival estimated at 71%. The median survival for CMV -/- patients was estimated at 1.31 years, with 2-year survival estimated at 48%. The difference between the two populations was highly statistically significant (p = 0.028 Log rank). The curves diverged early on: at 3 months Kaplan-Meier estimates of overall survival were 88 % versus 95%, at 6 months they were 68% versus 89%, at 9 months 56% versus 84%, and at 1 year 52% versus 79% (Figure 5.8a, Table 5.14).

CMV serostatus	n	6 month	12 month	2 year	5 year
At-risk	63	89%	79%	71%	63%
-/-	25	68%	52%	48%	43%

Table 5.14 Kaplan-Meier estimates of survival of sibling allo HSCT transplants, with respect to CMV serostatus

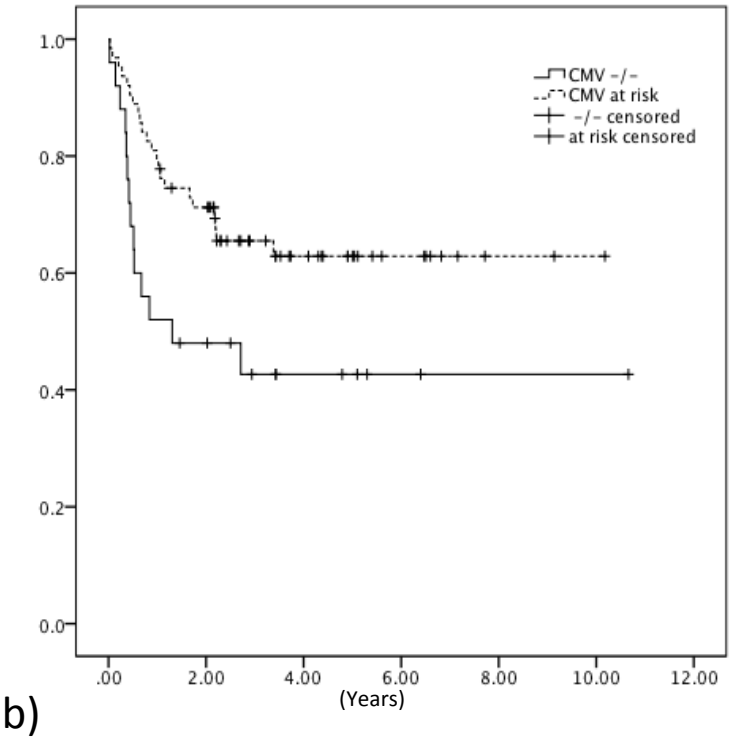
This difference was present regardless of whether the donor or recipient was CMV seropositive.

As shown in Table 5.15 and Figure 5.8b, the trend was for the CMV -/- group transplanted from sibling donors to do worse than the other groups. The risk of death at 12 months is double in CMV -/- patients compared to CMV-at-risk patients, although there were too few people in the -/+ group to make meaningful statistical comparisons.

CMV serostatus	*n*	6 month	12 month	2 year	5 year
-/-	25	68%	52%	48%	43%
-/+	7	71%	71%	71%	71%
+/-	20	85%	85%	79%	64%
+/+	36	92%	78%	64%	61%

Table 5.15 Kaplan-Meier estimates for survival based on CMV serostatus of donor and recipient in sibling allo HSCTs

a)



b)

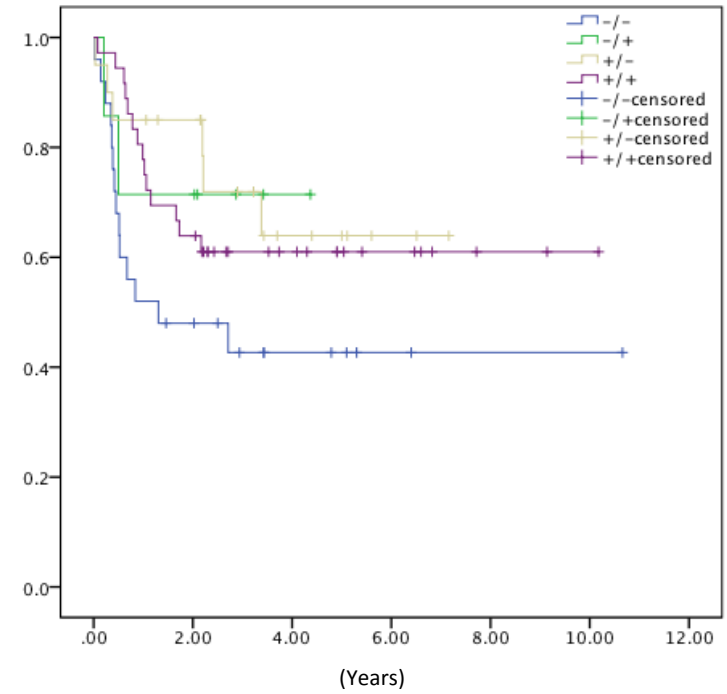


Figure 5.8: Overall survival is improved in CMV-at-risk patients transplanted from sibling donors.

a) Within patients transplanted from sibling donors, CMV-at-risk status (n = 63) is associated with an improved overall survival compared to CMV -/- transplants (n = 25) (p = 0.028)

b) Within the CMV-at-risk group, ther was no difference in +/-, +/+ or -/+ pairs.

The Overall Survival Difference is due to a decreased relapse rate in CMV-at-Risk Patients

The most common cause of death after transplant for AML is relapsed disease. Non-relapse mortality is defined as death from all causes other than relapse. A competing risk model was used to estimate relapse rates. Relapse and non-relapse mortality were treated as competing risks for an event (Pintilie, 2007).

The relapse rate in CMV -/- patients was double that of CMV-at-risk patients at 6 months and 1.5 times at 12 months (p = 0.046, log-rank, Figure 5.9a and Table 5.16).

CMV serostatus	**6 month**	**12 month**	**2 year**	**5 year**
-/-	22%	30%	37%	37%
At-risk	10%	20%	26%	29%

Table 5.16 Competing risk estimates of relapse according to CMV-at-risk status

The cumulative incidence of NRM was calculated with relapse as a competing risk. Non-relapse mortality is no different in the CMV -/- group compared to the CMV-at-risk group (p=0.327, Log rank) (Figure 5.9b and Table 5.17).

CMV serostatus	**6 month**	**12 month**	**2 year**	**5 year**
-/-	20%	23%	25%	29%
At-risk	16%	22%	23%	25%

