AN INVESTIGATION INTO THE IMPACT OF CYTOMEGALOVIRUS INFECTION IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKAEMIA

BY

HELEN MARIE PARRY

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College of Medical and Dental Sciences

University of Birmingham

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ABSTRACT

Chronic Lymphocytic Leukaemia (CLL) is associated with significant immunosuppression, with infection being the predominant cause of death. The immunosuppression is multifactorial and includes hypogammaglobulinaemia and T cell dysfunction. Expanded populations of effector memory T cells which are oligoclonal are postulated to arise in response to common antigenic stimulation. Increased expression of the inhibitory receptor programmed-death 1 (PD-1) has also been reported on the total CD4+ and CD8+ T cells in patients with CLL. Cytomegalovirus is a ubiquitous herpes virus which contributes to the oligoclonal populations of CD4+ and CD8+ T cells described. In the healthy elderly, CMV is associated with an immune risk phenotype and leads to an earlier death. But the impact of CMV on clinical outcome measures in CLL is unknown.

Using 60 CMV class I tetramer and for the first time 15 class II tetramer responses, I have characterised the phenotype and function of CMV-specific T cells in patients with CLL. Interestingly, increased expression of PD-1, was observed on CD4+ but not CD8+ CMV-specific T cells which remained constant over time and was not a result of recent T cell activation. Cytokine production of both CD4+ and CD8+ CMV-specific T cells was shown to be impaired in patients with CLL and PD-1 expression on CMV-specific CD4+ T cells contributed to this. Telomere lengths were also greatly reduced in CMV-specific T cells.

I have also used droplet digital PCR to successfully measure latent CMV viral load and found in advanced stage disease an increased CMV viral load was detectable. This most likely arises as a result of the increased immunosuppression and T cell dysfunction observed.

Despite these findings, this work reports no evidence that CMV infection impacts on clinical outcomes including time to first treatment or overall survival in two large independent cohorts of patients with CLL.

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DEDICATION

To my wonderful parents, husband and daughters for their endless support and love. Their interest in my work never fails to amaze me but I will never forget the day I overheard Adam telling a relative that I studied Chronic Myeloid Leukaemia…..

PUBLICATIONS

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ABBREVIATIONS

ATM   Ataxia telangiectasia mutated

B2M   Beta-2 microglobulin

BCL-2   B cell lymphoma-2

BCR   B cell receptor

BCSH   British committee for standards in haematology

BHH   Birmingham Heartlands Hospital

BR   Bendamustine and rituximab

Breg   B regulatory cell

BTK   Bruton tyrosine kinase

CCL   Chemokine ligand

CD   Cluster of differentiation

CDR   Complementary determining region

CLiP   Class II invariant-chain peptide

CLL   Chronic Lymphocytic Leukaemia

CMV   Cytomegalovirus

CT   Computerised tomography

CXCR   Chemokine receptor

ddPCR   Droplet digital PCR

DEPC   Diethylpyrocarbonate

DMSO   Dimethyl sulfoxide

dNTPs   Deoxy-nucleoside triphosphates

EBV   Epstein-barr virus

ELISA   Enzyme-linked immunosorbent assay

Eomes   Eomesodermin

FCR   Fludarabine, cyclophosphamide and rituximab

FCS   Fetal calf serum

FSC   Forward scatter

FISH   Fluorescence in situ hybridization

Fnabs   Functional antibody testing

FoxP3   Forkhead box P3

gB   Glycoprotein B

Hb   Haemoglobin

HD   Healthy donors

HIV   Human immunodeficiency virus

HLA   Human Leukocyte Antigen

IE   Intermediate early

IFN   Interferon

Ig   Immunoglobulin

IGHV   Immunoglobulin heavy-chain variable-region

IQR   Inter-quartile range

IRP   immune risk phenotype

Ii   Invariant chain

IL   Interleukin

IWCLL   International workshop on CLL

LCL   Lymphoblastoid cell line

LDT   Lymphocyte doubling time

LRF   Leukaemia research fund

mAb   Monoclonal antibody

MBL   Monoclonal B-cell lymphocytosis

MFI   Mean florescence intensity

MHC   Major histocompatibility complex

MIEP   Major immediate-early promotor

MRD   Minimal residual disease

NLC   Nurse like cell

ORF   Open reading frames

ORR   Overall response rate

OS   Overall survival

PBMC   Peripheral Blood Mononuclear Cells

PBS   Phosphate buffered saline

PCR   Polymerase chain reaction

PD-1   Programmed death 1

PET   Positron emission tomography

PFA   Paraformaldehyde

PFS   Progression free survival

Pi3K   Phosphoinositide 3-kinase

PMA   Phorbol-myristate acetate

PP65   Phosphoprotein 65

QEHB   Queen Elizabeth Hospital Birmingham

QPCR   Quantitative PCR

RAG   Recombinase activating gene enzyme

REC   Research ethics committee

RPM Revolutions per minute

RT Room temperature

SD Standard deviation

STELA Single telomere length analysis

TAP Transporter of antigen processing

Tbet T-box transcription factor

TCR T cell receptor

TDMH Tris-buffer, dNTPs, Magnesium and water

TGF Transforming growth factor

Th1 T helper type 1

Th2 T helper type 2

TNF Tumour necrosis factor

Treg T regulatory cell

TTT Time to first treatment

WHO World Health Organisation

ZAP-70 Zeta-chain associated protein -70

1 INTRODUCTION

1.1 The human immune system

1.1.1 Overview of the human immune system

The human immune system is comprised of the innate and adaptive immune response. It has evolved to destroy pathogens and recognize abnormal self antigens. The innate response is more primitive and is comprised of phagocytes (macrophages, neutrophils and dendritic cells) basophils, eosinophils, mast cells, γδ T cells and natural killer cells. It is referred to as the "non-specific" arm of the immune system, as it recognises foreign antigen through conserved, pathogen-associated molecules and does not require prior exposure to the pathogen in question (Murphy et al., 2012, Alberts et al., 2002). The human complement system is also part of the innate immune system, existing as a cascade of plasma proteins that act to opsonize foreign antigen and recruit inflammatory cells. The complement system also assists the antibody-mediated response and can subject infected cells to cytolysis (Dunkelberger and Song, 2009). The innate immune response delivers a fast and broadly effective response, but does not provide any immunological memory (Murphy et al., 2012).

In contrast, the adaptive immune system provides immunological memory to previously encountered pathogens and is antigen specific. It is composed of a humoral response mediated by B lymphocytes and a cell mediated response mediated by αβ T lymphocytes. The specificity of the adaptive response arises through receptors on the cell surfaces termed the B cell receptor (BCR) and T cell receptor (TCR), which recognise cognate antigen and are largely similar in structure (Goldsby et al., 2003). Although the initial response following primary exposure is slow, immunological memory permits a rapid, effective response should the same pathogen be encountered again (Murphy et al., 2012). The effectiveness of vaccination relies on an intact adaptive immune response to generate long term immunological memory (Ahmed and Gray, 1996).

1.1.2   Major histocompatibility complex (MHC)

The major histocompatibility complex is also known as the human leukocyte antigen (HLA). The two main types of MHC molecule are referred to as MHC I and MHC II. Both MHC molecules are found on the cell surface and contain a peptide-binding groove where antigen is presented (Townsend and Bodmer, 1989). MHC molecules are highly polymorphic due to the presence of a large number of alleles at each of the 9 loci on chromosome 6 that encode the MHC proteins. This increases the chance that peptide will be able to bind with good affinity and be presented. Humans inherit MHC alleles 'en-bloc', with one chromosome containing maternal alleles and the other containing paternal MHC alleles, with co-dominant expression (Murphy et al., 2012).

For T cell activation, the TCR interacts directly with the MHC molecule presenting the cognate peptide. MHC 1 expression is found on all nucleated cells in the human body and serves to present antigen from within the cell (either endogenous or pathogenic) to cytotoxic CD8+ T cells. For this, the proteasome processes protein into peptides, which are then transported via the 'transporters of antigen-processing' (TAP) to the endoplasmic reticulum for loading onto the MHC I molecule (Heath and Carbone, 2001). MHC II molecules are found on the professional antigen presenting cells, which are dendritic cells, macrophages and B cells. These endocytose exogenous antigen and present it via the MHC II binding domain to CD4+ T helper cells (Murphy et al., 2012). MHC II molecules are prevented from binding endogenous peptide in the endoplasmic reticulum by the presence of an invariant chain (Ii). This is later degraded before being exchanged for peptide ready for presentation to CD4+ T cells. Finally, additional antigen presentation (cross-presentation) can arise from dendritic cells phagocytosing antigen from other cells, followed by TAP-dependent processing and presentation via MHC-1 to cytotoxic CD8+ T cells (Heath and Carbone, 2001) (Figure 1.1).
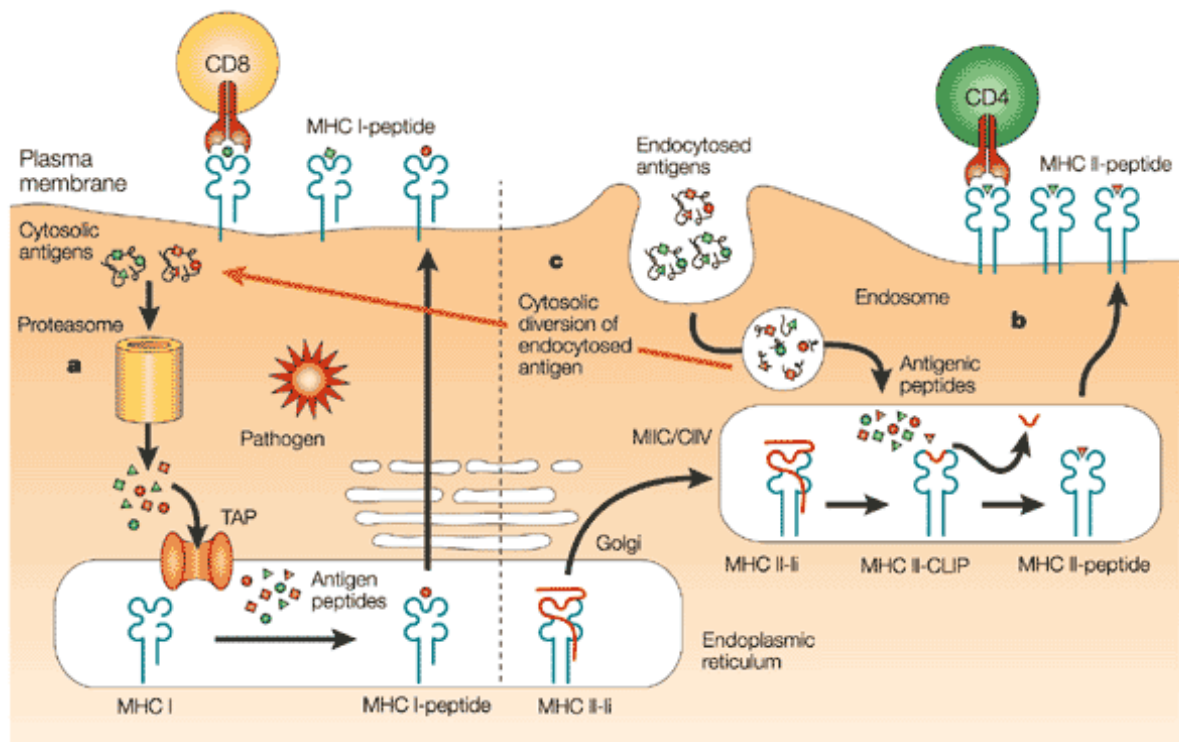
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Figure 1.1

The MHC class I and II antigen presentation pathways

MHC class I molecules present peptide that has been processed from endogenous proteins by the proteasome. The resulting peptides are transported via transporters of antigen-processing (TAP) to the endoplasmic reticulum where they are loaded onto the peptide binding groove of MHC I before being transported to the cell surface for antigen presentation to CD8+ T cells. MHC class II molecules are found on professional antigen presenting cells and present exogenous antigen. Peptides that have been endocytosed are then loaded onto the MHC II binding groove. The presence of an invariant chain (Ii) prevents endogenous peptides being presented. This is degraded to CLIP (class II invariant-chain peptide), which is then exchanged for the exogenous peptide and presented on the cell surface to CD4+ T cells. Adapted from (Heath and Carbone, 2001). Permission obtained from Nature publishing group (license number 3856960893508).

1.1.3   B cells

B cells arise from haematopoietic stem cells and each cell has a unique BCR. The BCR is also known as an immunoglobulin and is composed of a pair of identical heavy and light chains joined together by disulphide bonds. Each chain has a highly conserved constant region and a diverse variable region. The combination of the heavy and light chain variable regions determines the antigen binding specificity (Edelman and Gally, 1964). The genes encoding the variable regions of the heavy chain are found in 3 separate segments called the V, D and J segments; for the light chain variable region these consist of just V and J segments (Hozumi and Tonegawa, 1976). These are randomly combined by recombinase activating gene enzymes (RAG-1 and RAG-2) to increase the receptor repertoire (Oettinger et al., 1990, Schatz et al., 1989) (Figure 1.2). Further receptor diversity is gained through the addition of extra nucleotides by terminal deoxynucleotidyl transferase and somatic hypermutation (Goldsby et al., 2003).

Within the bone marrow, early B cells are first subjected to positive selection by a mechanism independent of antigen recognition and then by negative selection, whereby those with a BCR able to recognize self-antigen undergo deletion, anergy, or ignorance in order to achieve tolerance and prevent a response to self-constituents (Murphy et al., 2012). After exiting the bone marrow, the B cells circulate to the secondary lymphoid organs where they differentiate into mature, naïve B cells. Mature B cells that have undergone somatic hypermutation encounter their cognate antigen via their BCR and endocytose antigen in order to process it and present it to T follicular helper CD4+ T cells, which are located in the germinal centers of secondary lymphoid organs (LeBien and Tedder, 2008). Within the germinal centers, T follicular helper cells then give contact-dependent survival signals which permit the differentiation of B cells into memory B cells or plasma cells, which are then capable of

producing antibody with the same antigen specificity in future responses (LeBien and Tedder, 2008).

Regulatory B cells (Breg) are an additional type of B cell that support immunological tolerance. Breg cells secrete Il-10 and TGF-Beta and have an immunosuppressive impact on activated immune cells (Rosser and Mauri, 2015). They are also able to skew immune responses towards a regulatory phenotype and are thought to be important in the development and maintenance of T regulatory cells (Flores-Borja et al., 2013, Yoshizaki et al., 2012).
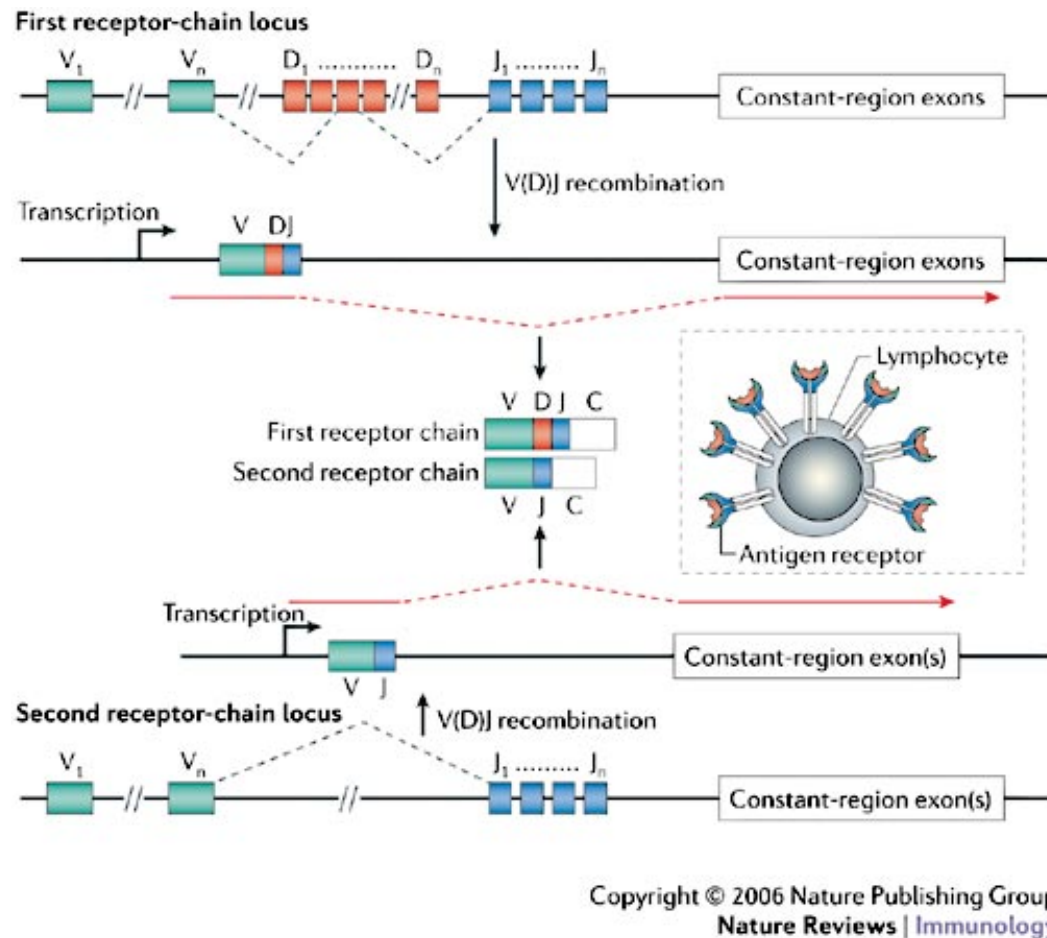
Figure 1.2

Gene rearrangement of V (D) and J segments for receptor generation.

The BCR and TCR are both composed of protein chains derived from independent loci. The

first receptor chain locus (the heavy chain in the BCR and beta chain in the TCR) is

composed of a recombination of gene segments known as the V, D and J segments. The

second receptor chain locus is a recombination of just V and J segments (this is the light chain

in the BCR and the alpha chain in the TCR). These loci make up the variable regions of the

receptor chains and following transcription are spliced (indicated by red dotted line) together

with the constant regions of the BCR or TCR to complete the chain. Adapted from (Nemazee,

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1.1.4 T cells

T cell progenitors also arise from haematopoietic stem cells in the bone marrow and migrate to the thymus where they undergo gene rearrangement of their alpha and beta chains to produce a TCR (Kondo, 2010, Hedrick et al., 1984). Similar to the BCR, the β chains contain rearranged V,D and J segements, whilst the α chains contain rearranged V and J segments (figure 1.2). These gene rearrangements permit up to 10^{15} unique receptors to be produced from a relatively small number of genes (Arstila et al., 1999). Each cell has receptors of only one specificity and around 30,000 TCRs on its cell surface (Murphy et al., 2012). At this stage T cells express both CD4+ and CD8+ receptors. Before exiting the thymus, the thymocytes are subjected to a process of positive and negative selection through interaction with thymic antigen presenting cells expressing MHC molecules (Klein et al., 2014). T cells recognizing MHC II, lose expression of CD8 and express just CD4, whilst those recognizing MHC I express just CD8 and lose CD4 (Murphy et al., 2012). Those recognizing self antigen with strong affinity are negatively selected for, whilst those with the potential to recognize non-self are positively selected for and become mature, antigen-naïve T cells (Klein et al., 2014). After exiting the thymus, naïve T cells enter the periphery but continue to re-circulate within secondary lymphoid organs where they can interact with cognate antigen. Professional antigen presenting cells migrate to the secondary lymphoid organs and present antigen via MHC molecules to the re-circulating lymphocytes. When naïve T cells encounter cognate antigen, they exponentially proliferate and differentiate into helper T cells (CD4+) or effector CD8+ T cells. CD8+ effector cells mediate their cytotoxicity through molecules known as perforin and granzyme or via the Fas/Fas ligand pathway (Lowin et al., 1994). CD4+ helper T cells consist of 2 main subtypes; T helper 1 type (Th1) and T helper 2 type cells (Th2). Th1 cells are helper T cells that produce IFNγ and promote CD8+ cytotoxic T cells responses. Th2

cells target extracellular parasites and can produce Il4, Il5 and IL10 and assist the humoral response (Murphy et al., 2012).

In humans, following resolution of infection, a pool of specific memory T cells persist, which are proficient at generating a rapid and effective response should re-infection or reactivation occur. This pool of T cells can be defined into memory and effector subsets based on combinations of CD45RA, CCR7 and CD27 expression (Sallusto et al., 2004, Sallusto et al., 1999). Naïve T cells are double positive, expressing CCR7 and CD45RA, whereas primed CD8+ T cells can be considered as belonging to one of three different subsets. Two of these lack expression of CD45RA; central memory cells (T_{CM}) and effector memory (T_{EM}) cells. T_{CM} cells express CCR7, while T_{EM} cells lack expression of CCR7. T_{CM} cells respond to antigen by proliferation and differentiate into T_{EM} cells. In comparison T_{EM} cells have poor proliferative capacity but great effector function and produce cytokines including IFN-γ (Larosa and Orange, 2008). In humans there is another memory subset, termed T_{EMRA}, which are CCR7 negative cells that co-express CD45RA. These cells are the most differentiated of memory cells and have poor proliferative capacity but are highly cytotoxic (Geginat et al., 2003) (Figure 1.3).