Table 5.17 Competing risk of NRM according to CMV-at-risk status

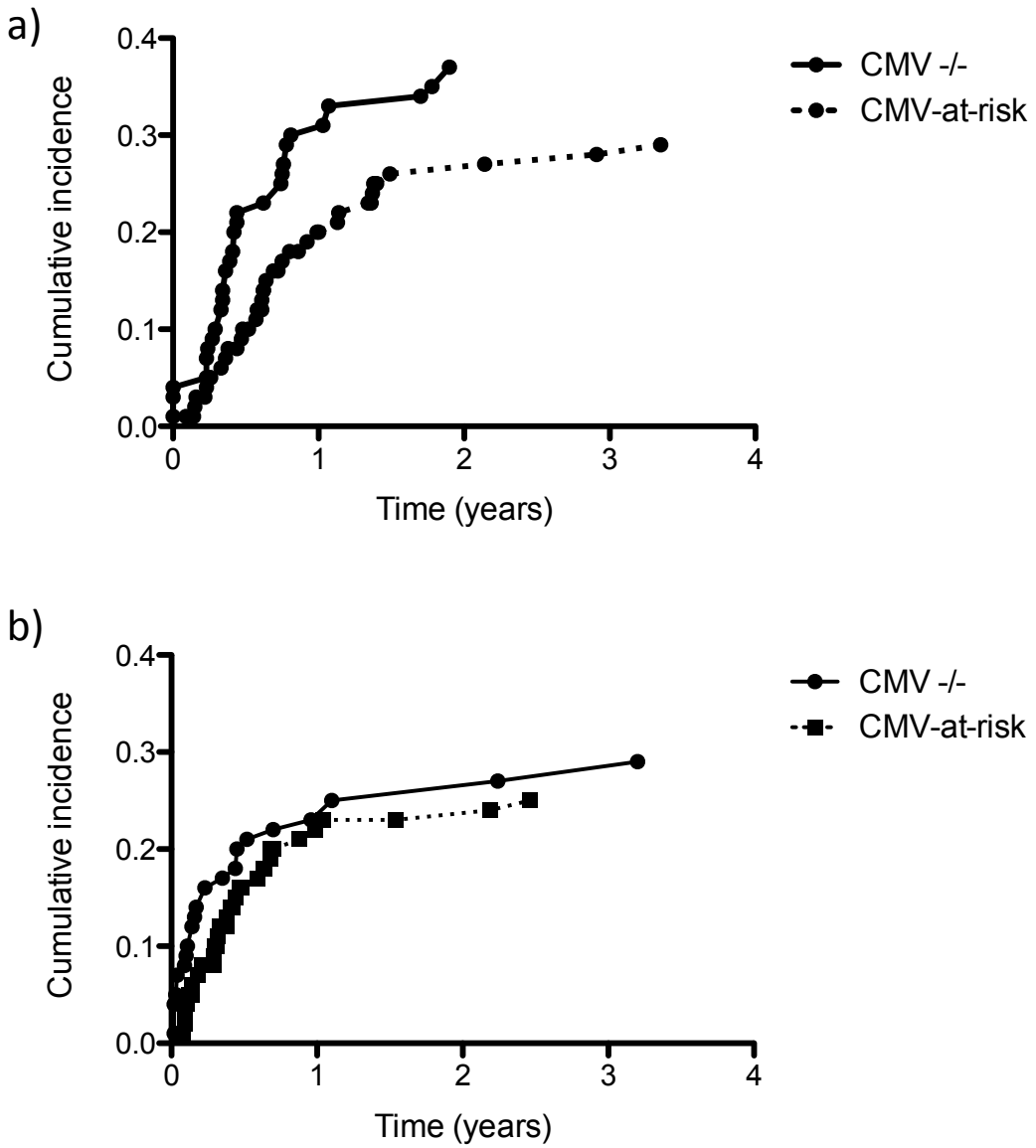


Figure 5.9: Relapse rates and NRM in recipients of fludarabine/melphalan/alemtuzumab-conditioned allo HSCT for AML

a) There was a significant (p = 0.046, log-rank) decrease in relapse rates in recipients of fludarabine/ melphalan/alemtuzumab conditioned alloHSCT for AML In CMV-at-risk versus CMV -/- patients

b) There was no significant difference in NRM between CMV-at-risk and CMV -/- patients

The effect of CMV serostatus on relapse was most marked in the group transplanted from sibling donors (p=0.03, log rank). Within the sibling transplant cohort, the relapse risk at 1 year for CMV -/- patients is double that of CMV-at-risk transplants. This difference persists up to 5 years from transplant (Figure 5.10a, Table 5.19). However, CMV-at-risk status made no difference to relapse risk in the unrelated donor setting (Figure 5.10b, Table 5.18).

Donor	***CMV serostatus***	**n**	**6 month**	**12 month**	**2 year**	**5 year**
Sibling	At-risk	63	6%	20%	22%	29%
	-/-	25	27%	39%	43%	43%
Unrelated	At-risk	81	11%	20%	28%	28%
	-/-	48	18%	25%	31%	31%

Table 5.18 Competing-Risk Estimates for Relapse, according to donor source and CMV-at-risk status

Within the cohort transplanted from sibling donors, relapse risk at 6 months post transplant was over 4 times higher in the first 6 months in CMV -/- patients compared to CMV-at-risk patients. After 6 months, the relapse rates were roughly the same in the CMV -/- patients and CMV-at-risk patients. A further 14% relapsed in months 6-12 in the CMV-at-risk patients compared to 12% of the CMV -/- patients.

These data suggest that CMV-at-risk status may protect from early relapse post sibling allo HSCT for AML and MDS.

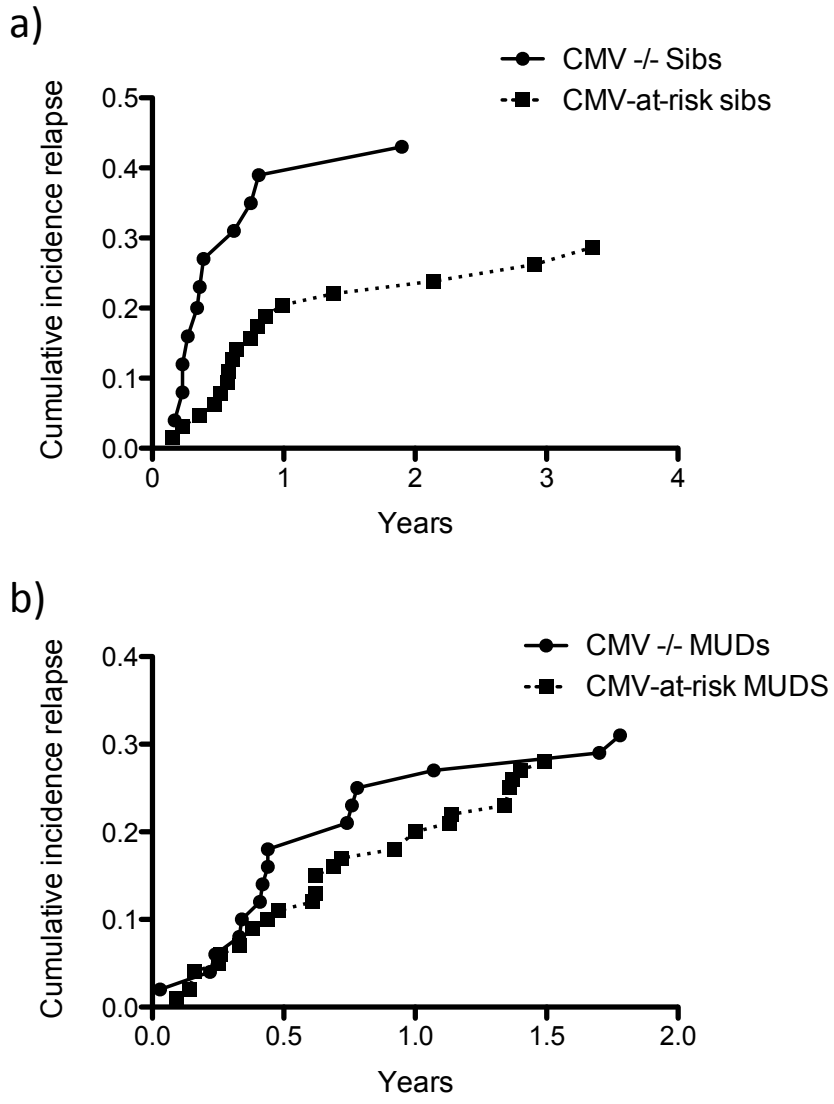


Figure 5.10: Competing risk analysis for relapse amongst recipients of allo HSCT for AML; the effects of donor source and CMV serostatus

a) There were significantly lower relapse rates in CMV-at-risk patients versus CMV -/- in recipients of sibling allo HSCT conditioned with fludarabine/melphalan/alemtuzumab (p = 0.03, Log-Rank)

b) There was no significant difference in relapse rates in recipients of unrelated transplants according to CMV-at-risk status

Non-Relapse Mortality and CMV

The competing-risk analysis demonstrated that donor source (unrelated versus sibling) was the most important factor in determining the NRM. CMV serostatus of patient or donor did not influence NRM, in either the sibling (Figure 5.11a and table 5.19) or the unrelated donor group (Figure 5.11b, and table 5.19).

Donor	*CMV serostatus*	n	6 month	12 month	2 year	5 year
Sibling	At-risk	63	5%	9%	9%	11%
	-/-	25	8%	12%	12%	12%
Unrelated	At-risk	81	25%	31%	33%	35%
	-/-	48	23%	27%	29%	36%

Table 5.19 Competing-Risk Estimates for Non-Relapse Mortality

These data show that CMV serostatus is an important predictor of outcome in recipients of RIC allo HSCT. Negative CMV serological status of the patient and donor is associated with a dramatic increase in relapse rate, in recipients of sibling donors. CMV seropositivity is not associated with higher NRM in either the sibling or unrelated donor setting.

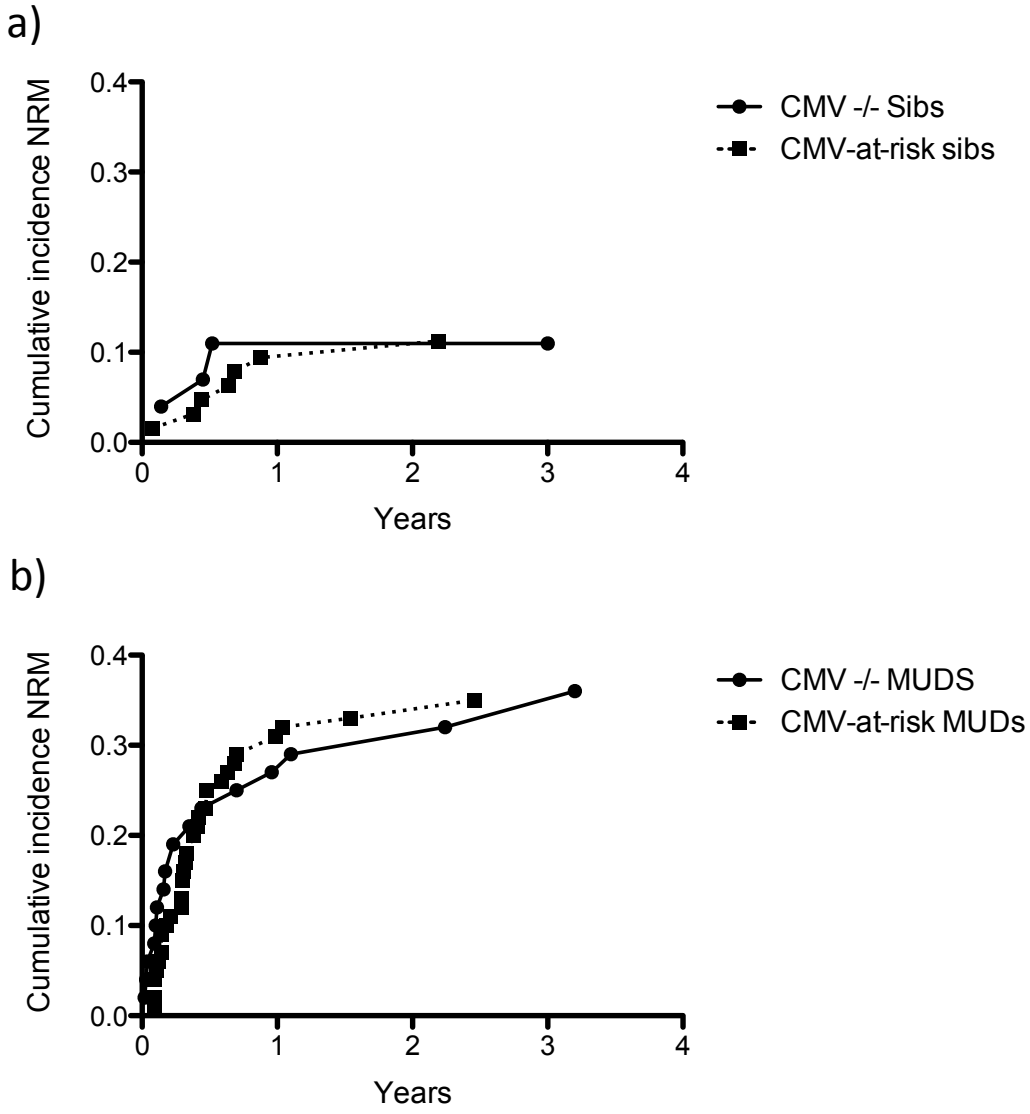


Figure 5.11: The relationship between CMV serostatus and NRM

a) CMV-at-risk status made no difference to the cumulative incidence of NRM in recipients of sibling or b) recipients of unrelated donor allo HSCT conditioned with fludarabine/ melphalan/ alemtuzumab.

CMV Reactivation

All patients have weekly CMV quantitative PCR performed post transplant, and pre-emptive anti-CMV treatment initiated on the basis of detectible CMV DNA. I was interested in analyzing whether CMV reactivation led to any difference in outcome.

Where data was available, 85/123 (69.1%) of CMV-at-risk patients within this cohort underwent an episode of CMV reactivation. There was no significant difference in the proportion if patients that reactivated CMV according to donor source. Reactivation was seen in 35 of 53 (66.0%) CMV-at-risk patients from sibling donors, compared to 50 of 70 (71.4%) transplanted from unrelated donors. 2 of 59 (3.4%) -/- patients, 2 of 14 (14.3%) -/+ patients, 30 of 40 (75%) +/- patients and 54 of 70 (77.1%) +/+ patients experienced at least one episode of CMV PCR positivity. Nearly all patients that reactivate CMV post transplant have the first episode of reactivation within 50 days post transplant. We examined overall survival in patients that survived to at least 50 days post transplant, in CMV-at-risk patients, to analyse if CMV reactivation was associated with a difference in overall survival, relapse incidence or NRM, using a competing risk method as previously described.

CMV Reactivation	**n**	**6 month**	**12 month**	**2 year**	**5 year**
No	33	73%	70%	51%	51%
Yes	85	86%	71%	59%	50%

Table 5.20 Kaplan-Meier Estimates of Overall Survival based on CMV reactivation

No difference was seen in in overall survival within patients that reactivated CMV versus those that did not, and CMV reactivation did not alter the relapse rate as calculated by a competing risk group (Figure 5.12a, Table 5.21).

CMV Reactivation	n	6 month	12 month	2 year	5 year
No	33	12%	24%	27%	27%
Yes	85	7%	19%	25%	28%

Table 5.21 Relapse rates, Competing risk method according to CMV reactivation

CMV Reactivation	n	6 month	12 month	2 year	5 year
No	33	18%	18%	18%	18%
Yes	85	9%	16%	19%	22%

Table 5.22 Non-relapse mortality according to CMV reactivation

There was also no increase in NRM in patients that reactivated CMV versus those that did not (Table 5.22). Within the cohort transplanted from sibling donors, there was a non-significant trend for improved overall survival in the group that reactivated CMV versus those that did not (Figure 5.12b and Table 5.23), but not in those transplanted from unrelated donors (Figure 5.12c) This was largely due to a decrease in the relapse rate in patients that reactivated CMV in recipients of sibling allo HSCTs. There was a non-significant trend for earlier relapse in patients that did not reactivate CMV versus those that did (Figure 5.12d and Table 5.24).

CMV Reactivation	n	6 month	12 month	2 year	5 year
No	17	77%	71%	58%	58%
Yes	24	97%	88%	79%	68%

Table 5.23 Kaplan-Meier estimates of overall survival in recipients of sibling allo HSCT based on CMV reactivation

CMV Reactivation	n	6 month	12 month	2 year	5 year
No	17	17%	28%	28%	28%
Yes	24	3%	17%	20%	25%

Table 5.24 Competing risk estimates of relapse risk in recipients of allo HSCT that reactivate CMV versus those that do not