Other types of T cells also exist and include T regulatory cells (Tregs). Tregs act to suppress the actions of other T cells and immune components. They can prevent potentially self-reactive T cells that have exited the thymus from causing auto-immune disease. 'Natural' T regulatory cells (Tregs) are produced in the thymus and express cell surface markers CD25, CD4 and the transcription factor Forkhead box P3 (FoxP3) (Hori et al., 2003). They mediate their immunosuppressive actions through direct cell contact and also via suppressive cytokine production. Further 'inducible' Tregs also exist and are produced in the periphery in response to antigen stimulation (Vignali et al., 2008).
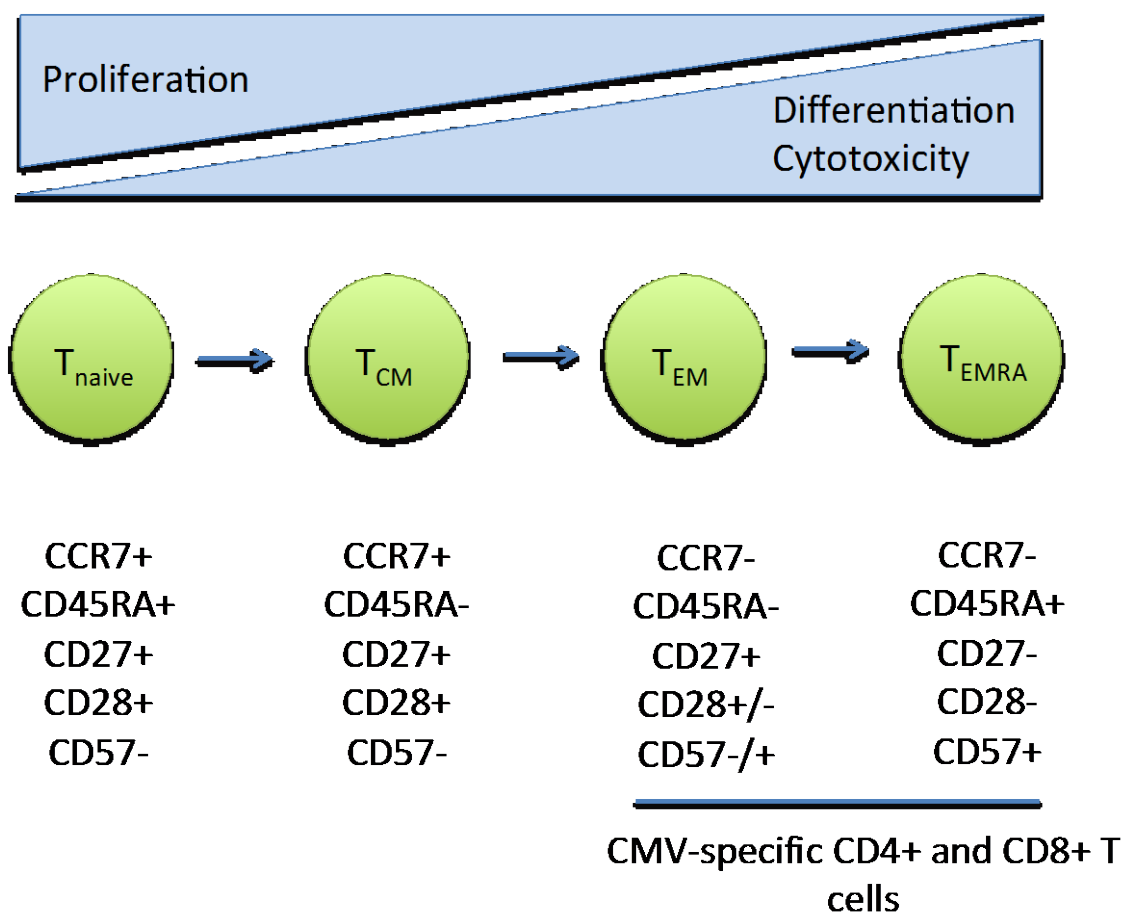
Figure 1.3

T cell memory subsets and their phenotypic markers

The phenotype of memory cells based on CCR7, CD45RA, CD27, CD28 and CD57

expression. As memory cells become more differentiated, their cytotoxic capacity increases

but their proliferative capacity diminishes (Mahnke et al., 2016).

1.1.5 Exhaustion and senescence

T cell exhaustion is used to describe cells that have been exposed to chronic, excessive antigen stimulation and is observed in the context of chronic viral infections and cancer. Exhausted T cells exhibit a hierarchal loss of effector functions and have increased expression of inhibitory receptors including programmed death 1 (PD-1), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and lymphocyte activation gene 3 (LAG-3) (Wherry et al., 2003, Legat et al., 2013). T cell exhaustion is reversible and immune responses can be reinvigorated through blockade of the inhibitory receptors (Wherry, 2011). Despite their name, exhausted T cells are not inert and still act to prevent pathogen or tumour proliferation but their functioning is suboptimal and thus ineffective at eradicating disease. They also have a distinct transcriptional profile to that of effector and memory T cells (Wherry and Kurachi, 2015).

Senescent T cells arise from repetitive T cell stimulation over time. Like exhausted T cells, they are viable cells with a low proliferative capacity and low telomerase activity. Senescent T cells differ from exhausted cells in their low expression of inhibitory markers, high expression of CD57 and preserved effector function (Wherry and Kurachi, 2015).

1.1.6 Tetramers

Tetramers permit the isolation and characterization of antigen-specific T cells in the laboratory. They consist of MHC-peptide complexes that contain streptavidin, which is tetravalent in structure (Altman et al., 1996). Typically, in-vivo interactions between T cells and self-MHC molecules presenting peptide are weak and very short lived. The presentation of 4 peptide-MHC molecules improves the avidity and extends the half-life of the interaction and thus improves the chances of detecting antigen-specific T cells (Dolton et al., 2015, Altman et al., 1996). This interaction is then identified by flow cytometry and the magnitude

of antigen-specific T cell responses can be assessed (Khan et al., 2002). MHC class I tetramers were first described in 1996 to identify HIV-specific CD8+ T cells (Altman et al., 1996) followed by class II tetramers in 1998 (Crawford et al., 1998). Although the use of MHC class I tetramers have been common place since their discovery, use of MHC class II tetramers has lagged behind due to difficult in stably producing MHC class II tetramer (which requires optimized folding conditions for each species and allotype) and low avidity (Vollers and Stern, 2008).

Class I tetramers are formed from B2microglobulin and the MHC class I molecule folding around the peptide of interest. This forms the monomer MHC/peptide unit, which is then biotinylated and purified. Four individually biotinylated monomers then combine with streptavidin to form the tetramer. The structure of a class I tetramer is illustrated in Figure 1.4 (Klenerman et al., 2002). Similarly, class II tetramers are formed from the folding of peptide around MHC class II molecules and biotinylated before combining with streptavidin.
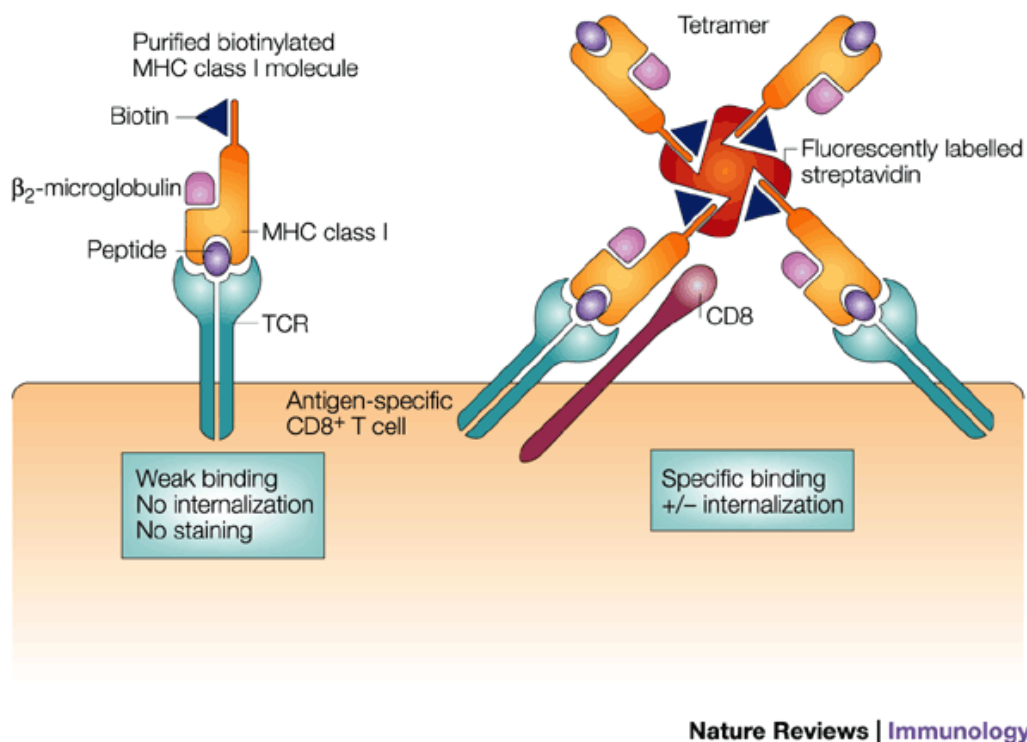
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Figure 1.4

The structure of a tetramer

Tetramers are composed of four monomeric MHC molecules with bound cognate peptide. T cell interactions with an individual MHC molecule (shown on the left), would fail to produce adequate binding and reproducible staining. When 4 MHC molecules are bound by fluorescent labeled streptavidin (shown on the right), the avidity of the bound T cells increases and can be visualised by flow cytometry. Adapted from (Klenerman et al., 2002) . Permission obtained from nature publishing group. (License number 3900150693292).

1.2 Chronic Lymphocytic Leukaemia

1.2.1 Overview of chronic lymphocytic leukaemia

Chronic Lymphocytic Leukaemia (CLL) is the most common leukaemia in Western Society, with an estimated UK incidence of 3400 cases per annum (CRUK, 2015). Clinically, CLL behaves heterogeneously and remains incurable with current chemotherapeutic options with the exception of allogeneic transplantation for a small minority of patients. Up to 80% of patients are incidentally detected by a persistent lymphocytosis and most patients are over the age of 65 years (Shanafelt and Kay, 2007). Given most patients are identified at an early stage, treatment is often not required at diagnosis and up to one third of patients may never require treatment during their lifetime (Shanafelt and Kay, 2007).

CLL is characterised by an unremitting proliferation of a mature B cell clone, which have specific immunophenotypic markers that differentiate it from other B cell malignancies. The malignant cells resemble antigen experienced B cells and demonstrate monoclonality through a predominance of either kappa or lambda light chain expression. Using sensitive immunophenotyping to screen healthy populations it has been shown that a CLL clone may be present in up to 12% of the population over the age of 40, where the lymphocyte count remains <5000/µl (Rawstron et al., 2002a, Ghia et al., 2004). This is now termed Monoclonal B lymphocytosis (MBL) and is known to precede all cases of CLL (Marti et al., 2005, Landgren et al., 2009). MBL can be split into low count (<0.5x 10^9/l lymphocytes) for which the risk of progression to CLL is negligible and routine follow up is not warranted, and high count >0.5x10^9/L where the risk is between 1 and 2 % per year, a situation akin to another clonal B cell disorder, Monoclonal Gammopathy of Undetermined Significance and its progression to Multiple Myeloma (Strati and Shanafelt, 2015).

In CLL, the clonal B cells, were originally thought to be indolent with a low proliferative capacity. The replicative ability of CLL cells was appreciated when Messmer D *et al* found the leukaemic cell birth rate to be between 0.1 and 1% of the CLL clone each day (Messmer et al., 2005). Traditionally, the bone marrow was thought to be the proliferative component of CLL with the peripheral component being quiescent. Further work has shown that in addition to the bone marrow, much of this proliferative activity actually occurs within secondary lymphoid organs and that the microenvironment of the lymph node is important for encouraging the growth and survival of the tumour cells particularly in pseudofollicles (Herishanu et al., 2011, Patten et al., 2008). Stimulated T cells and aberrant cytokine signaling participate in tumour evolution and provide anti-apoptotic support, which secures the longevity of the tumour clone (Ramsay and Rodriguez-Justo, 2013).

Predicting time to first treatment at diagnosis remains difficult based on the universally adopted staging methods developed four decades ago and although prognostic scoring systems which integrate biological and genetic features are now available, these are still not commonplace in clinical practice (Parikh and Shanafelt, 2016). For the majority of patients, treatment involves one or other conventional chemotherapy agents with or without monoclonal antibody therapy usually for a planned number of cycles, although the duration is often dictated by patient tolerance and toxicity, balanced against the need to achieve minimal residual disease status. Newer agents targeting the BCR signaling pathway offer efficacy for those with relapsed disease with a 71% overall response rate (Byrd et al., 2013) and trials are underway to assess their use in first line management. Despite the great responses seen to newer agents, CLL still remains incurable without an allogeneic transplant, a procedure that for most CLL patients remains inappropriate given the age profile and co-morbidities prevalent in this cohort of patients.

In addition to the overt clinical features of CLL, immunosuppression, which is multifactorial, is also problematic. It results from a combination of hypogammaglobulinaemia, immune cytopenias, a dysfunctional T cell repertoire and iatrogenic myelosuppressive therapy (Hamblin and Hamblin, 2008). Infection remains the commonest cause of death despite early use of antibiotic regimens and prophylactic immunoglobulin therapy to those deemed to be high risk (Hamblin and Hamblin, 2008). Overall CLL is highly heterogeneous, with a variable clinical course extending from a normal lifespan for some, to a progressive and refractory disease, with premature mortality in others (Chiorazzi et al., 2005).

1.2.2 Diagnostic features of Chronic Lymphocytic Leukaemia

The diagnosis of CLL is confirmed by peripheral blood film examination, demonstrating small, uniform mature lymphocytes with clumped chromatin, small nucleoli and little cytoplasm (Bain, 2006). Smear cells may be visible and are the result of pronounced mechanical fragility of the leukaemic cells, which become more predominant as the disease progresses.

To fulfill the diagnostic criteria set by the International Workshop on Chronic Lymphocytic Leukaemia (IWCLL) a clonal lymphocytosis of $>5x10^9$/L must persist for more than three months and demonstrate typical cell markers (Hallek et al., 2008). The characteristic phenotype includes B cell markers CD5, 19, 23 and 20. In addition, they express surface immunoglobulin weakly. The absence or weak expression of FMC7, CD79B and CD22 is also apparent (Dighiero and Hamblin, 2008, Hallek et al., 2008, Moreau et al., 1997, Matutes et al., 1994). Matutes *et al* first produced a 5 point scoring system for cell markers indicative of a diagnosis of CLL, which was later modified by Moreau *et al* (table 1.2) (Matutes et al., 1994, Moreau et al., 1997). Scores of 1 or 2 are indicative of alternative B cell disorders whilst scores of 4 or above are highly indicative of a diagnosis of CLL (Moreau et al., 1997).

The presence of anaemia should be investigated to exclude an autoimmune haemolytic component, which is known to occur in 5% of CLL cases (Moreno et al., 2010). Screening for hypogammaglobulinaemia is also commonly performed at diagnosis. Further prognostic tests including IGHV status and fluorescence in situ hybridization for chromosomal abnormalities may be undertaken at the clinician's discretion and where resources permit (Parikh et al., 2016).
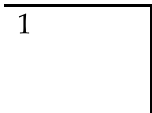
Prior to any treatment commencing, FISH testing should always be performed, including those patients who had no evidence of chromosome 17p deletion at diagnosis (Oscier et al., 2012, Zent and Burack, 2014). The presence of chromosome 11q or 17p deletion is known to carry an adverse prognosis and poor response to conventional alkylating agents and purine analogues. Mutation analysis for p53 and ATM mutation should also be undertaken, alongside bone marrow examination for clarification as to the origin of any cytopenias that are present and a CT staging scan to assess non-palpable disease and to monitor treatment response (Oscier et al., 2012).

Table 1.1

The modified CLL scoring system

A score of 4 or above is highly indicative of a diagnosis of CLL (Moreau et al., 1997).

Antigen

ive   1

1.2.3   Epidemiology of CLL

Chronic Lymphocytic leukaemia represents 11% of all haematological malignancies and accounts for 25% of all leukaemias (HMRN, 2013, Yee and O'Brien, 2006). It has an incidence of 7 per 100 000 persons in the UK and the majority of patients are over the age of 60 (HMRN, 2013). There is a male predominance for the disease, with a reported male: female ratio of 2:1 (Catovsky et al., 1989). The prevalence of CLL varies amongst ethnic populations, with higher rates observed amongst white Caucasian populations compared to Asian or African populations (Dores et al., 2007).

1.2.4   The pathogenesis of Chronic Lymphocytic Leukaemia

1.2.4.1 The cellular origin of CLL cells

Defining the cell(s) of origin of CLL has proven difficult. After identifying the presence of unmutated IgVH and mutated IgVH CLL cases, a two-cell model for the origin of cell was proposed (Chiorazzi and Ferrarini, 2011). This was also supported by a distinct difference in the IGVH repertoire described between mutated and unmutated CLL, where stereotypy is observed in around 30% of cases (Murray et al., 2008). However subsequent gene expression profiling has challenged this theory, as minimal differences were shown to exist between these 2 types of CLL (Klein et al., 2001). This has led to a theory that a common cellular origin is more likely. Indeed, the existence of a CLL-stem cell that has self-renewing capacities and can act as a reservoir for maturing CLL cells has been proposed. Such a stem cell could represent any cell type after the IGV rearrangement stage in B cell development (Chiorazzi and Ferrarini, 2011). However, marginal zone (MZ) B cells also pose as a potential candidate as these can express both mutated and unmutated IgVH genes and often can be self-reactive or polyreactive to common antigens encountered (Chiorazzi and Ferrarini, 2003).

Consistent with this, it is generally accepted that CLL cells are antigen experienced and have probably undergone several rounds of proliferation prior to a transforming event. However, immunophenotyping of CLL cells does not correspond with the CD5-CD23-CD22+ phenotype of MZ B cells (although notably CLL cells are constitutively activated, so these differences may reflect the activation status of the cell) (Chiorazzi and Ferrarini, 2011). Thus, the cellular origin of CLL (and also the potential for multiple cellular origins) is still the subject of ongoing research, with increasing emphasis on defining the origin of those cells found within the proliferation zones of the secondary lymphoid organs and bone marrow.

1.2.4.2 Genetic predisposition

The exact pathogenesis of chronic lymphocytic leukaemia remains unknown. Certain genetic susceptibility loci are known to exist for CLL and population based studies have clearly demonstrated the disease has a strong familial aggregation (Crowther-Swanepoel et al., 2010). First degree relatives of patients with CLL are almost 8 times more likely to develop CLL than the general population and the finding of MBL amongst first-degree relatives is also high at 13.5%, compared to 3.5% in an unselected population over 40 years of age (Goldin et al., 2004, Rawstron et al., 2002b, Rawstron et al., 2002a). Interestingly, genetic anticipation can be demonstrated, with the onset of symptoms in familial cases occurring approximately 10 years earlier than sporadic CLL cases (Wiernik et al., 2001, Goldin et al., 1999). Finally, the incidence of other lymphoid malignancies including hairy cell leukaemia is also increased in first-degree relatives, although not to the same degree as CLL (Goldin et al., 2009).

1.2.4.3 BCR stimulation

For those without a family history, no known association exists with radiation or chemical/pathogenic exposure. Instead it is thought to involve a combination of chronic antigen stimulation together with acquired B cell genetic aberrations and interactions with the

microenvironment. As discussed above, the phenotype of CLL cells represents that of antigen-experienced B cells, although ambiguity exists over the cellular origin of CLL (Chiorazzi et al., 2005, Seifert et al., 2012).