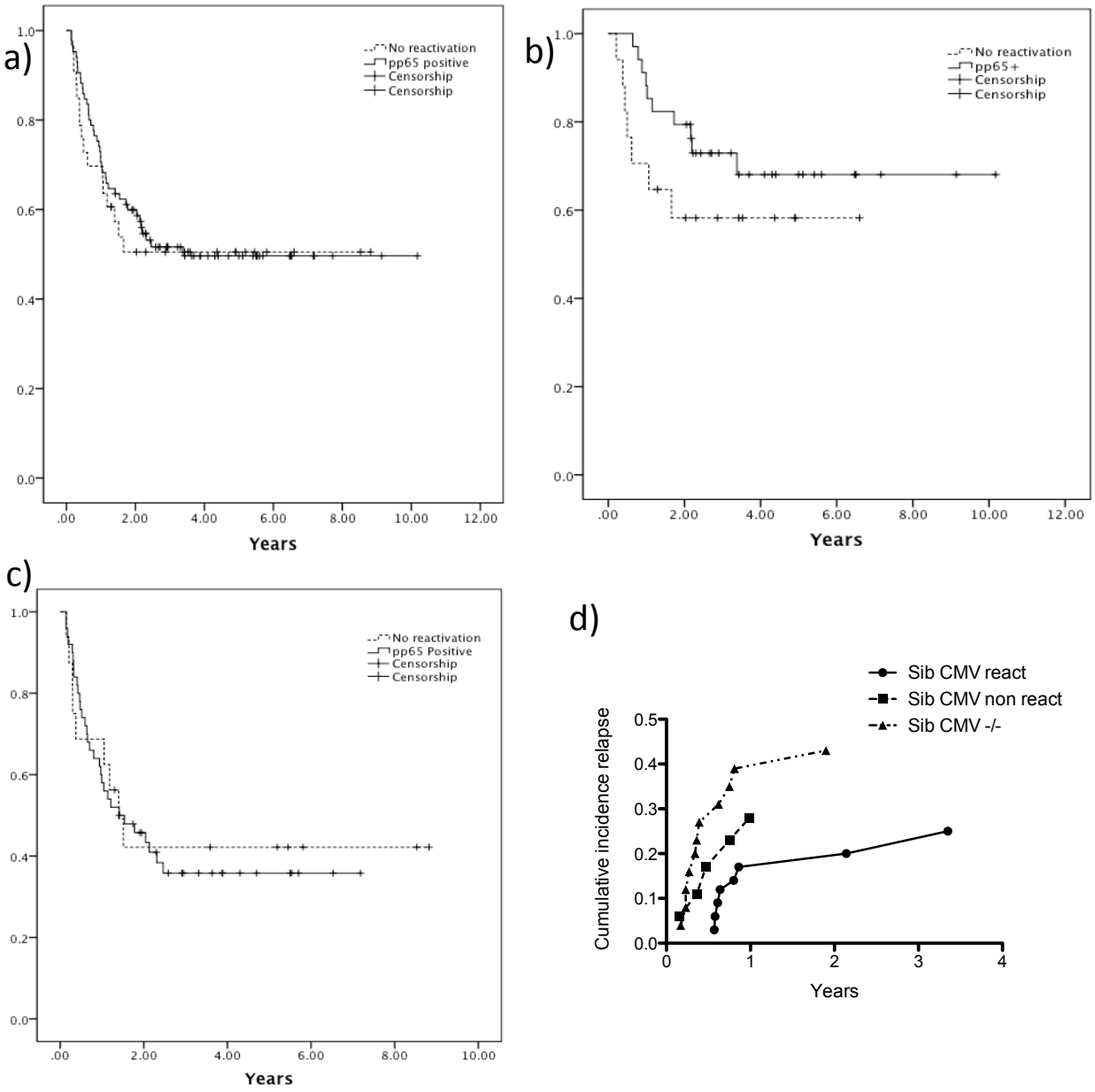


Figure 5.12: CMV Reactivation and Overall Survival

a) CMV reactivation, defined by the presence of CMV DNAemia makes no difference to overall survival in the whole group b) There was a non-significant trend for improved survival in patients transplanted from sibling donors associated with CMV reactivation c) There is no difference in survival comparing patients transplanted from unrelated donors with regards to CMV reactivation d) In recipients of sibling transplants there was a non-significant trend for lower relapse rates in patients that reactivate CMV over those that do not.

Graft-versus-Host Disease

Graft-versus-host disease (GvHD) is an important cause of morbidity and mortality post allogeneic HSCT. I was interested in analyzing whether the differences in immune reconstitution in different patient groups were reflected in differences in the incidence of GvHD post transplant.

There were no significant differences in the rates of GvHD in the different groups of patients with regards to CMV serostatus, where the data was available (Table 5.25).

Donor	CMV serostatus (Recipient/Donor)	Acute GvHD	Chronic GvHD
Sibling	-/-	3/14	2/14
	-/+	0/10	0/10
	+/-	3/13	5/13
	+/+	3/19	2/19
MUD	-/-	8/34	5/34
	-/+	2/5	0/5
	+/-	9/31	5/31
	+/+	8/34	6/34

Table 5.25 Numbers of patients with acute and chronic GvHD according to donor type and CMV serostatus

The association with CMV seropositivity and outcome is not confined to one centre

As the outcome data was collected from 3 different centres, we compared the effects of CMV-at-risk status on outcome in the different centres. The data from centres 2 and 3 was combined, and the outcome data from centre 1 was analysed separately.

In both datasets, there was an improved overall survival in patients that were CMV-at-risk, transplanted from sibling donors (Figures 5.13a and b), with no difference seen in the unrelated donor cohort (Figures 5.13c and d); see Table 5.26

Donor	***CMV serostatus***	***Centre***	**n**	**6 month**	**12 month**	**2 year**	**5 year**
Sibling	At-risk	1	42	88%	76%	67%	61%
		2, 3	21	91%	86%	81%	65%
	-/-	1	14	64%	43%	43%	43%
		2,3	11	73%	64%	55%	41%
Unrelated	At-risk	1	57	67%	53%	40%	32%
		2,3	24	71%	58%	44%	44%
	-/-	1	32	66%	53%	40%	15%
		2, 3	16	69%	50%	50%	42%

Table 5.26 Kaplan-Meier estimates of overall survival based on centre and CMV-at-risk status

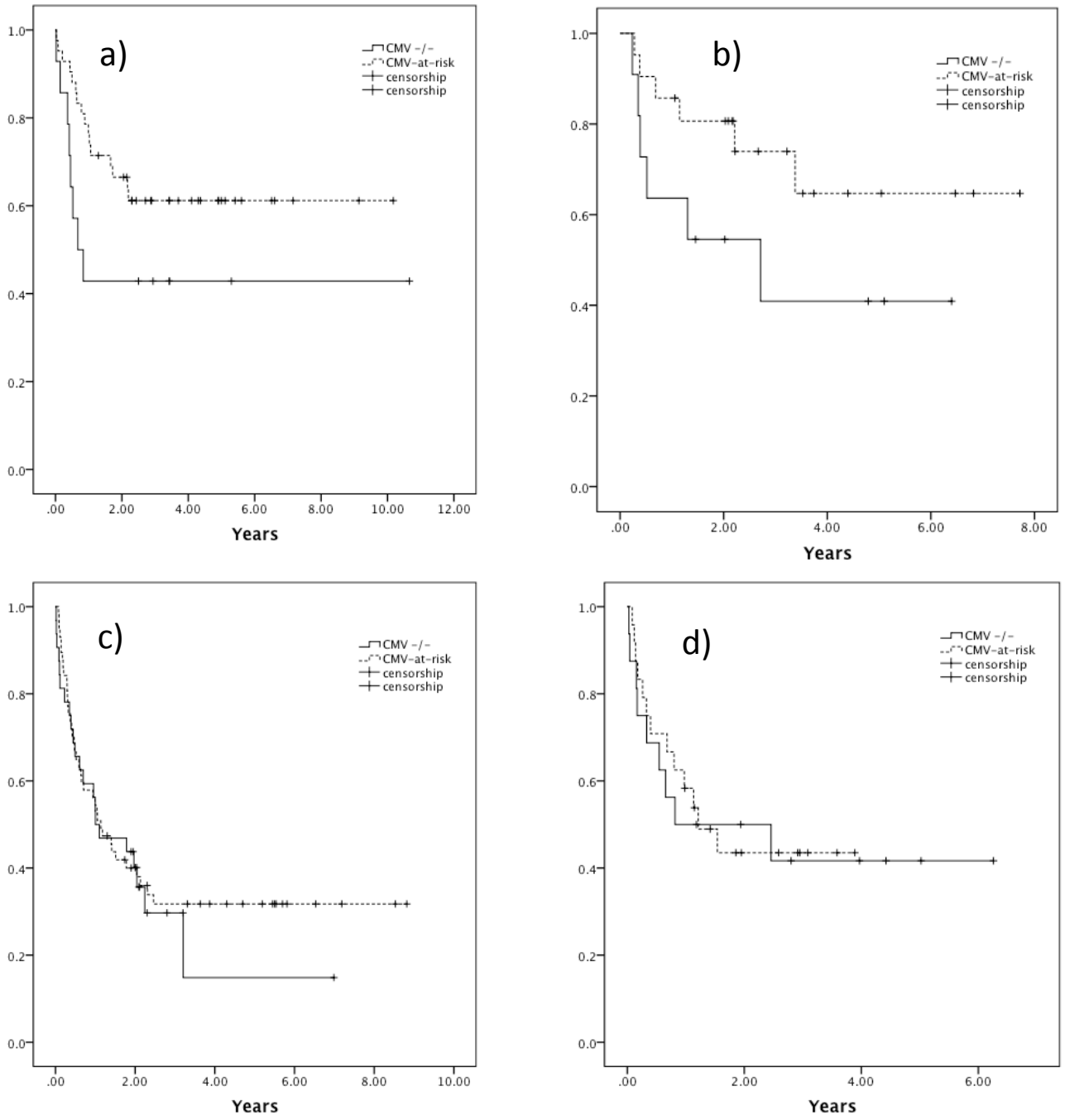


Figure 5.13: Association between CMV-at-risk status and overall survival is not a centre effect