The role of BCR signaling in CLL tumourgenesis has recently been shown in a transgenic mouse model (Iacovelli et al., 2015). Common antigen stimulation has been postulated to arise following work demonstrating a restricted immunoglobulin heavy chain repertoire amongst patients, in comparison to the usual vast Ig repertoire of healthy individuals (Chiorazzi and Ferrarini, 2003, Stevenson and Caligaris-Cappio, 2004, Agathangelidis et al., 2012). An example is the IGHV1-69 gene and its distinct 51p1-like allele, which is found in approximately 20% of all CLL cases (Potter et al., 2003). Candidate antigens required for chronic stimulation have included both self and common exogenous peptides that could be repeatedly available and have included Cytomegalovirus (CMV) (Herve et al., 2005, Lanemo Myhrinder et al., 2008, Hoogeboom et al., 2013, Steininger et al., 2012). Several groups have studied the use of recombinant antibodies (rAb), which have been produced to impersonate common immunoglobulins expressed in CLL clones and have been shown to recognise several self antigens, including non-muscle myosin heavy chain IIA and apoptotic peptides (Chu et al., 2008, Catera et al., 2008). More recently, Steininger et al found 6 rAb (encoded by IGHV1-69 or IGHV3-21) to react with the CMV structural protein, pUL32, including a rAb from the germ-line IGHV1-69 51p1 allele (Steininger et al., 2012). Despite these reports, the nature of antigens which can stimulate the BCR on CLL clones is still not defined and others have found evidence for autonomous BCR activation (Duhren-von Minden et al., 2012, Binder et al., 2013). More recently, the success of small molecules which target the BCR signaling pathway have given further support for the importance of BCR simulation in the pathogenesis of CLL and these are discussed in more detail in section 1.1.8.

1.2.4.4 The microenvironment

Survival of CLL cells is also dependent on interactions with the tumour microenvironment. "Pseudo-follicles" form in secondary lymph node organs and are identifiable as proliferation zones where 1-2% of the CLL population are renewed daily and stain positive for the proliferation marker ki67 (Messmer et al., 2005). The homing of CLL cells to the proliferation zones is dependent on cytokine and adhesion molecule interaction provided by various cell types including mesenchymal stromal cells and T cells within the microenvironment. Within the lymph nodes of patients with CLL, monocytoid cells termed "nurse-like cells (NLC)" also arise and secrete chemokines, which together with the mesenchymal stromal cells attract and traffic CLL cells to the lymph node. NLC have also been shown to present antigen to activate the BCR of CLL cells and can activate the nuclear factor kappa B signaling pathway, which is important for B cell activation (Herishanu et al., 2011, Burger et al., 2000, Binder et al., 2010).

Furthermore, CD38 expression is higher in CLL cells isolated from the proliferation zone and NLC are known to express CD38L (CD31) (Deaglio et al., 2005, Patten et al., 2008). Increased amounts of IL-4, IL-6, IL-10 and tumour necrosis factor alpha (TNFα) are also found and promote tumour cells with resistance to apoptosis in addition to providing proliferation support (Mainou-Fowler et al., 2001, Fayad et al., 2001, Cordingley et al., 1988). Follicular dendritic cells and endothelial cells are also important for tumour cell adherence and retention within the lymph nodes and can promote drug resistance and challenge efforts to attain MRD negativity (Maffei et al., 2012, Cols et al., 2012, Maffei et al., 2014).

1.2.4.5 T cells

Within the proliferation zones, an increase in T cells is seen and these appear activated (Schmid and Isaacson, 1994). The interaction of CD40 ligand (CD40L) on CD4+ T cells with

the CD40 receptor found on B cells, is essential for normal B cell responses to antigen and in the context of CLL, this CD40L interaction fosters survival of the tumour clone (Kitada et al., 1999). T cell help is recruited through the expression of CCL22 and CCL21 by CLL tumour cells and has been found to attract activated CD40L CD4+ T cells (Ghia et al., 2002). Furthermore, activated autologous T cells have also been shown to induce CD38 expression on CLL cells following co-culture and CD38 positive CLL cells are more likely to proliferate and expand, increasing the tumour burden (Patten et al., 2008).

The importance of CD4+ T cells in CLL biology is also highlighted through a xenograft model, where the adoptive co-transfer of even small numbers of autologous CD4+ T cells with CLL cells has been shown to produce disease resembling human CLL in a mouse, with depletion of CD4+ T cells culminating in loss of tumour replication. Using this model, Patten *et al* also demonstrated CD38 expression increased on CLL cells in culture, in the presence of activated T cells and this was reduced by partial depletion of CD4+ T cells. Furthermore, CD4+ T cells were shown to co-localise with ki67+ CLL cells on paraffin embedded sections, providing further evidence for the importance of CD4+ T cell interaction with the tumour clone (Patten et al., 2008).

In the peripheral blood compartment, patients with CLL actually demonstrate increased T cell numbers, which is predominantly due to an increase in the absolute CD8+ T cell number (Mackus et al., 2003). The T cell subsets are greatly restricted to populations of clonal and oligoclonal cells in CLL (Serrano et al., 1997, Rezvany et al., 2003). Loss of the normal immune synapse formation between antigen presenting cells and T cells is lost in CLL. Without adequate synapse formation, antigen recognition, immune surveillance and T cell cytotoxicity is impaired (Ramsay et al., 2008, Ramsay et al., 2012). Furthermore, the inhibitory receptors CTLA-4, PD-1, CD160 and CD244, are reported to be increased on T

cells in patients with CLL (Motta et al., 2005, Brusa et al., 2013, Riches et al., 2013, Nunes et al., 2012). A further compromise to immune surveillance in CLL is the finding that the number of T regulatory cells is also increased and the secretion of numerous cytokines by the CLL clone also contributes to the T cell anergy observed. (Piper et al., 2011, Jak et al., 2009, Fayad et al., 2001).

Finally, from clinical observations, T cell dysfunction is also apparent. Expansions of CD28-CD57+ cells are seen in CLL and have been associated with neutropenia, whilst increased frequencies of autoimmune disease are also well documented in patients with CLL (Moreno et al., 2010, Katrinakis et al., 1995). Second malignancies are increased in patients with CLL although it is unclear if immune dysfunction contributes to this.

1.2.5 Clinical features of Chronic Lymphocytic Leukaemia.

Patients usually present incidentally following full blood count analysis demonstrating a persistent lymphocytosis. Less frequently, presentation in the form of non-tender lymphadenopathy or general fatigue can occur and up to 15% of patients may present with constitutional B symptoms, comprising drenching night sweats, fevers or unintentional weight loss (Abbott, 2006). These symptoms often manifest at times of disease progression and are far commoner in Binet stage C patients compared to those with stage A disease. A history of susceptibility to infections including varicella zoster, influenza or bacterial pneumonia may also be present at diagnosis (Moreira et al., 2013). Physical findings include lymphadenopathy, splenomegaly or hepatomegaly and pallor or bruising from the presence of anaemia and or thrombocytopenia.

1.2.6 Clinical Staging in Chronic Lymphocytic Leukaemia.

In practice two clinical prognostic scoring systems are used today. Their ability to prospectively predict overall survival has been validated in numerous studies (Oscier et al.,

2010). The Rai system was first published in 1975 and identified 5 stages of disease (Rai et al., 1975) This was later modified in 1987 to identify 3 distinct patient subsets that differed in their survival (Rai, 1987). The simplicity of this scoring system (based on their clinical features and full blood count results alone) led to its universal acceptance and improved the accountability of results from prospective clinical trials (table 1.2).

The second staging system, known as the Binet classification, was developed later in 1981 and has been more widely adopted in Europe. Similarly, it classifies patients into 3 distinct groups (Binet et al., 1981). Stage A patients are those with less than three enlarged lymph node groups (where a lymph node group includes either cervical, axillary or inguinal lymph nodes, spleen or liver). Whilst those in stage B have more than three nodal groups and Stage C patients have anaemia (Hb <10) and or thrombocytopenia (plt < 100) (Table 1.2). As with the Rai scoring system each stage predicts survival outcome (Binet et al., 1981). Binet stage A or Rai stage 0 has an excellent prognosis with a median survival of greater than 10 years, whilst Binet stage B or intermediate Rai stage has between a 5 and 7 year median survival. Those patients falling into Binet stage C or high risk Rai stage have the worst prognosis with a median survival between 2 and 3.5 years (Cramer and Hallek, 2011). In 1989, The International Workshop on Chronic Lymphocytic Leukaemia (IWCLL) advocated integrating these two commonly used staging systems but despite this, each prognostic scoring system has remained largely separate in common clinical practice (Cheson et al., 1996).

Table 1.2

The two staging systems used in CLL (Binet et al., 1981, Rai, 1987)

Modified Rai staging

Risk	Clinical Features	Prognosis
Low	No clinical stigmata of disease. Isolated lymphocytosis and confirmatory phenotype (stage 0)	>10 years
Intermediate	Lymphocytosis and lymphadenopathy (stage I) and/or hepatosplenomegaly (stage II)	7 years
High	Lymphocytosis and anaemia (Hb<11g/dL) (Stage III) and /or thrombocytopenia (plt <100x10^9/l) (stage IV)	3-5 years

Binet staging

Stage	Clinical Features	Prognosis
A	Lymphocytosis and less than 3 areas of lymph node involvement	>10 years
B	Lymphocytosis and more than 3 areas of lymph node involvement	5 years
C	Lymphocytosis and anaemia and/or thrombocytopenia irrespective of lymph node involvement	3-5 years

Despite their use worldwide and recent endorsement by the IWCLL (Hallek, 2013), limitations exist (Zenz et al., 2010b). Neither prognostic scoring system discriminates between the origin of cytopenias in order to fulfill the requirement for poor risk or stage C disease. Moreno *et al* identified 73 Binet stage C patients and classified the cause of their cytopenia into "immune" or "infiltrative". Those with an immune origin for cytopenia, had on average a 45 month survival advantage (P=0.02) (Moreno et al., 2010). This work also demonstrated that patients with "immune" stage C disease still had a shorter survival than the stage A cohort and have associated poor prognostic risk factors such as zap70 and CD38 expression (Dearden et al., 2008).

Another potential future consideration includes the adoption of other nodal groups to the existing scoring systems. This is increasingly being noted with the advent of pre-treatment CT scanning. However, several studies have reviewed the addition of imaging in predicting prognosis but come to differing conclusions (Eichhorst et al., 2011, Blum et al., 2007, Muntanola et al., 2007).

1.2.7 Laboratory based prognostic markers in CLL

In addition to clinical staging, many laboratory based tests have been validated as indicators of poor prognostic disease in CLL and are increasingly important for early stage, asymptomatic patients where anxiety regarding the diagnosis remains high and where the Rai and Binet staging system fail to discriminate between those patients likely to progress quickly and those with indolent disease.

1.2.7.1 Lymphocyte Doubling Time

A lymphocyte doubling time (LDT) of less than 12 months confers poor prognostic risk and identifies individuals whose disease is progressing. In stage A patients, a LDT less than 12 months corresponded to a median of 61 months progression free survival, compared to those

whose LDT was greater than 12 months, where a median survival at 118 months still had not been reached (Montserrat et al., 1986, Shanafelt et al., 2004).

More recently, Nunes *et al* demonstrated LDT to be the most important prognostic parameter for time to first treatment in stage A CLL (Nunes et al., 2012). Although LDT is a widely accepted prognostic marker, it is likely that a combination of biological events are required before the tumour cell proliferation index increases and clearly LDT does not represent a means of detecting disease progression at diagnosis.

1.2.7.2 Beta-2 Microglobulin

Beta-2-Microglobulin (β_2M) remains a simple, cheap and reliable predictor of prognosis in CLL, particularly when adjusted for glomerular filtration rate (Delgado et al., 2009). It constitutes part of the MHC 1 molecule located on all human nucleated cells. Notably, an increase in β_2M is seen with bulky lymphadenopathy and bone marrow infiltration and is an independent predictor of overall survival. Of all the current serum markers, it is the most powerful at predicting outcome (Pratt et al., 2009, Hallek et al., 1996).

1.2.7.3 Immunoglobulin Heavy Chain Gene (IGHV) Mutations

IGHV mutation analysis is performed on fresh lymphocytes using RT-PCR. The patient's mutation status is determined by the percentage of variation in the B cell clone DNA sequence compared to the patient's germline and is defined as mutated when it exceeds 2% (Hamblin et al., 1999). Using this definition, approximately 50-70% of all CLL patients are found to have mutated IGHV (Hamblin et al., 1999, Damle et al., 1999).

In 1994, following a review of reported cases, a subset of patients with CLL with mutated variable heavy chain genes were first recognised (Schroeder and Dighiero, 1994). Subsequently, Fais *et al* also delineated a group of CLL patients whose clonal B cells did not appear antigen naïve. Instead, somatic mutations involving amino acid changes within the

complementarity determining regions (CDR1 and 2) of the immunoglobulin heavy chain variable domain (IGHV) were noted, suggesting antigen recognition had already taken place within the germinal centre and as such the CLL clones represented "memory" type B cells (Fais et al., 1998).

By 1999, two cardinal papers were published identifying a prognostic difference between those patients with a mutated and unmutated IGHV. Damle *et al* reported 18 of 23 patients (78%) with a mutated IGHV region required either no or minimum therapy and their median survival exceeded the study time. By comparison, 75% of the unmutated IGHV group required treatment (p=0.0001) and their overall median survival was reduced to 9 years (Damle et al., 1999). Shortly after, Hamblin *et al* studied another cohort of 84 CLL patients (38 unmutated, 46 mutated) and similarly found those with an unmutated IGHV had an advanced stage of disease (p=0.0009) and were more likely to have concurrent trisomy 12 anomaly. Kaplan Meier survival analysis found a difference in survival of 176 months, with those unmutated patients having the shortest median survival of only 117 months (Hamblin et al., 1999).

The mutation status of the IGHV has been found to associate with other known prognostic markers of CLL. Abnormalities in p53 functioning are strongly associated with absence of IGHV mutation (Lin et al., 2002), as is the expression of Zap70 (Crespo et al., 2003). Conversely patients with mutated IGHV, often have the favourable 13q14 deletion and longer survival (Oscier et al., 1997, Lin et al., 2002). One exception to this rule is patients specifically harbouring a mutated VH3-21 gene segment, whereby an increased frequency of p53 dysfunction is seen, with a poorer outcome (Lin et al., 2003). Although technically challenging and costly to perform, IGHV mutation analysis is now recommended at diagnosis where resources are available (Parikh et al., 2016) and within the context of clinical trials, its

use permits a confident and accurate distinction of two prognostic groups of patients with CLL.

1.2.7.4 Flow Cytometry

ZAP70: Zeta-chain associated protein (ZAP-70) is a tyrosine kinase protein that is usually expressed by T lymphocytes and NK cells and is important for T cell receptor signaling (Isakov et al., 1995). Its expression on CLL cells was identified following microarray analysis in an attempt to find surrogate markers for IGHV mutation status (Rosenwald et al., 2001).

Shortly after this, Crespo *et al* studied ZAP-70 expression on 56 CLL patients. Critically, they found an expression of ZAP-70 on more than 20% of leukaemic cells inferred poor prognosis and that expression coincided with the presence of unmutated IGHV. Conversely, of the 24 patients without 20% ZAP-70 expression, 21 had a mutated IGHV (P<0.001) (Crespo et al., 2003).

The detection of ZAP-70 expression using flow cytometry remains a reliable indicator of disease prognosis and it is simple to perform.

CD38: CD38 is an enzyme expressed on B cells and is important for cell adhesion, signal transduction and maintaining intracellular calcium (Cesano et al., 1998). Within the context of CLL, Damle *et al* first identified it as a prognostic marker in 1999 and noted an expression of >30% predicted a shortened time to chemotherapy and reduced overall survival in comparison to those CD38 negative (<30%) patients. CD38 expression also correlated with unmutated IGHV status when expression was >30% in all patients investigated (Damle et al., 1999). However, further work has found it to be independent of IGHV mutation status as a prognostic indicator (Hamblin et al., 2002).

Subsequent studies with larger patient numbers have confirmed CD38 as a prognostic marker for predicting progression free survival in stage A patients at diagnosis and have also demonstrated expression as low as 7% coincides with a poorer outcome (Kröber et al., 2002, Letestu et al., 2010). Importantly, this ability to distinguish amongst Binet stage A patients has led to recommendation that CD38 testing is performed at diagnosis, as unlike ZAP70 and FISH analysis, it remains an independent prognostic marker at diagnosis (Pepper et al., 2012). Furthermore, CD38 expression is not influenced by therapeutic interventions, although it can increase as disease progresses, and its presence at diagnosis provides a good indicator for the proliferative potential of an individual's B cell clone (Patten et al., 2008, Hamblin et al., 2002, Damle et al., 2007, Durig et al., 2002).

1.2.7.5 Cytogenetics

No single genetic defect is known to be necessary to cause CLL but a small number of recurrent genetic abnormalities arise in patients with CLL and have clear prognostic value (Zent and Burack, 2014). Previous difficulties in elucidating genetic aberrations using karyoptyping of metaphase cells have been superseded by the use of interphase fluorescence in-situ hybridization (FISH) and mutation analysis. It is known that 80% of patients with CLL have an identifiable genomic aberration and FISH is now recommended for all newly diagnosed patients, where resources permit (Parikh et al., 2016, Stilgenbauer et al., 2002, Döhner et al., 2000). Typical panels evaluate for the 4 commonest chromosomal abnormalities. These are deletion of 17p13, 11q22-23, 13q14 and trisomy 12. In addition, mutation analysis for *TP53* and *ATM* is also now standard clinical practice. Novel mutations including *NOTCH 1* (Rossi et al., 2012b), *SF3B1* (Quesada et al., 2012) and *BIRC3* (Rossi et al., 2012a) mutations also add prognostic value but are not currently in routine use outside of clinical trials. It is hoped that as availability and affordability of targeted next-generation

sequencing improves, detection of these genetic mutations will provide better prognostic information (Zent and Burack, 2014, Baliakas et al., 2015). The main genetic abnormalities are discussed below:

17p deletion/*TP53* mutation: Briefly, *TP53* is a tumour suppressor gene, found on the short arm of chromosome 17 (17p13). The product, p53, is an important cell cycle regulator, playing a key role in the DNA damage response and is commonly mutated in a variety of malignancies. Loss of *TP53* is known to occur in 5% of patients with CLL prior to any treatment, whilst a further 7 % have mutations in their *TP53*, which are undetectable by FISH analysis (Gonzalez et al., 2011). Amongst cytogenetic abnormalities, deletions in 17p are known to produce the shortest median time to treatment and both mutations and/or deletions of *TP53* provide resistance to conventional DNA-damaging chemotherapy and reduced overall survival (Zenz et al., 2010a, Döhner et al., 2000, Wattel et al., 1994). Alemtuzumab and more recently, the use of B cell receptor pathway inhibitors such as ibrutinib (a bruton tyrosine kinase inhibitor) should be used to treat patients with 17p deletion/p53 mutation, as its efficacy is independent of p53-mediated killing.

11q deletion/*ATM* mutation: The ATM (ataxia telangiectasia mutated) gene is located on chromosome 11q23. Like p53, it is also a cell cycle regulator and is important for the repair of damaged DNA by homologous recombination (Stankovic et al., 1999). Deletion of 11q22-23 is the second commonest genetic abnormality found in CLL and is associated with a reduced survival (Döhner et al., 2000). In approximately one third of CLL patients with an 11q deletion, the remaining ATM allele is also mutated (Austen et al., 2007). Deletion of 11q is associated with bulky lymphandenopathy, disease progression and reduced overall survival, although the adverse outcome appears to be partly overcome in the rituximab era (Stilgenbauer et al., 2002).

Trisomy 12 This is found in up to 20% of patients with CLL and confers an intermediate prognosis (Döhner et al., 2000). Trisomy 12 is recognised as a clonal driver mutation that occurs early in CLL evolution (Landau et al., 2013).

13q14 deletion Deletion of the long arm of chromosome 13 is associated with a more favourable outcome in patients with CLL and represents the commonest chromosomal abnormality found in over 50% of cases (Van Dyke et al., 2010). It is associated with loss of the micro-RNA (miR) genes miR15 and miR16, which leads to downregulation of multiple oncogenes including bcl-2 (Calin et al., 2002). Figure 1.4 demonstrates Kaplan-Meier curves for overall survival based on the aforementioned chromosomal abnormalities.

NOTCH 1, *SF3B1* and *BIRC3* **mutations**

Mutations in *NOTCH1* (seen in around 10% of CLL cases requiring treatment), *SF3B1* (seen in 17% of CLL cases requiring treatment) and *BIRC3* (seen in 4% of patients at diagnosis) are all risk factors for a shorter time to first treatment and overall survival (Oscier et al., 2013, Rossi et al., 2012b, Rossi et al., 2012a, Stilgenbauer et al., 2014). Activating mutations in *NOTCH1* are enriched in patients with chemotherapy resistant disease and have also been associated with an increased risk of transformation to diffuse large B cell lymphoma (Puente et al., 2011). The presence of *NOTCH1* mutations have also been found to occur more frequently in patients with trisomy 12 (Balatti et al., 2012) and from the CLL8 study, patients with *NOTCH1* mutations do not benefit from the addition of rituximab (Stilgenbauer et al., 2014).