a) There was improved overall survival in sibling, CMV-at-risk allo HSCT transplants in centre 1 and b) non-significant trend for improved overall survival in centres 2 and 3 combined. c) No difference in overall survival in CMV-at-risk unrelated donor transplants in centre 1 or d) in centres 2 and 3 combined

Multivariate analysis

A cox-regression analysis was performed on the dataset to assess the factors important in predicting outcome following transplant with this regimen. The donor source (Sibling versus unrelated) predominates as the main factor for predicting overall survival. This is mainly due to an increase in NRM within the group transplanted from unrelated donors (Hazard Ratio 3.44, p<0.001). Risk of death was over twice as high in the unrelated donor setting. CMV -/- status was associated with a 1.3 times risk of death compared to CMV-at-risk status, although this did not reach statistical significance within the whole group. CMV -/- patients were 1.5 times as likely to relapse, although this did not reach statistical significance in this multivariate model. (Table 5.27).

	Factor	***P value***	***Hazard ratio***	***95% Confidence Interval***
OS	Age	0.37	0.99	0.97 to 1.01
	Disease (AML vs MDS)	0.38	0.82	0.51 to 1.29
	CMV-at-risk	0.17	0.77	0.52 to 1.12
	Unrelated vs sibling	0.001	2.05	1.37 to 3.08
Relapse	Age	0.19	0.98	0.95 to 1.01
	Disease (AML vs MDS)	0.57	1.22	0.61 to 2.42
	CMV-at-risk	0.14	0.68	0.41 to 1.13
	Unrelated vs sibling	0.54	1.17	0.70 to 1.962
NRM	Age	0.87	1.00	0.97 to 1.04
	Disease (AML vs MDS)	0.012	0.46	0.25 to 0.83
	CMV-at-risk	0.37	0.78	0.45 to 1.35
	Unrelated vs sibling	<0.001	3.44	1.80 to 6.58

Table 5.27 Cox regression analysis for outcomes within the whole group OS=Overall Survival, NRM= non relapse mortality

Multivariate analysis within recipients of sibling allo HSCTs

Because donor source had such a large effect, I performed a subgroup analysis on recipients of sibling transplants. CMV-at-risk status was associated with improved overall survival and a decreases relapse risk. Age, and disease (AML vs MDS) did not make a difference to overall survival. The hazard ratio for relapse was 0.44 for CMV-at-risk patients amongst recipients of sibling transplants (Table 5.28).

	Factor	***P value***	***Hazard ratio***	***95% Confidence Interval***
OS	Age	0.96	1.0	0.96 to 1.04
	Disease (AML vs MDS)	0.15	0.52	0.22 to 1.26
	CMV-at-risk	0.024	0.45	0.23 to 0.90
Relapse	Age	0.81	0.99	0.95 to 1.03
	Disease (AML vs MDS)	0.50	0.73	0.30 to 1.83
	CMV-at-risk	0.039	0.44	0.20 to 0.96
NRM	Age	0.86	1.00	0.94 to 1.08
	Disease (AML vs MDS)	0.91	0.92	0.25 to 3.43
	CMV-at-risk	0.41	0.59	0.17 to 2.03

Table 5.28 Cox regression analysis for outcomes within the group transplanted from sibling donors

Within patients transplanted using T replete, myeloablative regimens, CMV serostatus makes no difference to overall survival

I went on to investigate whether the survival benefit from being CMV-at-risk extends to patients transplanted with T-replete regimens. All of the patients transplanted with T-replete regimens were from sibling donors.

The median survival for patients transplanted for AML with T-replete grafts from sibling allo HSCTs was 2.3 years for CMV -/- transplants versus 3.56 years for CMV-at-risk patients (p = 0.68 log-rank. N = 32 CMV -/- versus 25 CMV-at-risk, see Figure 5.14 and Table 5.29).

Donor	***CMV serostatus***	**n**	**6 month**	**12 month**	**2 year**	**5 year**
Sibling	At-risk	25	79%	61%	56%	44%
	-/-	32	71%	65%	57%	43%

Table 5.29 Kaplan-Meier estimates for overall survival in recipients of T-replete sibling allo HSCT for AML

It has previously been demonstrated that CMV seropositive patients transplanted from unrelated donors have a worse outcome (Craddock et al., 2001). In our dataset, there was a non-significant trend for worse outcomes if CMV-at-risk in a MUD setting for CML (median survival for CMV -/- (n = 9) not reached, versus 1.125 years for CMV at-risk (n = 11)).

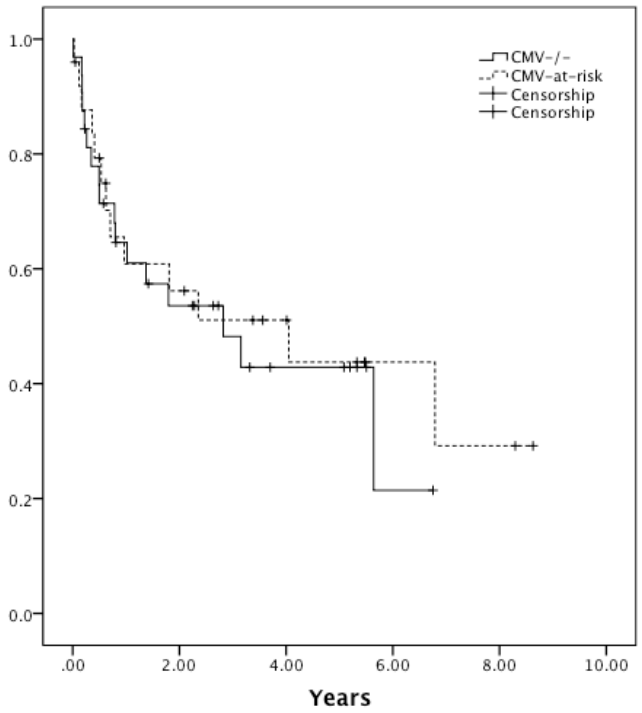


Figure 5.14: Overall survival from allo HSCT transplants for AML in recipients of myeloablative regimens

CMV-at-risk status is not associated with any difference in overall survival in recipients of myeloablative, T-replete sibling allo HSCT for AML

Discussion

Reduced intensity conditioned alloHSCTs were developed to decrease the morbidity and mortality associated with traditional myeloablative alloHSCTs. T cell depletion with alemtuzumab is added to these regimens to reduce transplant-related-mortality, particularly by reducing the incidence of GVHD (Chakraverty et al., 2002). Similarly, T-depletion with anti-thymocyte globulin (ATG) reduced chronic GVHD and NRM in recipients of unrelated donors (Socie et al., 2011).

However, relapse rates are increased in recipients of T-cell depleted grafts; in one study there was a 3-year relapse probability of 51% in alemtuzumab-conditioned patients versus 38% in T-replete grafts (Soiffer et al., 2011). In this study, there was no difference in non-relapse mortality comparing T-deplete with T-replete grafts. GvHD rates were lower in T-depleted grafts. Disease-free survival was reduced in T-cell depleted grafts, and there were no differences in the cause of death in the different treatment groups, with relapse being the most common.