The deletion or mutation of *BIRC3* is also enriched in patients with chemotherapy refractory disease. Furthermore, *BIRC3* is deleted in more than 80% of patients with a deletion in 11q and in this context, its value as an independent prognostic marker has been disputed (Rose-Zerilli et al., 2014). Finally, *SF3B1* encodes a protein required for producing mature RNA and

mutations in *SF3B1* have again been shown to infer a poor overall survival (Quesada et al., 2012, Stilgenbauer et al., 2014). Together, it is hoped that in the future, these key genetic mutations may form part of a new prognostic index score for patients with CLL at diagnosis in order to risk stratify early stage patients and direct patients towards the use of targeted therapies rather than conventional chemotherapy (Cortese et al., 2014, Rossi et al., 2013).

Figure 1.5

The overall survival of patients with CLL when stratified by chromosomal abnormalities.

A Kaplan-Meier curve of survival in CLL based on cytogenetics. The median survival is worst for those with chromosome17p deletion at 32 months, followed by deletion of chromosome 11q deletion at 79 months. Patients with trisomy 12 were found to have a longer survival than those with no genetic chromosomal abnormality, with a median survival of 114 compared to 111 months. Reproduced with permission from Copyright Massachusetts Medical Society.

1.2.8   Current treatment strategies for Chronic Lymphocytic Leukaemia

A consensus to treat patients only when they become symptomatic or have clear evidence of disease progression should be adhered to outside the setting of a clinical trial (Oscier et al., 2012). The watch and wait policy for early stage patients is based on several studies and a large meta-analysis (n= 2048) (CLL, 1999). As early as 1988, Shustik *et al* demonstrated in a small number study (n=59) that after 5 years of follow up, there was no survival advantage to those treated with monthly chlorambucil compared to observation only (Shustik et al., 1988). In 1998, Dighiero *et al* similarly studied stage A patients in 2 randomised trials with one trial comparing chlorambucil against no treatment (n=609; 11 years follow up) and the other comparing intermittent chlorambucil with prednisolone versus no treatment arm (n=926; 6 years follow up). In both studies there was no survival benefit for earlier treatment and in non-treated patients 49% had no further signs of disease progression more than 11 years later. As such the treatment of early stage CLL was deemed unnecessary (Dighiero et al., 1998). In addition to a lack of therapeutic efficacy, reports have highlighted the risk of chemotherapy related toxicity including myelodysplasia and acute myeloid leukaemia and concerns regarding tumour resistance following upfront chemotherapy have also been raised (Dighiero et al., 1998, Ricci et al., 2011, CLL, 1999).

Recently with the advent of monoclonal antibodies, Ferrajoli *et al* have investigated the impact of early treatment in asymptomatic patients with single agent Rituximab (CD20 monoclonal antibody) given weekly for 8 weeks at 375mg/m^2. Although given to asymptomatic early stage patients, recruitment targeted only patients with high β_2M and therefore those patients more likely to progress. This treatment regimen has been found to be safe and well tolerated. As yet any survival advantage of single agent rituximab over watch-and-wait is not known, but an overall response rate of 82% has been demonstrated and

tolerability proven (Ferrajoli et al., 2011). The CLL12 trial is now also underway which will randomise Binet stage A patients with high risk of disease progression to either ibrutinib (a novel bruton tyrosine kinase inhibitor discussed below) or watch and wait (Langerbeins et al., 2015).

Outside of such clinical trials, initiating treatment should be based on one of indications outlined by the IWCLL and listed in table 1.3. For optimal management, each case should be discussed in a multidisciplinary team meeting, overseen by a haemato-oncologist (Shanafelt et al., 2012). The majority of patients will respond well to the initial course of therapy but inevitably will relapse, with successive cycles of treatment becoming less effective and toxicity accumulating.

The depth of response achieved with each cycle is important, with MRD negativity producing an improved overall survival (Hallek et al., 2010). Encouragingly, the past decade has seen an increase in patient response to chemotherapy, duration of remissions, overall survival and the addition of new more targeted therapies including monoclonal antibodies and those targeting the B cell receptor pathway (Brenner et al., 2008).

Deciding on which treatment regimen to employ can be difficult, particularly as many patients with CLL are on average older than that those recruited into clinical studies and often have multiple comorbidities and often frailty. This limits both treatment choices and doses that are safe and requires the physician to balance therapeutic gain against toxicity and reduced quality of life. There is an increasing focus especially in trials on formally assessing co-morbidities, frailty and disability in order to optimally dose adjust but this field requires further study.

Table 1.3.

IWCLL indications for the treatment of CLL (Hallek et al., 2008)

Unexplained weight loss (>10% within 6 months)
Overwhelming fatigue
Fevers > 38 degrees for > 2 weeks without infection
>1 month of drenching night sweats without infection
Marrow failure: non-immune cytopenias
Autoimmune cytopenias that are steroid-refractory.
Symptomatic splenomegaly (>6cm below costal margin)
Symptomatic lymphadenopathy (>10cm in longest diameter)
LDT < 6months or lymphocytosis increasing >50% in 2 months.

1.2.8.1 Fit, young patients first line therapy

Providing no p53 mutation or deletion is present, the current gold standard treatment regimen for those deemed fit enough is FCR (fludarabine, cyclophosphamide and rituximab). From the German CLL8 trial of 817 treatment naïve patients with CLL, FCR has been shown to produce a superior overall survival compared to FC alone, with long-term remissions (HR 0.68; p=0.001). However, recruitment to this study involved relatively young patients (median age 61) who had no comorbidities (median cumulative illness rating score (CIRS) of 1) (Hallek et al., 2010, Fischer et al., 2016). Difficulties extrapolating these findings to the average cohort of elderly patients with CLL have occurred, as the degree of myelosuppression achieved using FCR is often too great for many patients with CLL, with prolonged neutropenia for up to 1 year post therapy seen in the FCR arm of the trial (Fischer et al., 2016). Reduced dose FCR regimen is an alternative option for some. The median PFS with FCR is between 4-5 years but this is significantly greater for patients with a mutated IGHV and many of these patients have PFS extending beyond 10 years. The addition of Rituximab or alternative anti-CD20 monoclonal antibodies to regimens has also improved the outlook for 20% of patients requiring treatment that have an 11q deletion (Hallek et al., 2010), but conversely the addition of an anti-CD20 mAb to those with NOTCH1 mutation, may not provide any additional benefit to FC alone (Stilgenbauer et al., 2014). The main question in 2016 is whether an ibrutinib plus rituximab combination can replace FCR for newly diagnosed patients and this is being examined in the UK FLAIR study currently.

1.2.8.2 Elderly or less fit patients first line therapy

First line therapy for unfit or elderly patients includes bendamustine or chlorambucil with rituximab or an alternative CD20 monoclonal antibody (mAb) such as ofatumumab (shown in COMPLEMENT-1 trial to have superior efficacy in combination with chlorambucil compared

to chlorambucil alone (Hillmen et al., 2015)) or obinutuzumab (shown to have superior outcome for unfit patients with bendamustine in CLL11 trial compared to bedamustine and rituximab (BR) (Goede et al., 2014)).

Considering chemotherapy agents for less fit patients, bendamustine has been shown to have superior PFS and response rates compared to chlorambucil and like chlorambucil, appears to be well-tolerated (Knauf et al., 2009). The CLL10 trial compared patients with bendamustine and rituximab (BR) to those given FCR but again in a young cohort (median age 61). Interim analysis showed no difference in overall response rate (ORR) or overall survival, although the complete response rate (FCR: 47.4% vs BR: 38.1%; p=0.031) and progression free survival (FCR: 85 vs BR: 78.2%; p=0.041) were more superior in the FCR arm. However when PFS was analysed in patients less or more than 65 years old, those randomized to the BR arm were significantly older (BR: 38.7% vs FCR: 30.5% were over 65 years old; p=0.042) and overall the BR arm had more cases with unmutated IGHV (BR: 53% vs FCR: 67.8%; p=0.003). In those over the age of 65, no clear benefit for FCR therapy was found. The FCR arm also had a much greater side effect profile with more infections and haematotoxicity observed (p<0.0001) and 47% of those over 65 in the FCR arm had at least one infection (Eichhorst et al., 2013)

In support of these findings, in 2014 the national comprehensive cancer network (NCCN) recommended that fit patients over 70 or those under 70 with co-morbidites should not receive FCR (NCCN, 2014). Instead, outside of a trial, bendamustine (or chlorambucil) and obinutuzumab (or an alternative anti-CD20 mAb) seems a reasonable alternative for elderly fit patients requiring first line therapy. Assessment of comorbidities, frailty and disability is becoming increasingly recognized as important in guiding treatment decisions.

1.2.8.3 Second line therapy

No clear guidance exists for second line therapy. Patients who relapse 2 or more years after initial treatment are often considered for retreatment with their initial treatment regimen providing it was well tolerated. Typically responses are less durable with each subsequent relapse and inevitably these patients will receive the newer novel agents such as ibrutinib if available or within a study. Patients deemed very high risk (those who are refractory or relapse within 6 months of first line therapy or those who are inherently resistant to chemotherapy mediated killing, with a p53 mutation +/or 17p deletion) were until recently considered for subcutaneous alemtuzumab (humanised anti-CD52 mAb) with high dose steroid and consolidated where possible with an allogeneic transplant if fit enough (Cortelezzi et al., 2012, Pettitt et al., 2012). The benefit of reduced intensity allograft and potential for cure has been shown in numerous studies and should still be considered for anyone fit enough with high-risk disease (Dreger et al., 2007, Dreger et al., 2010, Moreno et al., 2005). However, the more recent addition of the B cell receptor (BCR) signaling pathway inhibitors such as the BTK inhibitor (ibrutinib or acalabrutinib) (Byrd et al., 2016, Byrd et al., 2013) or the PI3K delta inhibitor, idelalisib (Furman et al., 2014) and the BCL-2 antagonist, venetoclax (Roberts et al., 2016), have changed the management for this challenging group of patients and when to intervene with transplantation is less clear (Dreger et al., 2014). Not only are these drugs orally available but they are showing dramatic efficacy in difficult-to-treat patient groups and those with high-risk genetic aberrations as detailed below. Finally, with the increasing survival of patients with CLL there is probably an increasing incidence of Richter's transformation, which typically presents as an aggressive, often focal tumour in a lymph node or as an extramedullary tumour, which is intensively active on PET imaging.

1.2.9   BTK inhibitors

For relapsed refractory patients on single agent ibrutinib the overall response rate was remarkable at 71% and was associated with a durable progression free survival (Byrd et al., 2013). A comparison with ofatumumab in previously treated relapsed/refractory patients with CLL, demonstrated a superior PFS with ibrutinib and also ORR (ibrutinib: 42.6% vs ofatumumab: 4.1; p<0.001) and importantly also demonstrated efficacy in those with 17p deletion with 83% of the 127 patients with 17p deletion showing PFS at 6 months (Byrd et al., 2014). Untreated patients with *TP53* aberrations have also been shown to have a RR of 97% and PFS of 91% at 24 months on ibrutinib therapy (Farooqui et al., 2015). Ibrutinib is generally well tolerated although an increased risk of bleeding, particularly in the presence of oral anti-coagulants and an increase risk of atrial fibrillation have been found (Byrd et al., 2014, Byrd et al., 2013). Newer, more selective second generation BTK inhibitors with an improved side effect profile are now appearing in clinical trials (Byrd et al., 2016).

1.2.10   PI3K inhibitors

In early studies, idelalisib as a single agent already showed promise with response rates of 72% observed amongst patients previously treated who had a short time to relapse (Brown et al., 2014). In 2014, 220 patients, of whom nearly 80% were over the age of 65 and had significant co-morbidity or were deemed not fit enough for chemotherapy, were investigated in a phase III study comparing rituximab and idelalisib versus rituximab and placebo. The study had to be stopped early due to the overwhelming difference in efficacy in the idelalisib arm, with 93% showing PFS at 24 weeks compared to 46% (p<0.001) and an ORR of 81% versus 13% (p<0.001) (Furman et al., 2014). Data for responses amongst prognostic groups is due to be published soon but oral reports again shown good efficacy against 17p del disease. Like ibrutinib, idelalisib is generally well tolerated in the relapse setting although temporary

transaminitis is common and reports of pneumonitis, colitis and hepatic dysfunction have also been reported (Furman et al., 2014). Unfortunately toxicities appear particularly increased in newly diagnosed patients with an excess of early deaths in Phase III studies (7.4% deaths in idelalisib arm vs 3.5% deaths in placebo arm) including from CMV and pneumocystis jirovecii leading to an interim recommendation to not initiate idelalisib as first line therapy (written correspondence). Further investigation of toxicity is ongoing and the long-term future of idelalisib remains uncertain.

1.2.11 BCL-2 antagonists

Results of the phase I trial using venetoclax in multiply relapsed disease have recently been published. Almost 90% had adverse prognostic features including poor cytogenetics and a 79% response rate was observed amongst the 116 participants, which included those with 17p deletion. Of note, 20% of participants had a complete remission with 5% reaching MRD negativity. Side effects included tumour lysis syndrome and a grade 3/4 neutropenia in around 40% but the authors concluded that the safety profile was manageable in this group of patients (Roberts et al., 2016).

1.2.11.1   *Concluding on the newer agents*

BCR pathway inhibitors and BCL2 antagonists have shown great promise and are changing the landscape of CLL management. With a tolerable side effect profile in the relapsed setting and evidence for their efficacy against 17p deleted disease and chemotherapy refractory/multiply relapsed patients with significant co-morbidities, the overall survival of patients with CLL is set to improve, particularly as new combinations of these agents are trialed with immunotherapeutic agents. However, the duration of therapy required and the long-term side effect profile are still unknown and despite such encouraging responses, resistance has already been reported and complete remissions are rare. In addition the

emergence of toxicities with the use of idelalisib in newly diagnosed patients has highlighted the need for caution. The search for a cure for CLL therefore continues.

1.3   Cytomegalovirus

1.3.1   Overview of cytomegalovirus

Cytomegalovirus is one of 8 ubiquitous human herpes viruses that infects populations worldwide (Mocarski ES, 2007). The virus is acquired through close personal contact and transferred through all bodily fluids, often at a young age. Its prevalence varies throughout the world and in general, healthy immunocompetent individuals are asymptomatic during primary infection. On occasion primary infection can be associated with an infectious mononucleosis type illness and a persistence of fatigue that can last several months (Hurt and Tammaro, 2007). The archetypical manner by which herpes viruses persist, is by maintaining a state of latency in their host for life. As a result, CMV has evolved strategies to avoid human immune surveillance.

In order for CMV to maintain latency, a rapid and intact cellular immune response is required to control the virus throughout life. This chronic, relentless T cell mediated viral suppression places an incredible burden on the immune system to maintain status quo (Khan et al., 2002). Conversely, a state of immunosuppression provides a niche for the uncontrolled replication of virus and the production of symptomatic disease. Such reactivation is a common phenomenon seen in progressive HIV infection, bone marrow transplantation and solid organ transplants. Florid and uncontrolled CMV replication can be in almost any tissues but frequently it results in colitis, pneumonitis or retinitis and requires anti-viral therapy (Navarro, 2016).

CMV may also result in congenital malformations and neurological impairment if primary infection or reactivation occurs during pregnancy and the virus crosses the placenta. The risk of end organ damage is greatest during the first trimester and commonly results in congenital

deafness and neurological impairment (Cannon et al., 2010). Given the ubiquitous nature of CMV and its clinical implications, the development of a vaccine against CMV has been highlighted as a major public health priority by the Institute of Medicine and although advances are being made, a range of different vaccines are still at the early stages of development with only a few in phase I or II clinical trials (Arvin et al., 2004).

1.3.2   The epidemiology of Cytomegalovirus

Human CMV can only infect humans. Although prevalent worldwide, rates of CMV infection vary, being highest in South America, Asia and Africa and lowest in Western Europe (Cannon et al., 2010). In countries with the greatest infection rates, acquisition of the virus usually occurs at a young age and can be transmitted via breast milk or through direct contact. In western society, prevalence increases with age, with seropositivity estimated to be 70% in people over the age of 60. People of lower socioeconomic status are also more likely to carry the virus. The differing rates of infection are likely to reflect differences in breast feeding, child care arrangements, cramped living conditions and differences in sexual practices (Cannon et al., 2010).

1.3.3   The structure of Cytomegalovirus

CMV is the largest of the human herpes viruses with a genome size of 236 kbp (Dolan et al., 2004). The central structure of the virus is icosahedral in shape and consists of a nucleocapsid containing double-stranded DNA. Between this and the viral envelope is the proteinaceous tegument layer (Mocarski ES, 2007) (Figure 1.5). The virus exists as an episome whilst latent (Bolovan-Fritts et al., 1999). 213 open reading frames (ORFs) have been identified for CMV (Sylwester et al., 2005). Interestingly, only 47 of these genes are important for viral replication. The majority of the remaining genes are thought to direct immune evasion or modify the hosts' cellular response. Indeed, deletion of certain ORFs results in increased viral

replication suggesting certain proteins encode a suppressor function on viral replication (Dunn et al., 2003). Many broadly distributed epitopes are responsible for the T cell immune response seen in humans, with at least 151 ORF known to be capable of generating CD4+ or 8+ T cell responses (Sylwester et al., 2005). Amongst the most immunodominant and most studied, is the phosphoprotein 65 (pp65) tegument protein that can be recognised by the immune system at all stages of viral replication and evokes both a humoral and cellular immune response (Kern et al., 2002).

1.3.4 The immune response to CMV

The humoral response to CMV occurs early in the primary immune response and the presence of IgG recognising CMV epitopes is the standard method for identifying prior CMV infection, using ELISA technique. Where a primary defect in the humoral system exists from birth, for example in the case of X-linked agammaglobulinaemia, reactivation of CMV is still not frequently encountered (Sullivan and Stiehm, 2014). This is fundamentally due to the presence of a dominant cellular immune response, a feature required by all infected humans and mice to prevent viral reactivation and used for therapeutic benefit in the correction of CMV viraemia in bone marrow transplant patients who receive CMV-specific CD8+ T cells (Cobbold et al., 2005).

T cell immune responses appear around 7 days following primary infection (Rentenaar et al., 2000). Usually, more than 2% and up to 50% of the CD8+ T cell repertoire is devoted to controlling lytic replication in health and unsurprisingly, CMV is speculated to be the most immunodominant antigen mankind encounters (Gillespie et al., 2000, Moss and Khan, 2004b, Wikby et al., 2002). This dominant CD8+ response is known to skew and invert the normal CD4:8 T cell ratio. The expanded populations of CD8+ T cells are oligoclonal and are of a late differentiated memory phenotype with low expression of the co-stimulatory molecules

CD27 and CD28. They typically are negative for CCR7 but often re-express CD45RA (Khan et al., 2002, Lachmann et al., 2012, Pourgheysari et al., 2007) (figure 1.3). This expansion of differentiated memory cells coincides with a restriction in the naïve memory T cell pool and increases with age. This finding is referred to as "memory inflation" (Karrer et al., 2003). Similarly, the CD4+ T cell response is known to increase with age and consist of effector memory cells that have increased expression of CD57 and a reduction in the expression of CD27 and CD28, a phenotype thought to be reflective of CD4+ T cells nearing senescence (Pourgheysari et al., 2007). Both virus–specific CD4+ and CD8+ T cells exhibit a cytotoxic phenotype with high levels of granzyme and perforin and have been found at even higher frequencies in immunocompromised individuals. There have also been reports of features of T cell exhaustion observed amongst T cells recognizing CMV, with loss of IL-2 production, poor proliferative capacity and increased PD-1 expression seen on CD4+ T cells (Antoine et al., 2012, Sester et al., 2008, Dirks et al., 2013).

Finally, it has been suggested that the dominant CMV T cell responses may impact on the ability to deal with heterologous infections and CMV positive elderly individuals have also been shown to have impaired vaccination responses compared to individuals who are CMV negative (Weng, 2006, Khan et al., 2004).
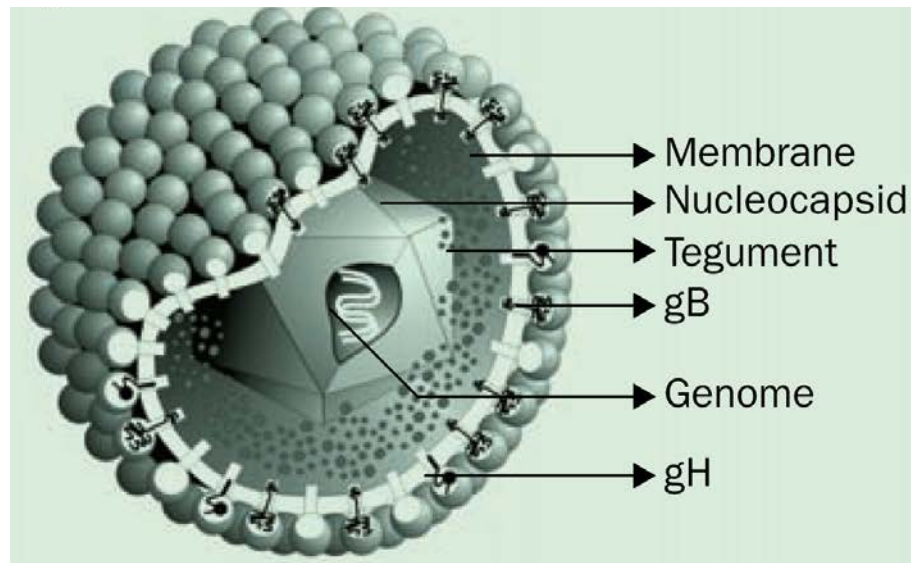
Figure 1.6

The structure of cytomegalovirus.

A 3-dimensional image of the structural components of cytomegalovirus including the

icosahedral nucleocapsid surrounded by the tegument and the outermembrane containing

glycoprotein structures including glycoprotein B (gB). Adapted from (Gandhi and Khanna,

2004). Permission obtained from Elsevier (license number 3857141295803).

1.3.5 Cytomegalovirus establishes latency in the myeloid lineage

Latency describes the ability of a virus to persist within a host without the production of infectious virions. The virus remains within a latent state, which continues until the necessary stimuli are provided that permit reactivation. Whilst latent, CMV conceals itself and remains exceedingly difficult to isolate (Jordan, 1983). Fundamentally, the existence of CMV is estimated to be of low frequency, with less than one in 10,000 peripheral blood mononuclear cells thought to be harbouring the virus (Slobedman and Mocarski, 1999).

Speculation regarding the cell type responsible for the carriage site of CMV was deduced from the ability of the virus to be transmitted through blood transfusion. It was also noted that the risk of transmission could be minimised by leucodepleting blood, suggesting leucocytes within the peripheral blood compartment were a host cell for viral latency (Verdonck et al., 1987, de Graan-Hentzen et al., 1989, Adler, 1983). More recently, sensitive PCR techniques have revealed that the virus persists within the myeloid lineage. Self-renewing CD34+ haemopoietic stem cells represent a reservoir for maintaining viral infection and it has been estimated that latent virus is present in 0.01% - 0.001% of myeloid progenitor cells within bone marrow (Kondo et al., 1994, Hahn et al., 1998). The primary cell for CMV carriage is thought to be the human monocyte, with no evidence for existence of the virus within neutrophils or lymphocytes apparent (Taylor-Wiedeman et al., 1991).

Transcription of intermediate early (IE) genes is required to initiate CMV replication and evidence for this is lacking amongst CMV infected monocytes suggesting the virus remains truly latent in this cell type (Reeves et al., 2005). From in-vitro studies, monocytes that have been infected in cell culture are also not permissive to replication and this has been shown to be due to inhibition of the major IE promoter through chromatin remodeling. The presence of heterochromatin protein 1 associating with the promoter is thought to be responsible for this

inhibition (Murphy et al., 2002, Reeves et al., 2005). It is hypothesized that once the host cell differentiates into a macrophage, if a suitable cellular environment is reached, viral replication can proceed (Sinclair, 2008).

1.3.6   Reactivation of Cytomegalovirus

Detecting CMV in health is extremely difficult (Steininger et al., 2004). Indeed, a positive CMV PCR is generally interpreted as evidence of clinically significant reactivation but the ability of CMV to subclinically reactivate in individuals with a competent adaptive immune system is also now recognised. The virus can be detected during stressful periods in health and also at sites of inflammation for example following a myocardial infarction or in rheumatoid arthritis (Núñez et al., 2012, Einsele et al., 1992). It is also thought to occur more frequently in the elderly as host immunity wanes (Leng et al., 2011b). Such repeated antigenic stimulation of T cells during a lifetime, is thought to account for the accumulation of CMV specific memory T cells and contributes to the immune phenotype changes seen with ageing (Weinberger et al., 2007). CMV can distribute throughout the body and infect numerous cell types. The differential environment of cell types places a substantial influence on the replicative ability of CMV (Mocarski ES, 2007).

Several sets of genes are expressed sequentially during lytic replication of CMV. Immediate Early genes (IE) are the first expressed during infection and act to prepare the cell environment for viral DNA production. Importantly, the differentiation of CD34+ cells and monocytes to mature DCs results in the association of acetylated histone 4 (H4) with MIEP (major immediate-early promotor). This modification is accompanied by the dissociation of repressor protein HP-1 from MIEP. Together this appears to instigate chromatin opening and permits the transcription of MIEP (Reeves et al., 2005). Activation of the MIEP is then associated with expression of the IE genes and sanctions a transcription cascade that leads to

viral replication. The cascade subsequently results in early (E) and late (L) gene expression. Functionally, the early genes are important in the coordination and production of viral DNA, whilst late genes are responsible for producing important structural proteins for the new virions produced (Mocarski ES, 2007) .

1.3.7 Cytomegalovirus and the ageing immune system

Immunosenescence describes the deterioration in the human immune system that occurs with ageing and is associated with an increased risk of infection (O'Connor et al., 2014). It is multifactorial in origin, but probably the most pronounced changes are seen within the CD8+ T cell compartment, where a restriction in the naïve T cell subset (mediated by thymic involution) and an expansion in effector memory T cells is seen contributing to inversion of the CD4:8 ratio (Linton and Dorshkind, 2004). An increase in pro-inflammatory cytokines also arises with ageing (Franceschi et al., 2000). These findings are exaggerated in individuals who are CMV positive. Several large population based studies have investigated the impact of CMV in healthy ageing and have associated it with an "immune risk phenotype" (IRP), which associates with mortality (Strindhall et al., 2012, Pawelec et al., 2001, Wikby et al., 2006). The Swedish OCTO study, recruited 109 healthy donors and first described the 2 year increased mortality seen in individuals aged over the age of 86, with an inverted CD4:8 ratio and reduced T cell proliferation (Ferguson et al., 1995). The inverted CD4:8 ratio was noted in 32% of individuals by the end of study. At risk individuals were found to be CMV positive with an expanded population of terminally differentiated memory CD8+ T cells. It was also later suggested that IRP could be simplified to just inversion of the CD4:8 ratio defined as 1 (Wikby et al., 2002) and this was subsequently confirmed in an independent UK cohort in 2003 (Huppert et al., 2003).

The Swedish NONA study recruited 138 individuals (with patients aged over 86) and reviewed cytokine production. Within only 2 years of follow up, the NONA study revealed individuals with an IRP at recruitment proceeded to develop an increase in the inflammatory cytokine IL-6, which was shown to independently predict 2 year mortality (Wikby et al., 2006). IRP was again associated with persistent CMV infection. Using the findings of IRP and evidence of low grade inflammation, 57% of deaths within the 2 years were predicted (Wikby et al., 2006).

The HEXA study has also looked at similar immune profile findings but in a randomly selected younger cohort with a mean age 66. 15% were noted to have inverted CD4:8 ratio which again correlated with CMV seropositivity (Strindhall et al., 2012). Due to the young age of participants, clinical outcome measures are not yet known in this cohort but it has been reported that the CD4:8 ratio inversion appears to be more frequent in males than females.

1.4 Cytomegalovirus and Chronic Lymphocytic Leukaemia

1.4.1 Overview

In certain populations, CMV seroprevalence in patients with CLL has been found to be higher than the age and sex matched control population (Steininger et al., 2009). Despite the immunosuppression associated with CLL, CMV-specific CD4+ and CD8+ T cells have also been shown to accumulate to a greater extent in patients with CLL compared to healthy age matched controls. Repeated or chronic exposure to CMV has been attributed to this finding (Akbar, 2010, Mackus et al., 2003, Walton et al., 2010, Pourgheysari et al., 2010). However, the frequency and duration of CMV reactivation in CLL remains undetermined. One study found up to 12% of patients were harboring CMV DNA in their leucocytes. However, this was in a selected cohort biased towards patients with an IGHV4-34 gene arrangement in their BCR and antibody titres of IGHV4-34 are known to be raised in individuals who are infected

with CMV (Kostareli et al., 2009). Another large study of 200 unselected patients found 3% had detectable CMV DNA (Vanura et al., 2013), whilst others studies have found little evidence for CMV reactivation using PCR methodology (Mackus et al., 2003, Pourgheysari, 2010).

There is also limited evidence for the prognostic impact of CMV infection in patients with CLL. Suggestions from a small cohort of 53 patients, with 23 deaths, point towards a poorer outcome, with up to 45 months shorter survival time on univariate analysis for CMV positive patients compared to those CMV negative for the virus (HR 0.67 (95% CI 0.25-1.81) p= 0.42). Interestingly, this report also noticed a tendency for increased infections within this cohort, although the authors did not make a firm conclusion as again, numbers were small (Pourgheysari, 2010).

A reversal in the CD4:8 ratio in healthy elderly adults clearly correlates with being serologically positive for CMV (Olsson et al., 2000, Wikby et al., 2002), and in CLL an inverted CD4:8 ratio has been associated with poor prognosis in early stage patients (Nunes et al., 2012). The HEXA study discussed in section 1.2.7 recruited donors of a similar age to patients diagnosed with CLL and also confirmed, at this younger age, that CMV is associated with an inverted CD4:8 ratio (Strindhall et al., 2012). Thus, there is compelling evidence that CMV impacts on the T cell repertoire in CLL but its impact on survival and need for therapy has not been fully evaluated. Without larger epidemiological and scientific studies in this field, any potential therapeutic benefit of anti-viral therapy in CLL may not be realised. Contributing literature relevant to this field will now be discussed in greater detail within the introduction of each chapter.

1.5 Aims of this study

There are 4 main questions addressed in this thesis:

1. Using class I tetramers, what is the impact of CMV infection on the phenotype and function of CD8+ T cells in patients with CLL and does CMV impact on the CD4:8 ratio in these patients.

2. Using novel class II tetramers, what is the impact of CMV infection on the phenotype and function of CD4+ T cells in patients with CLL.

3. Can CMV viral load be detected and quantified in patients with CLL and how does it relate to healthy donors and stage of disease.

4. Does CMV infection impact on survival or time to first treatment in patients with CLL and how does CMV impact on infection morbidity.

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2 MATERIALS AND METHODS

2.1   Purification of peripheral blood mononuclear cells and plasma

Peripheral blood monocyte cells (PBMC) and plasma were extracted from peripheral whole blood using a Ficoll density-gradient centrifugation under sterile conditions. Following venesection into lithium heparin lined blood tubes, blood was diluted 1:1 with RPMI (Invitrogen Gibco, Paisley UK) and layered onto ficoll-paque media (PAA, Buckinghamshire, UK). Next, samples were centrifuged at room temperature (RT) at 1800 rpm for 25 minutes (Allegra X-12R centrifuge, Beckman Coulter), with the brake off to maintain separation of layers during deceleration. 1.8ml of plasma was then retrieved from the top layer and stored at -20°C. The PBMC layer, which is visible as a band, was then extracted using a sterile transfer pipette and transferred to a fresh sterile falcon tube. Samples were then washed in RPMI and centrifuged for 10 minutes at 2000rpm. After discarding the supernatant, a further wash in RPMI and centrifugation for 10 minutes at 1600rpm occurred prior to ascertaining a mononuclear cell count using a haemocytometer. Cells were then either immediately used for stimulation assays or cryopreserved in freezing media (10% DMSO in FCS (Sigma-Aldrich Poole, UK, PAA)) and stored at -80°C overnight before transferring to -175°C.

2.2   DNA extraction

2.2.1   DNA extraction from a PBMC pellet

DNA was extracted from up to 5 x 10^6 pelleted cells using GenElute Mammalian Genomic DNA Miniprep kit, according to maufacturer's instructions (Sigma-Aldrich). Cells were resuspended in 200μl of resuspension buffer with 20μl of Proteinase K. 200μl of lysis solution C was then added and vortexed to homogenise the sample before incubating at 55°C for 10 minutes. 500μl of column preparation solution was added to a column provided with the kit and then centrifuged at 10,500 RPM for 1 minute. 200μl of 70% ethanol was then added to

the homogenised sample prior to loading on to the prepared column and centrifuging at 8000 RPM for 1 minute. The column containing the bound DNA was then removed and placed in a new collection tube. Using 500µl of wash solution, cells were then washed and centrifuged twice; first at 8000 RPM for 1 minute, then again at 13,000 RPM for 3 minutes. After the final wash, the DNA was eluted into a clean 1.5ml collection tube by adding 20µl of sterile DEPC treated water. A final incubation at RT for 5 minutes occurred prior to centrifuging at 8000 RPM for 1 minute. The final elution step was repeated to retrieve DNA into a final volume of 40µl. This was then stored at -20°C.

2.2.2   DNA quantification.

The Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA) was used to assess the purity and concentration of DNA. Briefly, following water calibration, 1µl of DNA sample was loaded on to the Nanodrop and using Nanodrop2000/2000c software, the 260/280 absorbance was measured, with a ratio between 1.6 and 1.9 deemed adequate for purity for further experiments. The concentration of DNA was given as ng/µl.

2.3 CMV ELISA

Using the previously published CMV ELISA testing kit (University of Birmingham) the presence and quantification of CMV IgG antibody was determined (Kilgour et al., 2013, Bartlett et al., 2012, Savva et al., 2013). The ELISA plate was first prepared the day before using mock and CMV-infected lysate. To do this, alternatively across the 96 well plate, a row of 50µl of mock-lysate, followed by 2 rows of 50µl CMV-lysate were applied and incubated at 4°C overnight. The next day, wash buffer was prepared as 0.5% Tween-20 in phosphate buffered saline (PBS) (Sigma-Aldrich) and dilution buffer prepared as 1% BSA (Sigma-Aldrich) in wash buffer. Using a known CMV lysate mixture generated from 3 healthy donors, 1 in 4 serial dilutions were used as a standard for titre calculations. After washing the

plate, a 1 in 600 dilution of patient serum to dilution buffer was added to the appropriate mock and lysate well corresponding to that patient sample and incubated for 1 hour at RT. After further washing, 100μl of 1 in 8000 dilution of IgG (SouthernBiotech, Alabama USA) was added to each well and incubated for 1 hour in the dark, RT. After repeat wash steps, 100μL of TMB (3, 3′, 5, 5′-tetramethylbenzidine ELISA peroxidase substrate) (Rockland, Limerick USA) was added and incubated for 10 minutes in the dark, RT. To stop the reaction, 100μl of 1mM HCl was added. Using the Viktor plate reader and PRISM version 6, CMV IgG titres were calculated through extrapolation of the standard curve titres.

2.4 HLA Typing

HLA typing was performed using PCR, as described by Bunce *et al*. (Bunce et al., 1995). HLA type was required for appropriate application of tetramers described in section 2.5. Table 2.1 contains the forward and reverse sequences of the primers used for each HLA type tested and the volume added per reaction, DEPC treated water was then added to each forward and reverse primer to reach a total volume of 4μl.

TDMH buffer was pre-prepared and stored at -20°C. To prepare the TDMH, 676 μl of 10x NH$_{4,}$ 135.2 μl of 10mM dNTPS, 270 μl of MgCl$_{2,}$ 162 μl of glycerol, 40 μl of cresol red and 1316.8 μl of H$_2$O were added and vortexed well prior to freezing.

A separate mastermix was made for each patient sample comprising enough for 10 reactions. For the mastermix, 140ng of DNA, made up to a total volume of 40μl with DEPC water, together with 70.8μl of TDMH, 0.75μL of BioTaq DNA polymerase (Bioline, London UK) and 7μl of control forward and 7μl of reverse primer. 9μL of mastermix was then mixed with 4μl of the HLA primer stock for each PCR reaction. Reaction conditions consisted of 1 minute at 96°C, followed by 5 cycles of 25 seconds at 96°C, 45 seconds at 70°C, 45 seconds at 72°C, followed by 21 cycles of 25 seconds at 96°C, 50 seconds at 65°C, 45 seconds at

72°C, followed by 4 cycles of 25 seconds at 96°C, 60 seconds at 55°C, and 120 seconds at 72°C. Samples were run on a 1% agarose gel containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich) for 35 mins at 140V against a hyperladder (Bioline) and the gel image obtained using the kodak TVC312A gel reader.

2.5 Tetramer preparation

2.5.1 CMV Class I Tetramer Preparation

9 CMV class I tetramers were kindly donated by Mrs J Begum to identify CMV-specific CD8+ T cells (Monomer details can be found in Table 2.2) (University of Birmingham). For this, 1µl of tetramer was added to $1\mathrm{x}10^{6}$ cells in 50µl of PBS buffer and incubated for 15 minutes at 37°C, prior to a further wash in MACS buffer.

2.5.2 CMV Class II Tetramer Preparation

For type 2 tetramer staining, 2 CMV tetramers (DYS and AGI) were generated by Benaroya Research Institute at Virginia Mason Seattle and stored for up to 6 months at 4°C. For tetramer staining, 1×10^{6} cells were resuspended in 50µl of sterile human serum and 0.5µl of the appropriate tetramer added for the individual's Class II HLA type. Cells were then incubated at 37°C degrees for 30 minutes prior to any surface or intracellular staining.

Table 2.1   **Primer sequences used for HLA typing analysis.**

Primer number	Sense primers	Antisense primers	HLA antigen	allele
48 and 49	5'-CCTGTGGCAGGGTAAGTATA	5'CCCGTAGTTGTGTCTGCACAC	DR7	DRB1*0701
76 and 151	5'-GGAGTACCGGGCGGTGAG	5'-CGTAGTTGTGTCTGCAGTAATTG	DR52B	DRB3*0201, 0202, 0203
286 and 431	5'-CGACGCCGCGAGCCAGAA	5'AGCCCGTCCACGCACCG	A1	A*0101, 0102
296 and 302	5'-GTGGATAGAGCAGGAGGGT	5'-CCAAGAGCGCAGGTCCTCT	A2	A*0201-17
193 and 221	5'-GGAGTATTGGGACCGGAAC	5'-TACCAGCGCGCTCCAGCT	B7	B*0702-0705,8101
312 and 221	5'-ACACAGATCTACAAGACCACC	5'-TACCAGCGCGCTCCAGCT	B703	B*0703
195 and 212	5'-GACCGGAACACACAGATCTT	5'-CCTCCAGGTAGGCTCTGTC	B8	B*0801, 0802, B51GAC, B*4406
195 and 220	5'-GACCGGAACACACAGATCTT	5'-CCGCGCGCTCCAGCGTG	B8	B*0801, 0802
63 and 64	5'-TGCCAAGTGGAGCACCCAA	5'-GCATCTTGCTCTGTGCAGAT	control	

Table 2.2   **Details of the CMV monomers used for tetramer work and their HLA restriction**

Monomer	HLA type peptide is restricted through	peptide	CMV protein
YSE	A1	YSEHPTFTSQY	pp65
NLV	A2	NLVPMVATV	pp65
VLE	A2	VLEETSVML	IE-1
RPH	B7	RPHERNGFTVL	pp65
TPR	B7	TPRVTGGGAM	pp65
QIK	B8	QIKVRVDMV	IE-1
ELR	B8	ELRRKMMYM	IE-1
ELK	B8	ELKRKMIYM	IE-1
DYS	DR7	DYSNTHSTRYV	gB
AGI	DR52B	AGILARNLVPMVATV	pp65

2.6   Immunophenotyping

2.6.1   Surface staining for immunophenotyping

For immunophenotyping experiments, all wash steps involved the addition of 3ml of MACS buffer (University of Birmingham), prior to centrifugation at 1800rpm for 5 minutes unless stated otherwise. PBMCs were defrosted and immediately washed in 13ml of PBS (University of Birmingham). After determining the cell concentration using a haemocytometer, 1×10^6 PBMC per tube were used for staining experiments. Live-dead fixable cell staining assay (Invitrogen) was added to each facs tube and incubated at RT in the dark for 15mins. Cells were washed before the addition of the appropriate tetramer if required as detailed in section 2.5. After repeat washing, surface antibodies were added for each panel and incubated for 15 mins at 4°C in the dark before a final wash and analysing on the LSRII™ (BD Biosciences). To identify correctly positive populations of cells, an unstained control and fluorescence-minus-one sample was performed for each experiment. Table 2.3 gives an overview of the panels used within this study.

Table 2.3

Antibody panels.

Red denotes the antibodies that are intracellular and therefore added after fixation and permeabilisation.