The alloimmune effect is likely to be particularly important in recipients of RIC allo HSCT, because the cytoreductive effects of the conditioning are insufficient to eradicate the malignancy (Soiffer et al., 2011), however there is currently little data demonstrating a GVL effect in the setting of RIC alloHSCT for AML. It has been demonstrated that lower ciclosporin dose post transplant is related to decreased relapse risk (Craddock et al., 2010).

It is thought that the immune reconstitution following allo HSCT is due to antigen driven homeostatic proliferation (Storek et al., 2008). The thymic production of naïve T cells is less efficient in older adults (Hakim and Gress, 2002, Small et al., 1999) and in this population antigen driven homeostatic proliferation will predominates as the

mechanism to reconstitute the T cell pool. Indeed, it is thought that the thymic reconstitution of naïve T cells may take 1 to 2 years, and may not occur at all in patients older than 50 years (Mackall et al., 1995, Roux et al., 2000). CD4 count has been shown to reconstitute more slowly in older adults (Berger et al., 2008). Herpes viruses, particularly CMV, are known to be very important drivers of antigen-specific immune reconstitution post allo HSCT (Cwynarski et al., 2001, Gratama et al., 2001), indeed, CMV infection post alemtuzumab-conditioned alloHSCT is associated with a 5-fold increase in CMV-specific CTLs (Lamba et al., 2005).

It is therefore possible that in the relatively older group of patients transplanted with RIC alloHSCTs that antigen-driven homeostatic proliferation is particularly important.

A higher absolute lymphocyte count at 3-4 weeks post T-cell depleted allo HSCT has been associated with an improved outcome (Savani et al., 2007b). All transplants in this study were from sibling donors, and were transplanted for haematological malignancies (AML, CML and ALL). The patients with overall lymphocyte counts >450 cells/μl at day 30 post SCT had improved overall survival, with lower relapse rates and less NRM. All patients received T-depleted grafts with DLI given. CMV serostatus was not included in the multivariate analysis in this study.

In paediatric patients, it has been demonstrated that faster immune reconstitution following allo HSCT was associated with a lower mortality risk. The main predictors were NK cell and CD4 T cell reconstitution. They demonstrated delayed T cell reconstitution in unrelated donor transplants compared to sibling transplant recipients (Bartelink et al., 2013).

CD4 T cell recovery is thought to be delayed in recipients of unrelated donor, as opposed to sibling, allografts, and may predict risk of death. In multivariate analysis, CD4 count at day 35 post allo HSCT was an independent predictor of NRM in 758 patients transplanted for a range of different diseases (Berger et al., 2008). Similarly, in a study of 69 patients post allo HSCT, CD4 T cell counts at 3 months were found to be predictive of NRM and were found to be higher in recipients of sibling allo HSCTs (Kim et al., 2006a),.

In paediatric recipients of unrelated cord blood transplants, detection of antigen-specific T cell responses to lysates derived from herpes-virus infected fibroblasts post transplant correlated with improved relapse-free survival, with a significantly lower relapse risk in patients with detectable herpes virus specific responses (Parkman et al., 2006). Similarly, in a study comparing recipients of double umbilical cord transplants with mobilized peripheral blood stem cell transplants, slower reconstitution of CD4 T cells, CD8 T cells and Tregs was associated with more infections, but no increase in NRM or relapse. This study only included unrelated donor grafts and recipients of cord transplants (Jacobson et al., 2012).

A decrease in the absolute lymphocyte count following CMV reactivation post allo HSCT has been demonstrated to be associated with worse outcomes (Fries et al., 1993, Einsele et al., 1993).

We have demonstrated that alemtuzumab causes delayed immune reconstitution post alloHSCT. CMV seropositivity is associated with significantly greater numbers of T cells at 3 months post allo HSCT. This effect is seen regardless of whether T-cell depletion was used, but is more pronounced in patients that receive alemtuzumab as part of their

conditioning. Because of these differences, I went on to analyse the outcome of a group of patients transplanted with RIC allo HSCT for AML.

In our cohort of patients, higher NRM was seen in recipients of unrelated donor grafts compared with sibling grafts. This is consistent with other publications: in a study published from the Center for International Blood and Marrow Transplant Research, the transplant related-mortality was 21%, 28% and 31% at 1,3 and 5 years for recipients of sibling allo HSCTs compared to 31%, 37% and 40% for unrelated donor transplants (Jagasia et al., 2012). In this study, CMV seropositivity of either recipient or donor was associated with higher NRM in recipients of unrelated grafts, but not in recipients of sibling alloHSCT.

In other studies, unrelated donors were 1.3 times more likely to die from transplant related mortality after allo HSCT for AML if there was more than one mismatch in HLA-A/B/C or DRB1, although relapse rates were lower in this group (Saber et al., 2012). Of note, only 9% of the patients transplanted from sibling donors, and 25% of patients from unrelated donors, received T cell depletion in this study. In a second study, patients transplanted for AML with myeloablative conditioning, those transplanted even from 10/10 (HLA-A, B, C, DRB1 and DQB1) fully matched unrelated donors had higher NRM compared to those transplanted from matched related donors, with comparable rates of relapse (Walter et al., 2010). Fewer than 10% of the patients in this study had T-depleted grafts.

CMV's influence on outcome post AlloHSCT

CMV reactivation post alloHSCT with alemtuzumab is known to be common, with published incidences in the region of 60% at one year (Lamba et al., 2005). CMV has traditionally been associated with increased morbidity and mortality post alloHSCT

(Craddock et al., 2001). Indeed, a policy of early PCR based detection and therapy is almost universally adopted post allo HSCT (Ljungman et al., 2004). Of note, all of the clinical studies thus far investigating the effect of CMV serostatus and reactivation in altering outcome following allo HSCT have been performed in very heterogenous populations with different diseases. Most of the studies with positive results have been in patients transplanted for myeloid diseases. Furthermore, there are currently no large studies examining the effects of CMV on immune reconstitution and overall survival on patients transplanted for myeloid malignancies with RIC regimens, or in the context of T cell depletion with alemtuzumab (see table 5.30). The majority of publications thus far examining the effects of CMV on survival advantages in myeloid malignancies post transplant have examined the effect of CMV reactivation, as opposed to CMV seropositivity (Green et al., 2013, Ito et al., 2013, Elmaagacli et al., 2011). The rate of CMV reactivation in our patient cohort was 69.1%, which was higher than that seen in other studies. Of note, the studies demonstrating a reduced relapse risk have all been studies post transplant for myeloid malignancies (Green et al., 2013, Ito et al., 2013, Elmaagacli et al., 2011). These studies demonstrated that CMV PCR positivity, rather than serostatus, was the important factor in reducing relapse risk. However, the majority of patients in these studies were transplanted with T-replete regimens. The majority of patients transplanted with T-depleted regimens experience at least one episode of CMV PCR positivity. In the patient cohort that I studied, a non-significant trend was seen for reduced relapse in patients that had episodes of CMV PCR positivity within the first 50 days post transplant.