Panel 1 - CD8 CMV T cell phenotype

Antibody	Colour	Clone	Company
CD3	Efluor450	OKT3	eBiosciences
CCR7	FITC	150503	R&D systems
CD45RA	AF700	HI100	BioLegend
CD4	APC-Cy7	RPA-T4	BioLegend
CD19	PE-CF954	HIB19	BD Biosciences
CD14	PE-CF954	MOPg	BD Biosciences
CD8	Amcyan	SK1	BD Biosciences
Class I tetramer	APC	N/A	University of Birmingham
Viability	Red dye	N/A	Invitrogen
PD1	Percp/Cy5.5	EH12.2H7	Biolegend

Panel 2 - CD4 CMV T cell phenotype

Antibody	Colour	Clone	Company
CD3	Amcyan	SK1	BD Biosciences
CCR7	FITC	150503	R&D systems
CD45RA	AF700	HI100	BioLegend
CD4	PE-CF954	RPA-T4	BD Biosciences
CD19	Pacific Blue	HIB19	eBioscience
CD14	Pacific Blue	HCD14	eBioscience
Class II tetramer	PE	N/A	Benaroya Research Institute
Viability	Bue stain	N/A	Invitrogen
TIM3	APC	F38-2E2	eBioscience
PD1	Percp/Cy5.5	EH12.2H7	Biolegend

Panel 3 - Activation panel for CD4 CMV specific cells

Antibody	Colour	Clone	Company
CD3	Amcyan	SK7	BD Biosciences
CD4	APC-CY7	RPA-T4	BioLegend
Viability	Red stain	N/A	Invitrogen
Class II tetramer	PE	N/A	Benaroya Research Institute
CD38	PE-Cy7	HIT2	BioLegend
CD69	AF647	FN50	Biolegend
PD1	BV421	EH12.2H7	BioLegend

Panel 4 - Cell sorting for telomere length analysis CD8 CMV specific cells

Antibody	Colour	Clone	Company
CD3	FITC	UCHT1	Beckman Coulter
CD8	Percp/Cy5.5	SK1	Biolegend
Class I Tetramer	APC	N/A	University of Birmingham
Viability	Red stain	N/A	Invitrogen

Panel 5 - Cell sorting for telomere length analysis CD4 CMV specific cells

Antibody	Colour	Clone	Company
CD3	APC-Cy7	SK7	BioLegend
CD4	Percp/Cy5.5	OKT4	eBioscience
Class II Tetramer	PE	N/A	Benaroya Research Institute
Viability	Red stain	N/A	Invitrogen

Panel 6 - Stimulation panel for CD4 CMV specific cells

Antibody	Colour	Clone	Company
CD3	Amcyan	SK7	BD Biosciences
Class II tetramer	PE	N/A	Benaroya Research Institute
Viability	Red stain	N/A	Invitrogen
CD4	APC-Cy7	RPA-T4	BioLegend
IFN-gamma	AF700	4s.B3	BioLegend
TNF-alpha	PE-CY7	MAb11	eBioscience
Il-4	APC	8D4-8	BioLegend
Il-5	FITC	9906	R&D systems
Il-10	BV421	JES3-9D7	Biolegend
PD1	PerCP/Cy5.5	EH12.2H7	BioLegend
Fix/Perm: 4% PFA and Saponin			

Panel 7 - Stimulation panel for CD8 CMV specific cells

Antibody	Colour	Clone	Company
CD3	APC-Cy7	SK7	Biolegend
Class I tetramer	APC	N/A	University of Birmingham
Viability	Red stain	N/A	Invitrogen
CD8	Amcyan	SK1	BD Biosciences
IFN-gamma	AF700	4s.B3	BioLegend
TNF-alpha	PE-CY7	MAb11	eBioscience
Fix/Perm: 4% PFA and Saponin			

Panel 8 - Cytotoxicity panel for CD4 CMV specific cells

Antibody	Colour	Clone	Company
CD3	Amcyan	SK7	BD Biosciences
CD4	APC-Cy7	RPA-T4	BioLegend
Viability	Red stain	N/A	Invitrogen
Class II tetramer	PE	N/A	Benaroya Research Institute
Granzyme B	AF647	GB11	BioLegend
Perforin	FITC	dG9	eBioscience
Fix/Perm: 4% PFA and Saponin			

Panel 9 - T regulatory panel for CD4 CMV specific cells

Antibody	Colour	Clone	Company
CD25	Pe-CY7	M-A251	BD Biosciences
Foxp3	AF700	PCH101	eBioscience
CD3	eFluor450	OKT3	eBioscience
Viability	Red stain	N/A	Invitrogen
Class II Tetramer	PE	N/A	Benaroya Research Institute
PD1	Percp/Cy5.5	EH12.2H7	BioLegend
CD4	APC-Cy7	RPA-T4	BioLegend
Transcription factor kit (ebioscience)			

Panel 10 - Transcription factor staining for CD4 CMV specific T cells

Antibody	Colour	Clone	Company
CD3	BV605	OKT3	BioLegend
CD4	APC-Cy7	RPA-T4	BioLegend
Class II tetramer	PE	N/A	Benaroya Research Institute
Viability	Red stain	N/A	Invitrogen
Eome	FITC	WD1928	eBioscience
Tbet	Percp/Cy5.5	4B10	Biolegend
PD1	BV421	EH12.2H7	BioLegend
Transcription Factor kit (ebioscience)			

Panel 11 - Cytotoxicity panel for CD8 CMV specific cells

Antibody	Colour	Clone	Company
CD3	APC-Cy7	SK7	Biolegend
CD8	Amcyan	SK1	BD Biosciences
Viability	Red stain	N/A	Invitrogen
Class I tetramer	APC	N/A	University of Birmingham
Granzyme B	PE	GB11	BD Biosciences
Perforin	FITC	dG9	eBioscience
Fix/Perm: 4% PFA and Saponin			

Panel 12 - Sorting for myeloid subsets

Antibody	Colour	Clone	Company
Viability	Red stain	N/A	Invitrogen
CD34	PE	563	BD Biosciences
CD56	percp/Cy5.5	B159	BD Biosciences
CD16	Pe-Cy7	3G8	Biolegend
CD14	FITC	M5E2	BD Biosciences

2.6.2 Intracellular staining with 4% paraformaldehyde and saponin.

Prior to fixation, cells were defrosted, counted and stained with tetramer and surface antibodies as described in section 2.6.1. Details of the antibodies used for each intracellular experiment are found in Table 2.3 (panels 6-8 &11). After incubating for 15 minutes at 4°C with surface antibody, cells were washed in MACS. Discarding the supernatant, cells were then resuspended in 100μL of 4% PFA (Sigma-Aldrich) and left at RT in the dark for 15 minutes. After a further wash in MACS, cells were resuspended in 0.5% saponin (Merck millipore) and incubated for 5 minutes at RT in the dark. Subsequently, the appropriate intracellular antibodies (highlighted in red within table 2.3) were then added and left for 30 minutes at RT in the dark prior to washing in MACS and analysing on the flow cytometer.

2.6.3 Intracellular staining with FoxP3 transcription factor kit.

Prior to fixation, cells were defrosted, counted and stained with tetramer and surface antibodies as described in section 2.6.1. Details of the antibodies used for the each intracellular experiment are found in Table 2.3 (panels 9 & 10). After incubating for 15 minutes at 4°C with surface antibody, cells were washed and the supernatant discarded. Using the transcription factor staining kit (eBioscience) cells were resuspended in 500μL of diluted 4x fixation concentrate. For this, 125μL of fixation solution was added to 375μl of the diluent provided with the kit. Cells were then incubated for 30 minutes at RT in the dark. Meanwhile, a 1 in 10 dilution of the 10 x permeabilisation buffer was prepared with MACS buffer. After fixation, the cells were washed in 2 ml of the diluted permeabilisation buffer, centrifuged at 1800rpm for 5 minutes and the supernatant discarded. A further 100μL of diluted permeabilisation buffer was then added and cells left for 15 minutes at RT in the dark. Intracellular antibodies were then added for the respective panels (indicated in red in Table 2.3) and left for 30 minutes in the dark at RT prior to a further wash in 2ml of diluted

permeabilisation buffer. After further centrifugation, the supernatant was discarded and cells resuspended in 200µl of MACS and ran on the LSRII.

2.7 Stimulating CMV-specific T cells.

Following the method described by Te raa *et al* for CD8+ T cells and following optimization experiments described in chapter 4 for CD4+ T cells, 2×10^6 PBMCs were defrosted and washed before being stimulated with the appropriate concentration of peptide for NLV, TPR or ELK as described in chapter 3 or DYS and AGI as described in chapter 4, for 6 hours at 37^0C in the presence of 1µl of protein transport inhibitor cocktail (eBioscience) (te Raa et al., 2014). B:T cell ratios were not manipulated for this work and as such represented that found in-vivo. After stimulation, cells were washed, stained and incubated at 37^oC with class I or II tetramer, as described in section 2.5. Following this, surface staining as detailed in panels 6 for CD4+ cell stimulation or panel 7 for CD8+ cell stimulation, were performed prior to intracellular staining as described in section 2.6.2 using PFA and saponin. Cells were then washed and ran on the LSRII.

2.8 Cell sorting for telomere length analysis

Cells were stained with the appropriate tetramer at 37^0C as described above, followed by Live-dead fixable cell staining assay (Invitrogen). Surface antibodies were then added as detailed in Table 2.3 (Panels 4 and 5). Tetramer positive cell populations were sorted alongside the corresponding CD4+ or CD8+ tetramer negative populations into 1.5ml eppendorfs, which had been coated with MACS buffer, using the MoFlow sorter (Beckman Coulter). Sorted cells were then centrifuged at 1300 RPM for 5 minutes and the supernatant discarded. Cell pellets were then stored at -80^oC and transported on dry ice to Cardiff University for further analysis. Single molecule PCR was performed amplifying the telomere

lengths as described by Baird *et al* and is discussed in more detail in chapters 3 and 4 (Baird et al., 2003).

2.9   Digital and QPCR to detect CMV viral load amongst myeloid cells

2.9.1   Cell enrichment for myeloid cell subsets: CD14, CD34 and CD16.

Using excess cells obtained from apharesis of healthy stem cell donations, enrichment of CD14, CD16, CD34 and dual positive CD14/16 cells were obtained by cell sorting (MoFLow sorter, Beckman coulter). This was required in order to assess CMV viral load in myeloid subsets. For this experiment, cells were defrosted and washed in PBS prior to surface staining as described in section 2.6.1 using panel 12. Unfortunately due to the limited number of cells and donor material available, the purity for each sorted population was not obtained.

For extraction of CD14 positive cells from healthy donor PBMC, positive selection using CD14 magnetically labeled beads was used and an average enrichment found to be 98.73% (SD 0.39) by flow cytometry (Miltenyi Biotec, Surrey, UK). DNA extraction was then performed on the enriched cell populations according to the protocol for GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, St. Louis, MO USA) and DNA concentration and purity checked using the Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA).

2.9.2   CMV plasmid controls for standard curve generation for QPCR.

Human CMV HHV5 kit (PrimerDesign) for quantitative PCR (QPCR) amplification of glycoprotein B was purchased and used for all CMV PCR reactions including digital PCR. The plasmid control from the kit was reconstituted and aliquoted for storage at -20°C. For each experiment, plasmid dilutions were prepared fresh and were diluted to produce the

following copies per reaction: 50000, 10000, 2500, 500, 250, 100, 50,10, 5 and 1 (Parry et al., 2016b).

2.9.3   Droplet Digital PCR.

The QX100 droplet digital PCR system was used to generate digital PCR results (Bio-rad). For this, a reaction mixture consisting of 5μl of either CD14 positive DNA (10ng/μl) or plasmid standard were used and made up to a volume of 8μl with PCR grade water. This was then mixed with 10μl of 2 x ddPCR supermix for probes (Bio-rad), 1μl of reconstituted FAM labeled CMV primer and probe (Primer Design) and 1μl of HEX labeled RPP30 copy number assay for ddPCR which was used as an internal control (Bio-Rad). The reaction mixture was then loaded into a disposable plastic cartridge for droplet generation (Bio-Rad). 70μl of droplet generation oil (Bio-Rad) was also added before loading the cartridge into the droplet generator (Bio-Rad). Following droplet generation, samples were then transferred into a 96 well PCR plate (Eppendorf) and PCR amplification carried out using the T100 thermocycler (Bio-Rad). PCR conditions consisted of 10 min at 95°C, prior to 40 cycles at 94°C for 30seconds and 60 seconds at 60°C and a finally 1 cycle at 10min at 98°C, ending at 12°C. After amplification the plate was loaded onto the droplet reader (Bio-rad) and results analysed by QuantaSoft software (Bio-Rad) to give the number of virus copies per μl of PCR reaction. A positive and negative control together with a well containing just water, was included in each experiment. Results were obtained in triplicate and averaged. Each mammalian cell contains 2 copies of the internal control RPP30. Therefore the absolute quantification of RPP30 obtained by digital PCR was divided by 2, in order to determine the actual cell number present. The absolute CMV viral load was then divided by this figure to obtain the CMV load per cell in each well (Parry et al., 2016b).

2.9.4 Quantitative PCR.

For QPCR, the 7500 Real Time PCR system was used (Applied Biosystems, Life Technologies). Using the same amount of starting DNA as used for the ddPCR assay, a reaction mixture consisting of 5μl of standard plasmid or 5μl of CD14 positive DNA (10ng/μl) was made up to a volume of 9μl with PCR grade water, together with 10μl of 2 x Taqman Universal mastermix II (Applied Biosystems) and 1μl of FAM labeled CMV primer and probe were loaded into a 96 well PCR plate (Eppendorf). PCR amplification consisted of 2 min at 95°C, prior to 50 cycles at 95°C for 10 seconds and 60 seconds at 60°C. A positive and negative control plus water well were included in each experiment and the standard curve repeated in triplicate and averaged. Samples were only considered positive if present in triplicate (Parry et al., 2016b).

2.10 Functional Antibody testing

Functional antibody testing was performed by The Clinical Immunology Service at the University of Birmingham for work presented in chapter 6. This was performed in order to ascertain specific IgG responses to antigens of commonly encountered pathogens. Responses were then used to assess vaccine responsiveness and protective immunity against these common pathogens in accordance with criteria outlined by the WHO. For this work, 1 ml of patient serum was collected and stored at -20°C until testing. A 1:100 dilution of serum was then used to determine IgG antibody levels to 19 vaccine antigens: 12 pneumococcal (Pn) polysaccharides (serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F), four meningococcal polysaccharides (serogroups Men A, C, W and Y), *Haemophilus influenza-b* (Hib), tetanus toxoid and diphtheria toxoid, using a bead based 19-plex antibody. The polysaccharide antigens were first conjugated to poly-L-lysine (Sigma -Aldrich). All antigens, including the toxoids, were then conjugated onto carboxylated microspheres

(BioRad) specific for different bead regions. Patient samples were incubated with the conjugated beads alongside a serial dilution of standard reference sera. A subsequent incubation with mouse anti-human IgG phycoerythrin (PE)-conjugate (Southern Biotech) took place before analysis on a Luminex-100 instrument (BioRad). Data analysis was performed using Bio-plex Manager 4.1.1 software (BioRad). This created a standard curve of median fluorescence intensity (MFI) against antibody concentration for the reference standard serum, which the software used to assign specific antibody concentrations to the test sera (Whitelegg et al., 2012).

2.11 Ethical approval

For the study of patients with CLL, ethical approval was obtained from West Midlands regional ethics committee (REC) (#10/H1206/58). For healthy donor controls, ethical approval was also obtained from West Midlands REC (#2002/073). For the use of waste material for donor stem cell bags ethical approval was obtained from West Midlands REC (#05/Q2707/175). Additional ethics was obtained to investigate the impact of CMV on patient outcome from South East Wales REC (#02/4806) and from the Human Subjects Institutional Review Board at the Mayo Clinic and the University of Iowa. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

2.12 Statistical Analysis

The statistical analysis used for each specific chapter is detailed in the methods section of that particular chapter. In general, normality testing was performed using d'Agostino-pearson omnibus normality test. Where normality was confirmed, Student's T-tests were used to compare 2 datasets and where normality wasn't shown, non-parametric Mann-Whitney testing was performed. For 3 or more datasets, where normality was shown, Anova testing

was performed. For comparison of more than 2 non-parametric datasets, Kruskal-Wallis test with post hoc Dunn's testing was performed. For correlation analysis, pearson's correlation co-efficient where data was parametric and spearman's correlation for non-parametric data was used. Linear regression was used to examine the relationship between variables as detailed in chapter 5. For functional antibody work, the geometric mean and SD was utilised to present the data. Survival analyses employed the Kaplan-Meier method. Curves were compared using the log rank test. Dot plot graphs throughout are presented as the median with the IQR. Prism version 6 was used to produce illustrations and all statistical analysis other than the multivariate analysis presented in chapter 6. For multivariate analysis, Cox proportional Hazards model were constructed using SPSS version 23 and data on the selection of variables modeled is presented in chapter 6.

3 CD8+ CMV-SPECIFIC IMMUNE RESPONSE

3.1 Introduction

Chronic lymphocytic leukaemia is associated with an increase in the absolute numbers of circulating CD8+ T cells and expanded populations of differentiated memory CD8+ T cells (Serrano et al., 1997, Goolsby et al., 2000, Briggs et al., 1990, Mackus et al., 2003). The proliferative capacity, cytotoxicity and cytokine production of these CD8+ T cells is known to be impaired in patients with CLL and microarray analysis of the gene expression profile of CD8+ T cells has demonstrated multiple pathway defects in the cytoskeleton network and vesicle trafficking, both of which are pivotal for T cell activation and effector functioning through immunological synapse formation (Ramsay et al., 2008, Gorgun et al., 2005, Scrivener et al., 2003, Riches et al., 2013).

Evidence for clonal CD3+ T cell populations was first reported in 1990 in patients with CLL (Wen et al., 1990). Analysis of the T cell receptor V alpha and beta gene usage and TCR sequencing supported the existence of populations of clonal and oligoclonal CD8+ cell populations (Farace et al., 1994), as did analysis of the size of the CDR3 region of the TCR transcript in patients with CLL (Alatrakchi et al., 1998). These oligoclonal CD8+ T cells have been shown to proliferate when exposed to autologous dendritic cells presenting the cognate immunoglobulin derived VH-DR3 region, and secrete IFN-γ in response to autologous dendritic cells that had endocytosed apopototic bodies from dying CLL cells (Kokhaei et al., 2003). However, there is no definitive evidence to support the hypothesis that these clonal T cells are CLL-specific and unstimulated CD8+ T cells from patients with CLL have failed to activate in the presence of autologous CLL tumour cells (Krackhardt et al., 2002, Buhmann et al., 1999).

Chronic activation of CD8+ T cells by commonly encountered pathogens could be an alternative mechanism leading to the clonal T cell expansions seen. CMV is common,

maintains latency, frequently reactivates in its host with increasing age and is known to drive CD8+ T cell differentiation to a CCR7-CD45RA+/- phenotype (Gamadia et al., 2001). In healthy elderly CMV seropositive individuals, CMV-specific CD8+ T cells have been shown to constitute up to 25% of all circulating CD8+ T cells and the CD8+ T cell response to CMV is thought to be greater than that directed against any other pathogens (Khan et al., 2002). Paradoxically this response increases further in states of immunosuppression (Gamadia et al., 2001). Mackus *et al* first identified an increase in the frequency of CMV-specific CD8+ T cells in patients with CLL compared to age matched healthy donors. Using HLA A2 NLV and HLA B7 TPR tetramers, Mackus *et al* showed an increase in CMV-specific T cell in CLL patients (n=10) compared to controls (n=6) (P=0.0225) and found up to 15% of CD8+ T cells were directed against CMV. PCR detected the virus in one patient. Mackus *et al* described their phenotype to be consistent with a cytotoxic effector memory cell being CD27- and CD45RA+ (Sallusto et al., 1999, Mackus et al., 2003). Positive CMV serology also increased the relative and absolute number of these cells compared to patients who were CMV negative, leading the authors to conclude that the expanded populations of CD27-CD45RA+ cells were unlikely to be tumour-specific (Mackus et al., 2003).

Subsequent studies have investigated whether CMV is responsible for the global impairment of CD8+ T cell function reported in patients with CLL. Features of CD8+ T cell exhaustion have been described in CLL *in vitro* and in murine models (Gassner et al., 2015, McClanahan et al., 2015, Riches et al., 2013). These studies have appraised expression of the T cell inhibitory receptors PD-1, CD160 and CD244 (Riches et al., 2013), poor immunological synapse formation (Ramsay et al., 2008) and loss of proliferative capacity (Riches et al., 2013). Riches *et al* reported that the increased expression of inhibitory receptors on CD8+ T cells was present irrespective of CMV serological status and that PD-1 expression was

decreased in CMV positive patients compared to negative on CD8+ T cells and attributed this decreased expression to a skewed CD8+ T cell differentiation in CMV positive individuals, with a greater ratio of T_{EMRA}: T_{EM} cells observed in CMV positive individuals. This work also demonstrated that the production of Th1 cytokines was increased in CD8+ T cells in patients with CLL when stimulated with PMA and ionomycin and that the defects in CD8+ T cell cytotoxicity reported were due to a lack of polarized expression of CD107a and granzyme B at the immunological synapse. No difference was reported in CD8+ cytokine production, proliferation or cytolytic activity in CMV positive and CMV negative patients with CLL (Riches et al., 2013).

The aim of this work was to specifically identify CD8+ CMV-specific T cells and assess their contribution to the phenotype and function of the CD8+ T cell repertoire in patients with CLL. The results from this chapter were also used to provide a comparison for experimental data presented in chapter 4, where the CD4+ CMV-specific immune response is interrogated in patients with CLL.

3.2 Methods

3.2.1 Recruitment of donors

120 patients with CLL were recruited from outpatient haematology clinics at BHH and QEHB. All patients were either untreated or more than 6 months post treatment and all had a lymphocyte count >1.5x 10^9/L at the time of sample collection. 34 healthy donors were also recruited to this study. Following a 50 ml blood donation, plasma and PBMCs were extracted over a Ficoll-density gradient as described in chapter 2. DNA was extracted from a PBMC pellet for PCR to identify the class I HLA type of donors.

3.2.2   CD8 CMV specific T cell immunophenotyping

PBMCs were defrosted and stained as described in chapter 2. For this chapter, flow cytometry panel 1 was used for analysis of memory phenotype and PD-1 status, panel 11 for analysis of cytotoxicity and panel 4 for cell sorting of CD8+CMV-specific T cells for telomere length analysis.

3.2.3   Peptide stimulation assay

Following identification of an ELK, TPR or NLV tetramer response, $2x10^6$ cells were incubated at 37^oC for 6 hours with either 0.25μg/μl TPRVTGGGAM (TPR) peptide or NLVPMVATV (NLV) or ELKRKMIYM (ELK) at 0.5μg/μl. Tetramer and antibodies were then applied as detailed in panel 7.

3.2.4   Statistical analysis

Unless otherwise stated, non-parametric unpaired Mann-Whitney testing was performed to compare 2 groups within this chapter. Additional statistical testing is described in the figure legends.

3.3   Results

3.3.1   Identifying CD8+ CMV specific T cell responses

120 patients were recruited and 27 CMV negative donors were identified. A further 61 patients were found to be CMV positive and had at least one appropriate HLA type for examining CMV specific CD8+ T cell responses. To examine the CD8+ CMV-specific T cell response, 9 different tetramers were used depending on the class I HLA type identified; (22 donors were HLA A1 (for which VTE and YSE tetramers were applied), 38 donors were HLA A2 (for which NLV and VLE tetramers were applied), 33 donors were HLA B7 (for which TPR and RPH tetramers were applied) and 21 donors were HLA B8 (for which ELK,

ELR and QIK tetramers were applied). A total of 114 tetramer stains were performed, of which 60 tetramer responses were identified from 45 patients; 33 from patients with a pp65 response (tetramers NLV, YSE, TPR and RPH), 24 from patients with an IE-1 response (ELK, ELR, QIK and VLE) and 3 patients with a pp50 (VTE) response. A true tetramer response was verified through the lack of background staining by gating all CD3+ T cells, against CD8+ T cells. The median age of the patients with CLL with identifiable CD8+ tetramer responses was 73 (IQR: 66.5-80). Table 3.1 provides the characteristics of the 45 patients with identified tetramer responses used in this chapter.

Using the same tetramers and for comparative analysis, 15 age matched healthy donors (HD) were also identified to have CD8+ CMV specific T cell responses (12 pp65 and 3 IE-1 and 0 pp50 responses), with a median age of 80.5 (IQR: 75.5-83.8). Figure 3.1A shows the gating strategy for identifying tetramer responses.

3.3.2 The magnitude of the CMV specific CD8+ T cell response is not increased in patients with CLL compared to healthy donors.

Next, the CMV-specific CD8+ T cell response was compared between patients with CLL and healthy donors. In contrast to published findings reported by Mackus *et al*, an increase in the CMV-specific CD8+ T cell response was not observed amongst patients with CLL (median HD: 2.10 vs CLL 1.00%; p=0.527) (Mackus et al., 2003). This result was somewhat surprising but may be explained by the fact that the healthy donors were on average 7 years older (Figure 3.1B). Indeed, amongst all donors (CLL and HD) a significant increase in the CMV-specific CD8+ T cell response was observed with increasing age (R^2=0.06; p=0.035). Furthermore, when a comparison of CMV-specific CD8+ T cell responses were made between those under the age of 80 and those over, a greater response was observed in those

aged 80 or over (median age in <80 years was 0.95 vs 2.3 in those over the age of 80; p=0.039) (Figure 3.1C).

3.3.3 CMV specific CD8+ T cell responses increase with stage of disease in patients with CLL.

Having demonstrated no difference in the magnitude of CMV-specific CD8+ T cell responses in HD and patients with CLL, I next assessed whether CMV responses increased with disease severity. For this, the magnitude of IE-1 and pp65 tetramer responses was assessed by Binet stage of disease in patients with CLL. Patients with tetramer responses that were classed as Binet stage C demonstrated the greatest CMV-specific CD8+ T cell responses with a median frequency of 2.8% compared to stage A patients who had the lowest frequency at 0.55% whilst stage B patients demonstrated an intermediate frequency of 1.05% (Figure 3.2). Overall the difference between the 3 groups did not reach significance (p=0.051) but post-hoc Dunn testing revealed a significant difference between stage C patients and stage A patients (p=0.018). As age impacts on CD8+ CMV-specific T cell responses, the ages of patients were then compared by stage of disease. No difference was observed in the age of patients by stage of disease, such that stage of disease appears to impact on the magnitude of CD8+ T cell responses independently of age (Median stage A: 73 vs Stage B: 67 vs Stage C: 79; p= 0.638 Kruskal-wallis test).

Table 3.1

Clinical characteristics of CLL patients with a CMV-specific CD8+ T cell response

Median age at diagnosis (years)	73 (IQR 66.5-80)
Male sex	30/45 (67%)
Follow up time at the point of sample collection (years)	6 (IQR: 2.8-9.2)
Previous history of treatment	13 (29%)
Time since treatment finished (years)	2 (IQR: 0.5-5)
Binet stage A	25 (56%)
Binet stage B	6 (13%)
Binet stage C	14 (31%)
Missing	0
Number of deaths	9 (20%)
CD38 positive	9 (20%)
CD38 negative	18 (40%)
CD38 missing	18 (40%)
IGHV mutated	5 (11%)
IGHV unmutated	4 (9%)
IGHV missing	36 (80%)
FISH	
Normal	19 (42%)
13q-	2 (4%)
12+	2 (4%)
11q-	4 (9%)
17p-	4 (9%)
Other	0
Missing	14 (31%)

Figure 3.1

CMV-specific CD8+ T cell response increases with age

A) Demonstrates the gating strategy for identifying CD8+ CMV-specific T cell responses. After excluding doublets, live PBMCs were identified, followed by CD3+ cells positive for CD8 and tetramer. B) No significant difference was observed in the frequency of CMV-specific CD8+ T cells in patients with CLL compared to HD (Mann-Whitney). C) A significant positive correlation was observed with age and the magnitude of CMV-specific CD8+ T cell responses (p=0.035) (Pearson's correlation co-efficient). This was further observed when comparing those donors over the age of 80 to those below the age of 80 (p=0.039) (Mann-Whitney).

A

B

C
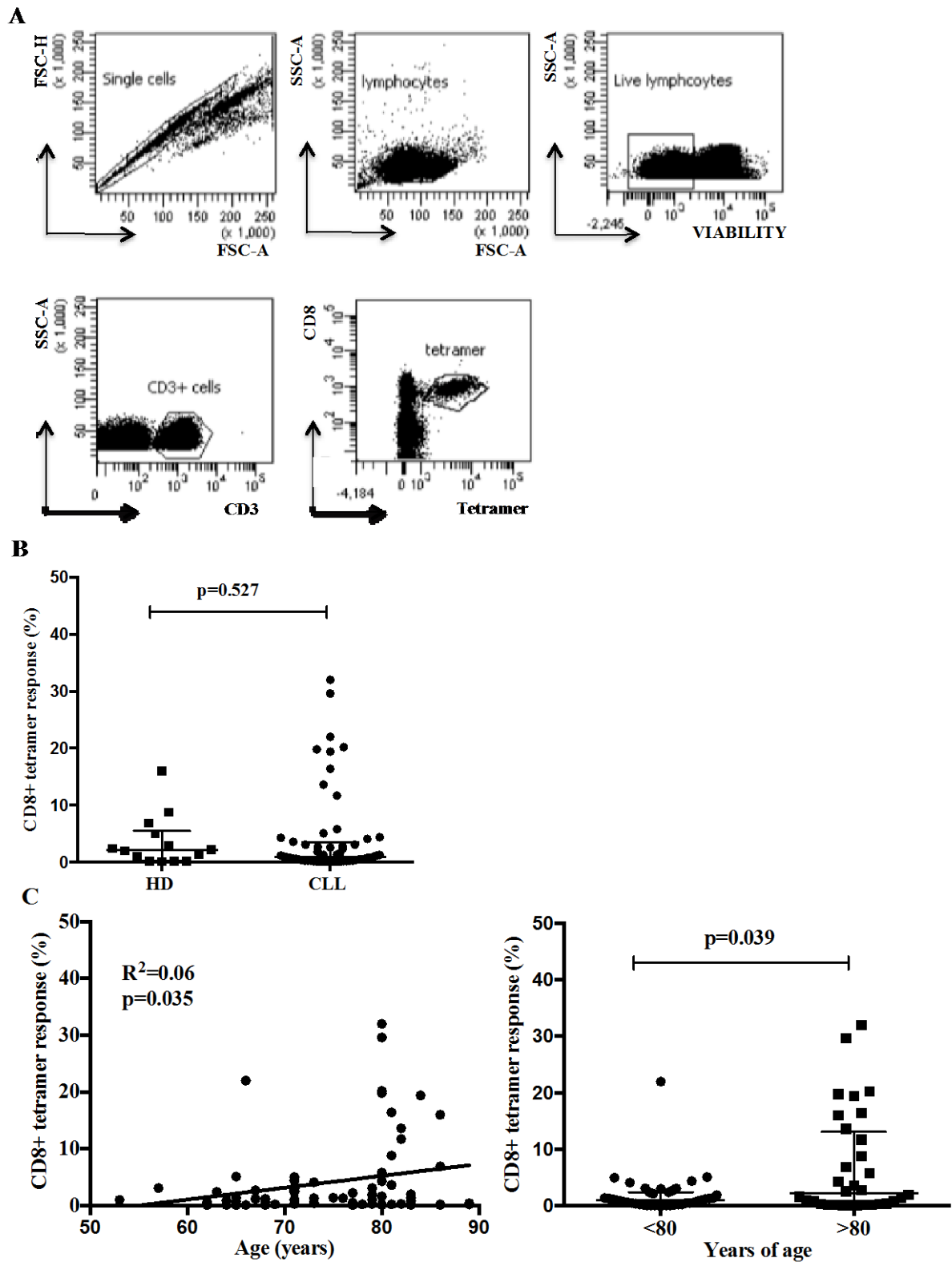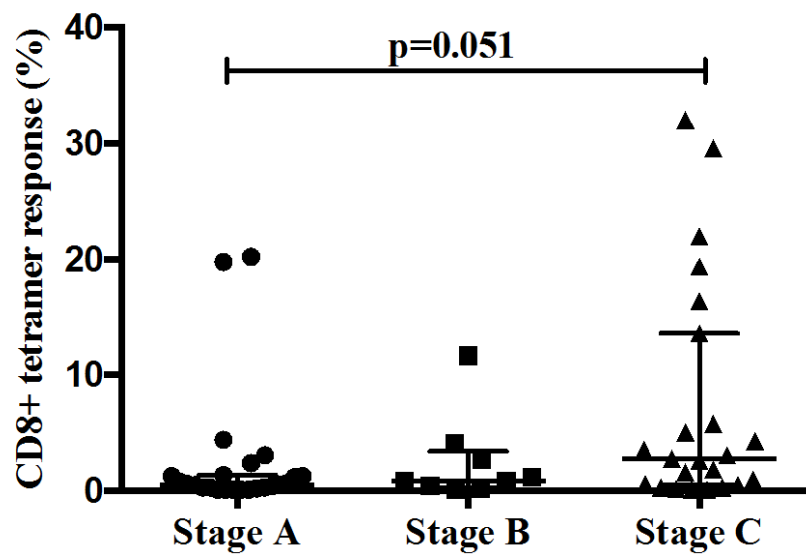
Figure 3.2

CMV- specific CD8+ T cell responses increase with advanced stage disease

The IE-1 and pp65 CD8+ T cell responses analysed by stage of disease is shown. An increase

in the frequency of response is seen with stage of disease, with stage C patients demonstrating

a greater frequency of CMV-specific CD8+ T cells than stage A patients (p=0.051) (Kruskal-

Wallis test).

3.3.4   CMV-specific CD8+ T cells acquire a T_{EMRA} phenotype.

The phenotype of the identified CMV-specific CD8+ T cells was next investigated. Many cell markers have been used to identify memory cell subsets as described in chapter 1 and illustrated in figure 1.3. In order to reduce heterogeneity between publications Maecker *et al* suggest utilising CCR7 and CD45RA to define memory subsets (Maecker et al., 2012). Using CCR7 and CD45RA surface staining, memory subsets were defined as naïve (CCR7+, CD45RA+), central memory (T_{CM}) (CCR7+, CD45RA-), effector memory (T_{EM}) (CCR7-, CD45RA-) and terminally differentiated effector memory cells (T_{EMRA}) (CCR7-, CD45RA+). An example of representative staining in a healthy donor can be seen in Figure 3.3A. Firstly, a comparison of memory subset distribution between all HD and patients with CLL regardless of CMV serostatus did not reveal any difference in the total CD8+ T cell populations, with similar proportions of each memory subset observed (median HD: $T_{naïve}$ 6.5%; T_{CM} 5%; T_{EM} 54.3%; T_{EMRA} 29.5% vs CLL: $T_{naïve}$ 3.4%; T_{CM} 3.1%; T_{EM} 50%; T_{EMRA} 35.1%) (Data not shown).

Next, CD8+ T cell memory subsets were compared between CMV positive and CMV negative individuals. In healthy donors, no statistical difference was observed in the frequencies of memory subsets when comparing CMV positive to negative donors, although a trend towards greater frequencies of T_{EMRA} cells and reduced $T_{naïve}$ cells was observed in CMV positive donors (CMV negative vs CMV positive: T_{EMRA}: 26.3% vs 31.8% (p=0.306); T_{naive}: 7.25% vs 2% (p=0.137) and T_{EM}: 51.2% Vs 59.6% (p=0.251)) (Figure 3.3B). Next CLL patients who were CMV positive (n=61) and CMV negative (n=27) were investigated. Patients with CLL who were CMV positive had a greater frequency of T_{EMRA} cells compared to patients who were CMV negative (CMV positive T_{EMRA} 36.9% vs CMV negative T_{EMRA}: 23.5 %; p=0.051) (figure 3.3C). A trend towards a reduced frequency of naïve CD8+ T cells

and increased T_{EM} CD8+ T cells was also observed amongst CMV positive patients compared to CMV negative patients, although these did not reach statistical significance (median CMV negative T_{naive}: 6.4% vs CMV positive T_{naive}: 3%; p=0.181 and CMV negative T_{EM}: 43.5 % vs CMV positive $T_{EM:}$ 51%; p=0.101).

Next, the CMV-specific CD8+ T cell memory phenotype was investigated. CMV specific CD8+ T cells were either T_{EM} or T_{EMRA} in phenotype in both HD and patients with CLL. In HD, a greater proportion of T_{EMRA} cells were observed compared to T_{EM} amongst CMV-specific CD8+ T cells (84.7% T_{EMRA} vs 14.5% T_{EM}; p=0.0001) (figure 3.3D). Healthy donors were also seen to have more T_{EMRA} cells than patients with CLL (HD: 84.7 vs CLL 50.7; p=0.047) (data not shown). The frequency of T_{EM} and T_{EMRA} CD8+ CMV-specific T cells was similar in patients with CLL, with a marginally greater proportion of T_{EMRA} cells observed (T_{EMRA} 50.7% vs T_{EM} 43%; p=0.751) (figure 3.3E). No difference between CMV-specific T cell memory phenotype and the background CD8+ T cells were observed in CMV positive patients (Figure 3.3E).

In summary, CMV-specific CD8+ T cells in both healthy donors and patients with CLL have an differentiated effector memory phenotype. However, in patients with CLL a greater proportion were found to be T_{EM} in phenotype (43%) compared to healthy donors where T_{EM} phenotype made up only 14.5% of the CMV-specific CD8+ T cells.

Figure 3.3

The memory phenotype of CMV-specific CD8+ T cells

(A) represents a typical plot of CD8+ T cell staining for memory subset analysis using CCR7 and CD45RA. (B) Demonstrates the frequency distribution of memory T cell phenotypes in CMV positive and negative healthy donors. (C) Demonstrates the frequency distribution amongst CMV positive and negative patients with CLL. CMV positive donors had a greater frequency of T_{EMRA} memory cells (36.9%) compared to patients who were CMV negative (23.5%) (p=0.051). (D) Demonstrates the frequency distribution amongst the total CD8+ T cell population and also the CMV-specific CD8+ T cell populations in HD. The CMV-specific CD8+ T cell phenotype was predominantly T_{EMRA}. (E) In contrast, patients with CLL demonstrated no difference in memory subset distribution between CMV-specific CD8+ T cells and the total CD8+ T cell population, including the frequency of T_{EMRA} cells (CMV-specific CD8+ T cells: 50.7% vs total CD8+ population 39.6%; p=0.104) (Mann-Whitney).
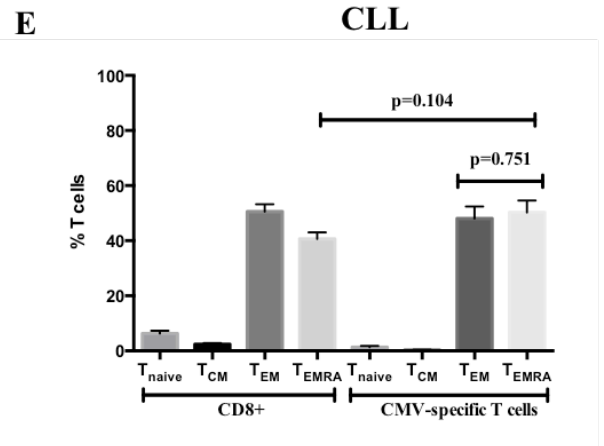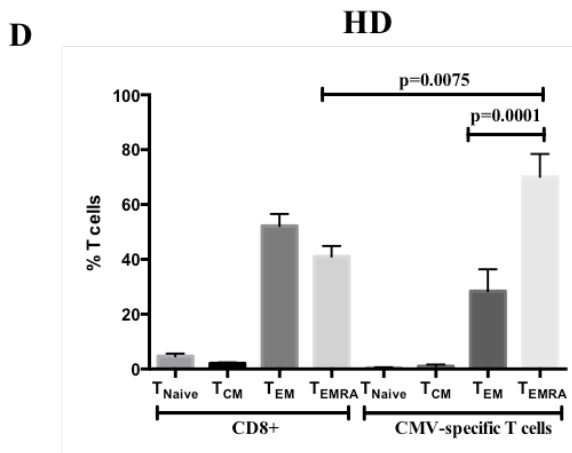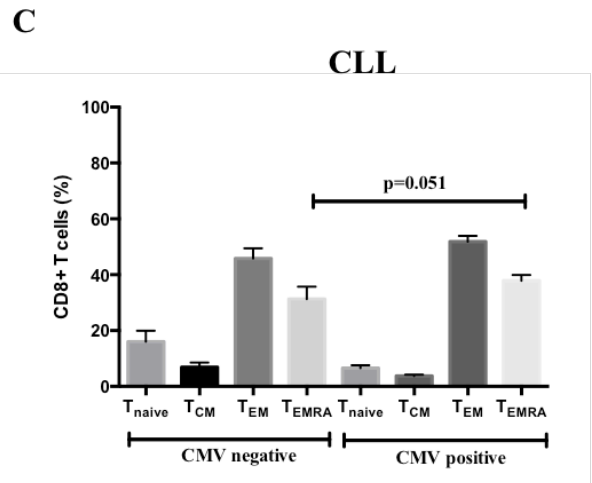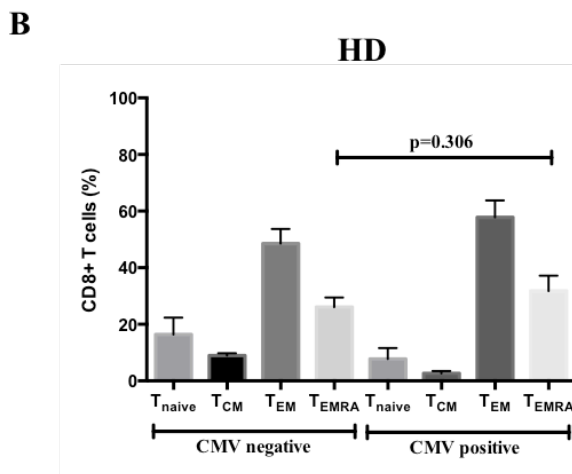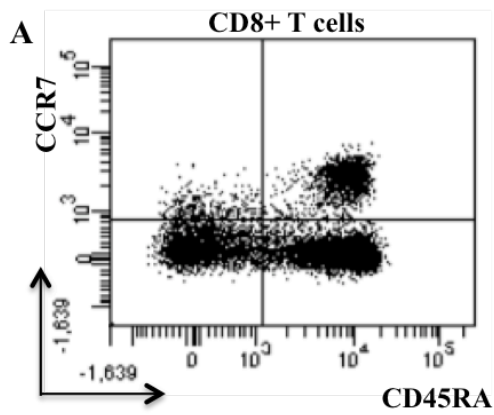
3.3.5   CMV specific CD8+ T cells contain granzyme and perforin.