Publication	Subset of patients	n	Effect seen
(Craddock et al., 2001)	Cy/TBI conditioned MUDs allo HSCT for CML	106	Recipient CMV seropositivity associated with higher TRM and overall mortality
(Meijer et al., 2002)	Acute leukaemia, CML, aplastic anaemia	48 MUDs 205 MRDs	Recipient CMV seropositivity adversely affected outcome in MUD transplants
(Nachbaur et al., 2001)	Acute leukaemia	103	In HLA A2 positive recipients, there was a lower relapse risk and better OS in sibling allo HSCT associated with CMV seropositivity
(Nachbaur et al., 2006)	Acute leukaemia, MUDs, T-depletion with ATG	48	Donor CMV seropositivity associated with lower relapse risk (20% vs 52%), no OS difference
(Erard et al., 2006)	ALL, AML, CML, all T-replete	768	No difference in HLA-A2 individuals transplanted from sibling donors of different CMV serostatus
(Kollman et al., 2001)	Donor registry, acute leukaemia, CML, aplastic anaemia	6978 of which 1373 AML	No effect of donor CMV serostatus on outcome
(Ljungman et al., 2003)	EBMT megafile analysis	7018	In recipients of MUD grafts, beneficial effect of transplantation from CMV seropositive donors (improved EFS, no difference in relapse)
(Kim et al., 2006b)		76	CMV reactivation associated with improved survival
(Behrendt et al., 2009)	Paediatric transplants, all T-replete	140	CMV seropositivity of either donor or recipient associated with reduced relapse rate and similar TRM, improved OS
(Craddock et al., 2011)	Primary refractory AML from MUDs 132/168 with myeloablative regimens	168	CMV seropositivity associated with improved overall survival and leukaemia free survival
(Elmaagacli et al., 2011)	AML, myeloablative, T-replete grafts	266	CMV pp65 antigenaemia associated with significant decrease in relapse risk post allo HSCT
(Ito et al., 2013)	CML	110	CMV reactivation <d 100 associated with decreased relapse, no OS benefit
(Green et al., 2013)	Various AML, 95% T-replete	2566, AML 761	CMV pp65 antigenaemia associated with decreased D100 relapse risk in AML patients (HR 0.56), not in other conditions studied. Increased NRM in reactivating group, no OS difference

Table 5.30 Summary of publications on CMV and its effect on outcome following allo HSCT

A decrease in relapse risk has been outweighed by increased NRM in some studies, as detailed in the table above. We did not see increased NRM in our CMV-at-risk patients compared to the CMV -/- population. No other studies, however, have focused on a group of patients uniformly T-cell depleted with alemtuzumab. It is possible that the potential morbidity caused by CMV reactivation is offset by chronic antigenic stimulation of the immune system and earlier T-cell reconstitution.

Potential mechanisms of a beneficial effect from CMV could be due to alterations in a number of lymphocyte subsets. NK cell reconstitution has been shown to be accelerated by CMV seropositivity (Hokland et al., 1988). The proportion of well-differentiated TEMRA CD8 T cells is accelerated by CMV seropositivity (Storek et al., 2008), and we have shown that this remains true in the context of alemtuzumab conditioned RIC alloHSCT. In our data, the effect of CMV on terminally differentiated T cells seemed greatest in the cohort transplanted from sibling donors.

It has been shown that a higher NK cell count on day 30 post stem cell transplant is associated with improved overall survival, and less relapse, in patients receiving sibling alloHSCT for myeloid malignancies, but not for other indications (Savani et al., 2007a).

In patients transplanted for ALL and AML, the majority with T-deplete conditioning, it has been suggested that increased γδ T cell reconstitution within the first year after transplant is associated with a lower relapse risk, with a five year leukaemia-free survival of 54.4% versus 19.1%. No association with CMV was found in this study, although the γδ T cells predominantly expressed the Vδ1 chain (Godder et al., 2007).

In patients undergoing alloHSCT, LGL lymphocytosis has also been observed. From 201 consecutive patients receiving an allograft, 6 developed an LGL lymphocytosis,

associated with recurrent viral infections, especially CMV. 5/6 of these patients achieved a long-term complete remission. In this setting, all of the patients' LGLs were CD8/CD57+ve. Most were polyclonal (Mohty et al., 2002).

CMV seropositivity has been shown to be associated with increased numbers of Vδ2 –ve γδ T cells post allo HSCT. (Knight et al., 2010). We demonstrated similar increases in Vδ1 γδ T cells associated with CMV seropositivity in patients following allo HSCT.

Vδ1 γδ T cells have been shown to have in vitro activity against ALL blasts, demonstrated by proliferative activity during co-culture, and expression of the activation markers CD69 and HLA-DR (Lamb et al., 2001). More recently it has been shown that Vδ2 negative γδ T cells are capable of cross-reactivity against CMV and leukaemia cells (Scheper et al., 2013). They measured γδ T cells post stem cell transplant, and found an expansion of nonVδ2 γδ T cells associated with CMV reactivation, which had a well differentiated phenotype. Expanded, patient derived nonVδ2 γδ T cells were able to recognize and secrete IFNγ in response to CMV-infected fibroblasts. Vδ2negative, and not Vδ2 positive γδ T cells from these patients were able to recognise leukaemia cell lines as well as primary AML blasts. Cloned CMV-reactive Vδ1 γδ T cells were able to cross-recognise tumour cell lines and primary AML blasts, in a TCR dependent manner. They also found that CD8αα was an important co-receptor for tumour responsive Vδ1 γδ T cells.

Conclusions

T cell depletion with alemtuzumab dramatically decreases the number of lymphocytes at 3 months post alloHSCT. The patients undergoing this procedure are nearly all older than 45 years old, and their capacity for thymic production of naïve T cells is likely to be low. Reconstitution of the immune system in this group of patients is likely to be more dependent on antigen-driven homeostatic proliferation than in younger patients. CMV drives the reconstitution of certain lymphocyte subsets, particularly CD8 and Vδ1 γδ T cells. In the absence of CMV, immune reconstitution is poor.

We have demonstrated that in patients that undergo RIC HSCT with FMC conditioning for AML that CMV seropositivity is associated with an improved outcome from transplant. This is conferred by a decreased relapse risk, with no extra NRM. The decreased relapse rate seems to be restricted to recipients of sibling allo HSCTs. The NRM is significantly higher in recipients of unrelated donor transplants; it is possible that this difference could mask any potential benefit of CMV seropositivity within this group.

The reconstitution of atypical, innate-like lymphoid subsets such as Vδ1 γδ T cells may contribute to this decrease relapse risk in sibling RIC allo HSCTs.

Chapter 6
General Discussion

I examined the association of CMV serostatus with immune profiles in two groups of patients undergoing treatment for myeloid malignancies.

CMV seropositivity is associated with significant alterations in the immune phenotype of patients treated with imatinib. These changes are consistent with changes that are seen in healthy CMV seropositive people. I did not find convincing evidence, however, of ongoing CMV reactivation. Within the imatinib-treated group, an increase in γδ T cells was associated with the achievement of deep responses to treatment.

Dasatinib is a TKI with activity against *Src* family kinases, which has been shown to have profound in vitro immunosuppressive effects and inhibits T cell receptor mediated activation of T cells. In patients treated with dasatinib I demonstrated evidence of CMV reactivation, as evidenced by an increase in the CMV-specific T cell response, and serological evidence of recent CMV reactivation. There is an associated expansion of the CD8 T cell compartment, particularly of terminally differentiated CD8 T cells.

CMV reactivation is associated with an expansion of Vδ1 γδ T cells. These also express a terminally differentiated memory phenotype. On examination of their T cell receptors, we have demonstrated a remarkable oligoclonality within the Vδ1 γδ T cell population in patients with expansions. Comparing different clones within the sorted Vδ1 γδ T cell fraction, there are only small differences in TCR sequences from one clone to another. The oligoclonality, suggests an antigen-driven expansion.

It has been shown that Vδ1 γδ T cells may recognize endogenous antigen in a TCR dependent manner (Willcox et al., 2012). It is possible that CMV reactivation induces over-expression of antigen on infected cells and the Vδ1 γδ T cell expansions are in response to this. However, there are no conserved regions in the CDR3 regions of TCR sequences from one patient to the next.

Although we did not see any activity of sorted Vδ1 γδ T cells against tumour cell lines, other authors have reported activity against a wide variety of tumour targets (Halary et al., 2005) including leukaemia cells (Scheper et al., 2013).

It has recently been reported that patients with increases in their overall lymphocyte count respond better to TKI treatment with dasatinib (Kumagai et al., 2014).

Ideally, I would like to prospectively examine outcomes from patients treated with dasatinib and assess their response according to CMV serostatus. Unfortunately, in the UK dasatinib is currently not NICE approved for treatment of CML as first line therapy.

In recipients of allo HSCTs, the role of CMV is controversial; it is conventionally associated with increased morbidity. There is an emerging literature suggesting that CMV reactivation may be associated with a decreased relapse risk in patients transplanted for AML (Elmaagacli et al., 2011, Ito et al., 2013, Green et al., 2013). Whilst these studies demonstrated a reduced relapse risk, there was no overall survival benefit from CMV reactivation. Of note, most of the published literature to date has been in patients with T-replete transplants.

I examined a patient cohort that was aggressively T-depleted using the anti-CD52 monoclonal antibody alemtuzumab, and found that CMV seropositivity was associated with a significantly higher number of T cells at 3 months post RIC alloHSCT, particularly of CD8 and Vδ1 γδ T cell subsets. This group of patients is likely to be more dependent on antigen-driven proliferation as they are a relatively elderly population compared to patients transplanted with myeloablative regimens, in whom this mechanism of immune

reconstitution may be more important than conventional thymic reconstitution (Storek et al., 2008).

In this cohort of patients treated for AML, CMV seropositivity was associated with a decreased relapse risk and an improved overall survival. The benefit from protection from relapse seemed to be seen largely within the first six months of transplant, suggesting that early immune reconstitution is very important in preventing relapse. We did not see the effect in patients transplanted from unrelated donors, but their NRM was significantly higher than sibling transplants, which may have abrogated the effect.

It is possible that this survival benefit is due to the expansion of a lymphocyte subset, which has dual anti-CMV and anti-tumour effects. Vδ1 γδ T cells would seem to be ideal candidates for this effect, and worthy of further investigation, particularly as it is becoming clearer what the T cell receptors recognize.

Further investigation of the Vδ1 γδ T cell receptors sequenced may reveal common antigens recognized by this subset of lymphocytes. The TCRs may recognize herpes virus peptides and be important in their control. It is also possible that they recognize over-expressed self-antigen, as is the case with a recently described interaction between a Vδ4 γδ T cell and EPCR (Willcox et al., 2012), potentially leading to dual anti-infective and anti-tumour roles (Halary et al., 2005). As these TCR/antigen interactions are not thought to be MHC restricted, they may be very useful as DLI post allo HSCT.
It is also possible that this data may inform donor selection for allogeneic stem cell transplant. Current strategy is to match CMV seronegative patients with CMV seronegative donors; in the context of RIC allo HSCT, this may not be the best strategy and may need to be re-evaluated.

Chapter 7
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Appendix

Taqman primers and probes

EBV POL

Forward primer CTT TGG CGC CGA TCC TC

Reverse primer AGT CCT TCT TGG CTA GTC TGT TGA C

Pol probe (FAM) – CAT CAA GAA GCT GCT GGC GGC C-(TAMRA)

β2m

Forward primer GGA ATT TTG GGA GAG CAT C

Reverse primer CAG GTC CTG GCT CTA CAA TTT ACT AA

B2m probe (VIC) (VIC)-AGT GTG ACT GGG CAG ATC CAC CTT C-(BHQ)

Appendix: Reagents for HLA-PCR

Primer Set 1 (A1 = A*0101, 0102)

Sense: 5' CGA CGC CGC GAG CCA GAA

Anti-Sense: 5' AGC CCG TCC ACG CAC CG

Primer Set 2 (A2 = A*0201-17)

Sense: 5' GTG GAT AGA GCA GGA GGG T

Anti-Sense: 5' CCA AGA GCG CAG GTC CTC T

Primer Set 3 (B7 = B*0702-0705, 8101)

Sense: 5' GGA GTA TTG GGA CCG GAA C

Anti-Sense: 5' TAC CAG CGC GCT CCA GCT

Primer Set 4 (B7 = B*0703)

Sense: 5' ACA CAG ATC TAC AAG ACC AAC

Anti-Sense: 5' TAC CAG CGC GCT CCA GCT

Primer Set 5 (B8 = B*0801, 0802, B51GAC, B*4406)

Sense: 5' GAC CGG AAC ACA CAG ATC TT

Anti-Sense: 5' CCT CCA GGT AGG CTC TGT C

Primer Set 6 (B8 = B*0801, 0802)

Sense: 5' GAC CGG AAC ACA CAG ATC TT

Anti-Sense: 5' CCG CGC GCT CCA GCG TG

Control Primer Set (PIC1, PICA)

Sense: 5' ATG ATG TTG ACC TTT CCA GGG

Anti-Sense: 5' TTC TGT AAC TTT TCA TCA GTT GC

* All Primers supplied by Invitrogen

BioTaq DNA Polymerase	Bioline	BIO-21040
DEPC Treated H_2O	Ambion	AM9906
Cresol Red Sodium Salt	Sigma Aldrich	114480
Glycerol	Sigma-Aldrich	G8773
dNTP	Bioline	BIO-39025
Agarose	Invitrogen	16500100
Hyper Ladder IV	CS	
Tris Base	Sigma-Aldrich	93362
Boric Acid	Sigma-Aldrich	B7901
EDTA	Sigma-Aldrich	E9884

Ethidium Bromide

BUFFERS:

2mM dNTP Stock Mix

dNTP A (Bioline)	250µl
dNTP B (Bioline)	250µl
dNTP C (Bioline)	250µl
dNTP D (Bioline)	250µl
PCR grade H_2O (Life Technologies)	11.5mls

Freeze in 1ml aliquots at -20°C

Cresol Red Stock Solution (10mg/ml)

Cresol Red Sodium Salt (Sigma)	30mg
PCR grade H2O (Life Technologies)	3mls

Store at room temp for up to 3 months

TDMH Buffer

Bioline 10 x NH_4 Buffer	676µl
Bioline 50mM $MgCl_2$ Solution	270µl
Ambion PCR Grade H_2O	776µl
Bioline 2mM dNTP Stock Mix	676µl
Glycerol (Sigma)	162µl
Cresol Red Solution (Sigma) 10mg/ml	40µl

Freeze in 50ml aliquots at -20°C

10 x TBE Buffer (Geneflow)

dH_20	800ml
Tris Base	108g
Boric Acid	55g
EDTA	9.3g

Adjust to 1litre with additional dH_2O

Isolation of mRNA from sorted γδ T cells

The sample was first defrosted and centrifuged at 14,000 rpm for 7 minutes at 4C in a microcentrifuge, and the supernatant removed. The cells were lysed by adding 0.4 ml of the BME-containing lysis buffer (OL1) to the cell pellet, and the mixture vortexed for 20 seconds. The lysate was then transferred to a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged at 14,000 rpm in a microcentrifuge, 18 to 25C, for 2 minutes. The flow-through was transferred into another 1.5 ml tube. Buffer ODB was then added to the lysate, and they were vortexed. 20 μl of Oligotex bead suspension was added and the mixture incubated for 10 minutes at room temperature, in order to hybridise the oligo(dT) on the beads with the polyA+RNA. The beads were then washed: They were firstly pelleted by centrifugation at 14,000 rpm for 5 minutes, 18 to 25C. The supernatant was removed. 350 mcl of buffer OW1 was added, and the mixture vortexed for 30 seconds to resuspend the beads. The sample was then placed in a small spin column placed in a 1.5 ml collection tube, which was centrifuged for 1 minute at 14,000 rpm. The flow-through was discarded. The spin column was then placed on another collection tube, and buffer OW2 added. This was centrifuged at 14,000 rpm for 1 minute. The collection tube was discarded. This step was then repeated. In order to elute the mRNA, the spin column was placed into a 70C heating block. 20 μl of pre-heated (70C) buffer OEB was placed into this column, and the mixture was pippetted up and down to mix. This mixture was then centrifuged at 14,000 rpm for 1 minute at 18 to 25C. This step was repeated to ensure maximum yield.

Manufacture of cDNA

cDNA was then synthesized, using a SMARTer PCR cDNA Synthesis kit (Clontech). 1 mcl of the 5' CDS oligo(dT) primer was added to a tube with 6 μl of the mRNA. This was

placed in a heating block at 70C for 1 minute, then at -20C for 1 minute. The following components were added: 3 μl 5*RT buffer, 1 μl DTT, 1 μl SMART II oligo, 1 mcl RNaseOUT, 1 mcl dNTP mix and 1 mcl Superscript II RT. The mixture was vortexed and placed in a heating block at 42C for 90 minutes. This mixture was then spun at top speed in a microcentrifuge, 10 μl of Tricine buffer added, and the mixture mixed with a pipette. The RT was deactivated by placing the mixture in a heating block at 72C for 7 minutes, and then the mixture was spun briefly at top speed. cDNA clean up was performed using a NucleoSpin Extract II procedure. cDNA binds to a silica membrane in the presence of chaotropic salt in the NT binding buffer.