Having demonstrated phenotypic differences in memory subsets in HD and patients with

CLL, I next went on to assess if there was any difference in the expression of perforin and

granzyme B amongst CMV-specific CD8+ T cells. Using intracellular staining described in

chapter 2, a total of 25 donors (15 CLL and 10 HD) were investigated. In all donors, the

majority of CMV-specific CD8+ T cells expressed both perforin and granzyme B (median

83.6% (IQR: 63.7-92.2) consistent with published literature (Gamadia et al., 2001).

Expression of perforin and granzyme amongst CMV-specific CD8+ T cells was significantly

greater than that observed on the total CD8+ T cell population (median 57.3% IQR: 44.9-

75.4; p=0.0007) (Figure 3.4A). When comparing HD and patients with CLL, the granzyme B

and perforin content of CMV-specific CD8+ T cells was not significantly different (median

CLL: 87.4% vs 73.3%; p=0.13) (Figure 3.4B). However, a greater difference was observed

between CMV-specific CD8+ T cells and the total CD8+ T cell perforin and granzyme B

expression in patients with CLL (p=0.001). A similar trend was seen in HD although this did

not reach statistical significance (p=0.325).
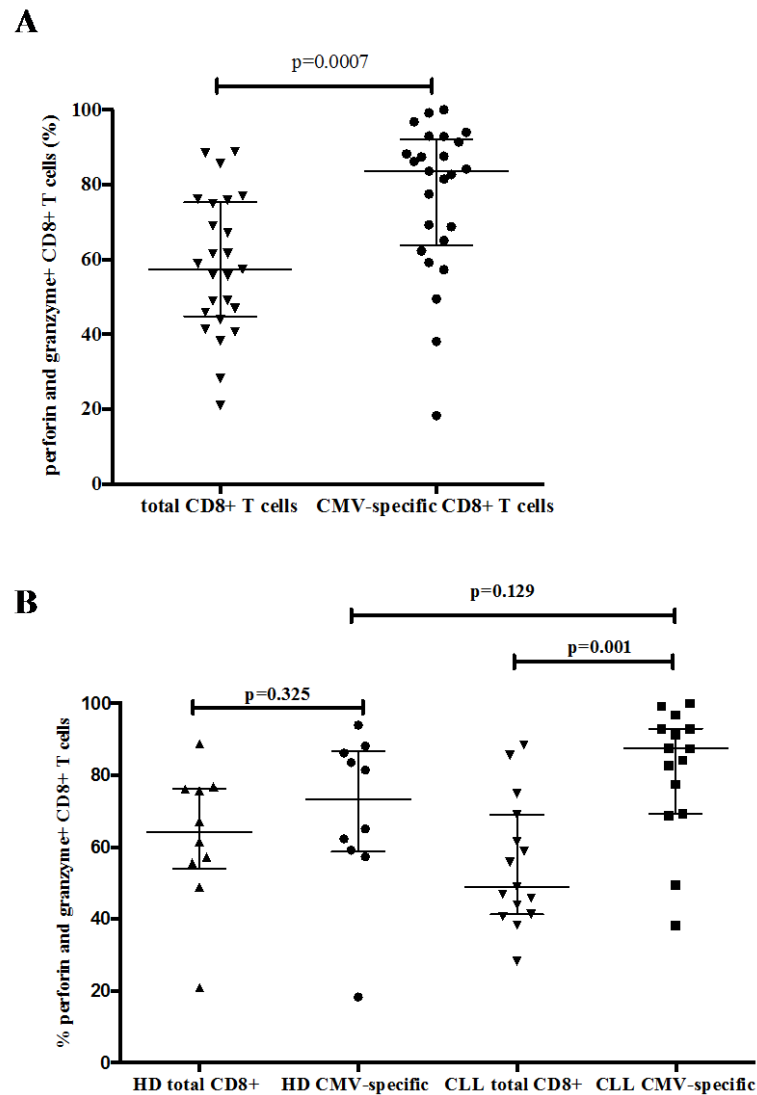
Figure 3.4

Perforin and granzyme B expression are high in CMV-specific CD8+ T cells

A) Demonstrates a dot-plot of perforin and granzyme B expression on CD8+ T cells from all

donors (CLL and HD). Expression was found to be greatest on CD8+ CMV-specific T cells

compared to background CD8+ T cells (83.6% vs 57.3%) (Mann-Whitney). When healthy

donors and patients with CLL were analysed separately, a greater difference was noted in

patients with CLL between the perforin and granzyme B expression in CMV-specific CD8+ T

cells compared to the total CD8+ T cell population (p=0.001), as illustrated in dot-plot (B)

(Mann-Whitney).

3.3.6 CD8+ CMV-specific T cell function.

To further assess the functional capacity of CMV-specific CD8+ T cell responses, I next stimulated PBMCs from HD or patients with CLL with cognate peptide for 6 hours and then performed tetramer staining, followed by intracellular staining as detailed in chapter 2. In order to ascertain the optimal concentration of peptide at which surface tetramer staining could be observed alongside maximum cytokine production, three peptides were titrated (ELK, TPR and NLV) in a healthy donor. Cytokine response was measured as the percentage of double positive CMV-specific CD8+ T cells producing IFN-γ and TNF-α. For NLV and TPR, the optimal concentration was determined to be 0.25μg/ml, whilst for stimulation with ELK a concentration of 0.5μg/ml was found to be optimal. An example of the titration graph for NLV is shown in figure 3.5A. Following peptide titrations, PBMCs from donors with known tetramer responses were stimulated with the appropriate peptide, alongside an unstimulated control for 6 hours at 37°C. Minimal cytokine expression was seen, if any, in unstimulated tetramer positive cells and where detected, this percentage was subtracted from the percentage expression found in stimulated cells. This subtraction allowed only the inducible cytokine production following peptide stimulation to be ascertained.

All CMV-specific CD8+ T cells that produced IFN-γ also produced TNF-α. TNFα was occasionally produced in the absence of IFN-γ and this was most notable in patients with CLL. In HD, on average 0.7 % of CMV-specific cells were seen to make TNF-α only, whilst amongst patients with CLL, 4.8% produced TNF-α alone (p=0.019). Overall, the proportion of CD8+ CMV tetramer+ T cells producing both IFN-γ and TNF-α was decreased in patients with CLL (n=10) compared to HD (n=10) (35.95% vs 55.7%; p=0.029 Figure 3.5C) suggesting CLL cells are suboptimal at producing cytotoxic cytokines following induced stimulation.
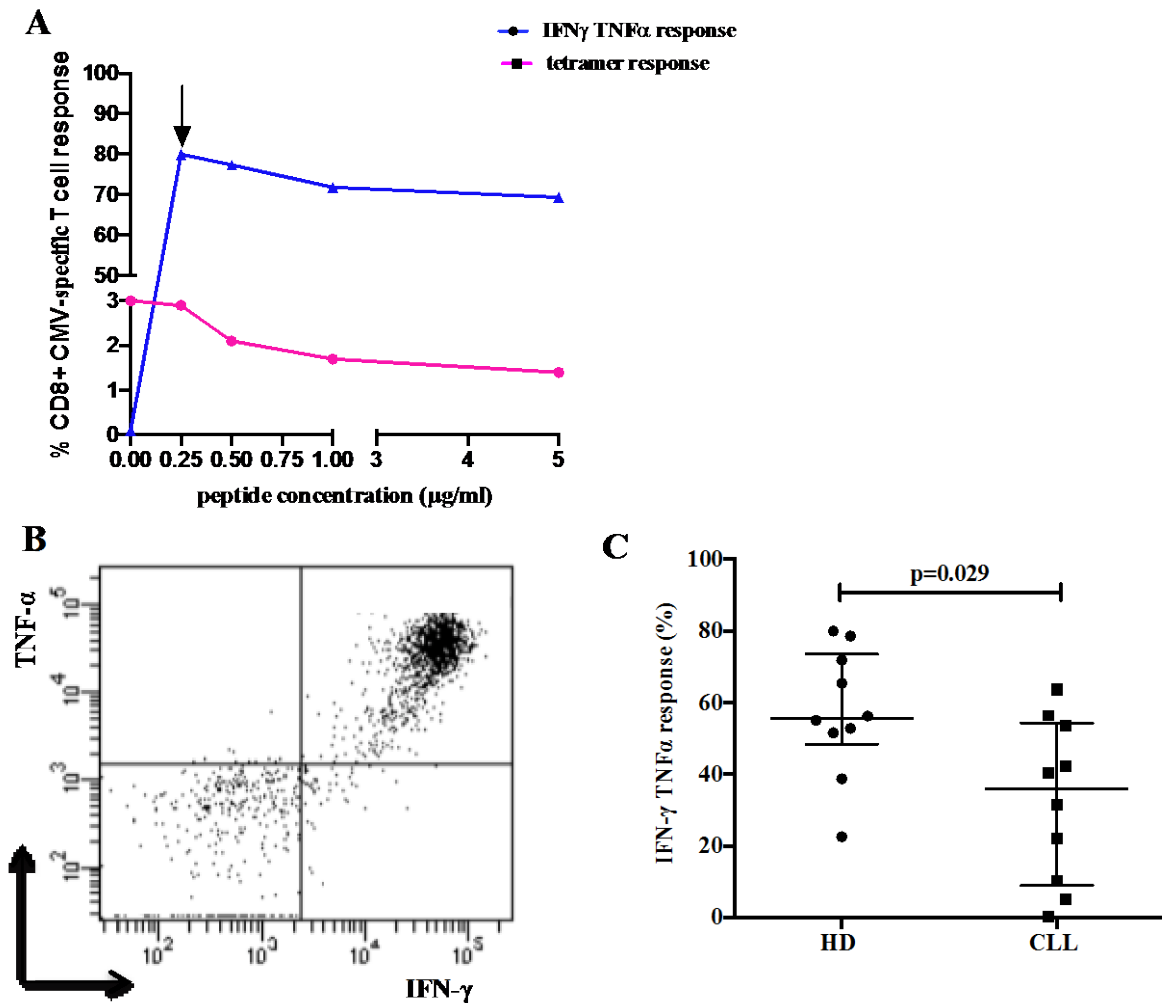
Figure 3.5

CMV-specific CD8+ T cells produce less IFN-γ and TNF-α than healthy controls.

A) An example of the optimization of peptide concentration for CMV-specific T cell stimulation using NLV peptide. An arrow indicates the optimal concentration used for further experiments, which was derived from the peptide concentration that provided the maximum cytokine response, whilst retaining tetramer staining. B) An example plot of CD8+ CMV-specific T cell production of IFN-γ and TNF-α. C) A comparison of responses from 10 HD and 10 patients with CLL, revealed a poorer IFN-γ and TNF-α response from stimulated CMV-specific CD8+ T cells in patients with CLL (p=0.029) (Mann-Whitney).

3.3.7 PD-1 expression is increased on CD8+ T cells of patients with CLL.

Given that CMV-specific CD8+ T cells derived from patients with CLL have inferior IFN-γ and TNF-α in response to CMV peptide stimulation, I next examined whether these cells exhibited the inhibitory cell marker PD-1. An example of PD-1 staining is shown in figure 3.6A. Increased expression of PD-1 has been reported on CD8+ T cells in patients with CLL and has been associated with T cell exhaustion. PD-1 expression was found to be greater on CD8+ T cell from patients with CLL (n=72) compared to HD (n=20) (Median HD: 8.5% vs CLL: 15.85; p=0.0004) (figure 3.6B).

3.3.7.1 PD-1 expression on CD8+ T cells does not correlate with stage of disease

To assess if the increase in PD-1 expression in patients with CLL is possibly driven by tumour load in the peripheral blood, PD-1 expression was examined on CD8+ T cells in patients with CLL according to stage of disease. No difference was observed between patients with advanced stage of disease and those with stage A CLL (Kruskal-wallis testing for Stage A: 16.45% vs Stage B: 14.51% Vs Stage C: 19.35%; p= 0.247) (figure 3.6C).

3.3.7.2 PD1 expression on CD8+ T cells is not influenced by prior treatment.

A further comparison of the PD-1 expression on CD8+ T cells in patients with CLL was undertaken to ascertain if a history of prior chemotherapy treatment impacted on PD-1 expression in this cohort. Amongst patients with CLL, no difference was observed in those patients who had previously been treated (14.9%) compared to those who had received prior chemotherapy (16.6%); (p=0.583) (figure 3.6D).

3.3.8 PD1 expression is decreased on CMV-specific CD8+ T cells.

Having shown that tumour load and previous therapy did not impact on PD-1 expression, I next investigated the impact of CMV seropositivity on PD-1 expression. The frequency of PD-1+ CD8+ T cells was compared between patients with CLL who were CMV positive to those who were CMV negative. CMV sero-status was determined using in-house ELISA method as described in chapter 2. No significant difference in PD-1 expression was observed between CLL patients based on serostatus (CMV negative CD8+PD-1+ T cells: 16.10 vs CMV positive CD8+PD-1+ T cells: 16.25; p=0.582). Neither was a difference observed by CMV serostatus on CD8+ PD-1 T cell expression in HD (CMV negative: 5.15% vs CMV positive: 10.15%; p=0.254) (data not shown).

Analysis of PD-1 expression on the 60 CMV-specific CD8+ T cell responses identified in patients with CLL was next undertaken. PD-1 expression was decreased on CMV-specific CD8+ T cells in patients with CLL compared to the total CD8+ T cell populations (CLL: CD8+ T cells: 15.3% (IQR: 6.68-26.3) vs CMV-specific CD8+ T cells: 7.35% (IQR: 2.15-12.93); p=<0.0001). In comparison HD CMV-specific CD8+ T cell responses demonstrated a greater expression of PD-1 compared to the total CD8+ T cell population, although this did not reach statistical significance on the 15 responses examined (CD8+ T cells: 8.8% (IQR:5.1-12.9) vs 12.5 (IQR: 4.4-27); p=0.254). Finally, PD-1 expression on CD8+ CMV-specific T cells derived from CLL patients was significantly decreased compared to HD CMV-specific CD8+ T cells (p=0.043) (figure 3.6E).

Figure 3.6

PD1 expression on CD8+ T cells and CMV-specific CD8+ T cells.

A) Example of PD-1 staining observed on CD8+ T cells and CMV-specific CD8+ T cells in patients with CLL. B) The percentage of PD-1 expression on CD8+ T cells in patients with CLL and HD is shown. The percentage of PD-1 expression on CD8+ T cells in patients with CLL according to disease stage (C) and previous therapy (D) is shown. E) The percentage of PD-1 expression on CMV-specific CD8+ T cells in patients with CLL and HD is shown (Mann-Whitney testing used).
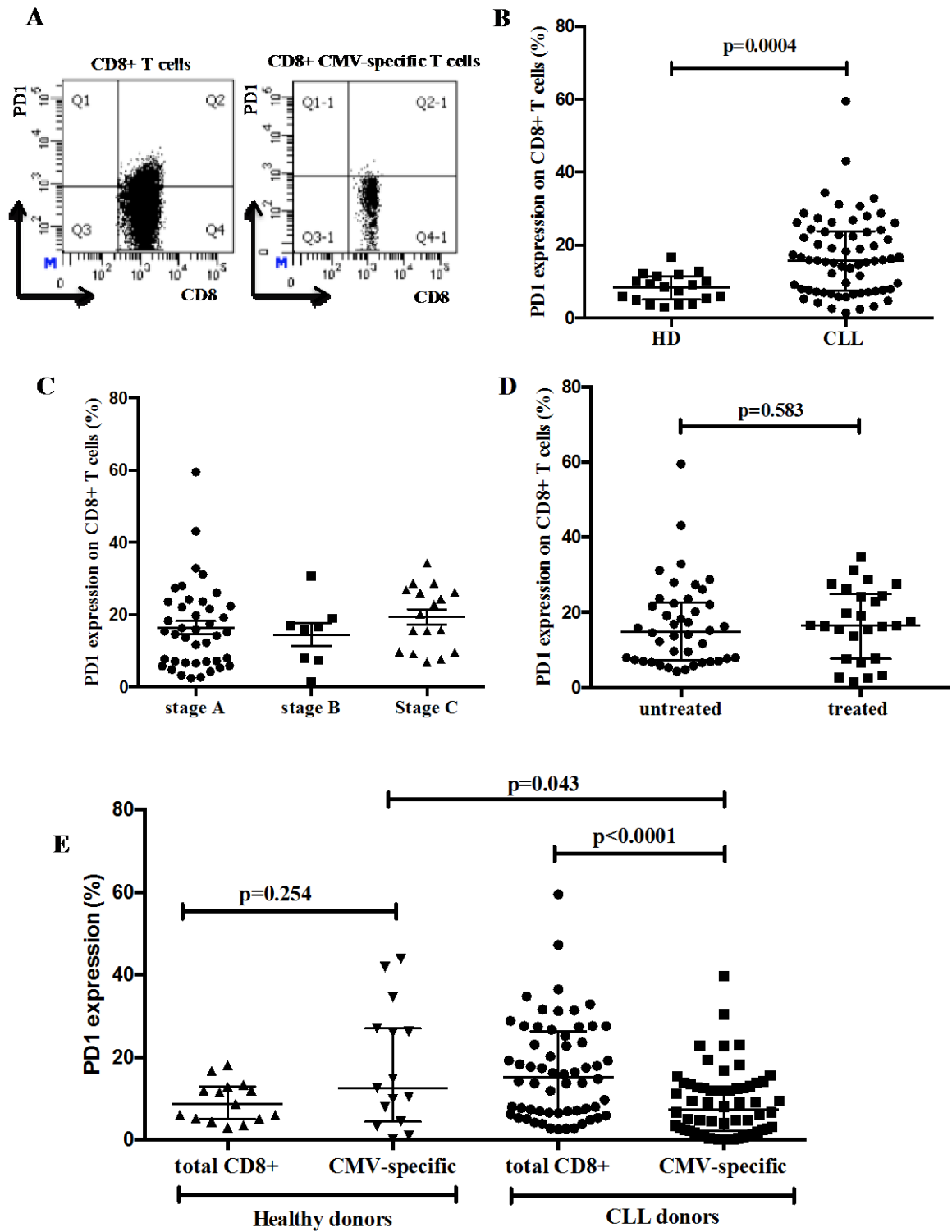
3.3.9 Telomere lengths of CD8+ CMV-specific T cells are reduced compared to the total CD8+ T cell population.

Given the degree of T cell differentiation of CMV-specific CD8+ T cells described in section 3.3.4, I next investigated their telomere lengths as an indication of whether these cells had undergone multiple rounds of cell division and compared these to the total CD8+ T cell population. Somatic cells are known to lose between 50 and 100bp in their telomere lengths with each round of cell division until a critical shortening in length is reached. Once this point is reached, cells undergo apoptosis or become senescent (Hills et al., 2009). Single telomere length analysis, is a single cell PCR technique whereby the XpYp telomere at the end of the chromosome is examined. Multiple single cell telomere lengths from a cell population are used to determine the mean telomere length of the cells of interest.

Here, 3 patients with CLL with known CMV-specific CD8+ T cell responses were identified. PBMCs were then sorted for tetramer positive cells, alongside tetramer negative CD8+ T cells at the University of Birmingham and then transported and analysed at the University of Cardiff by Professor Duncan Baird. CMV-specific CD8+ T cell populations were found to have reduced mean telomere lengths compared to the tetramer negative CD8+ T cell population in patients with CLL. A mean difference of 1.38kb was observed (maximum 1.6kb) (n=3; p=0.06) (Donor 1 CD8: 4.39 kb vs tetramer: 2.79 kb p= <0.0001; donor 2 CD8: 3.42 kb vs tetramer: 3.15 kb p=0.12; donor 3 CD8: 3.25 vs tetramer: 1.89 p= <0.0001). (Figure 3.7).

A

B

	Tet	CD8	Tet	CD8	Tet	CD8
	CLL donor 1		CLL donor 2		CLL donor 3	
Mean (kb)	2.79	4.39	3.15	3.42	1.89	3.25
SD (kb)	1.22	2.17	1.08	1.55	0.59	1.49
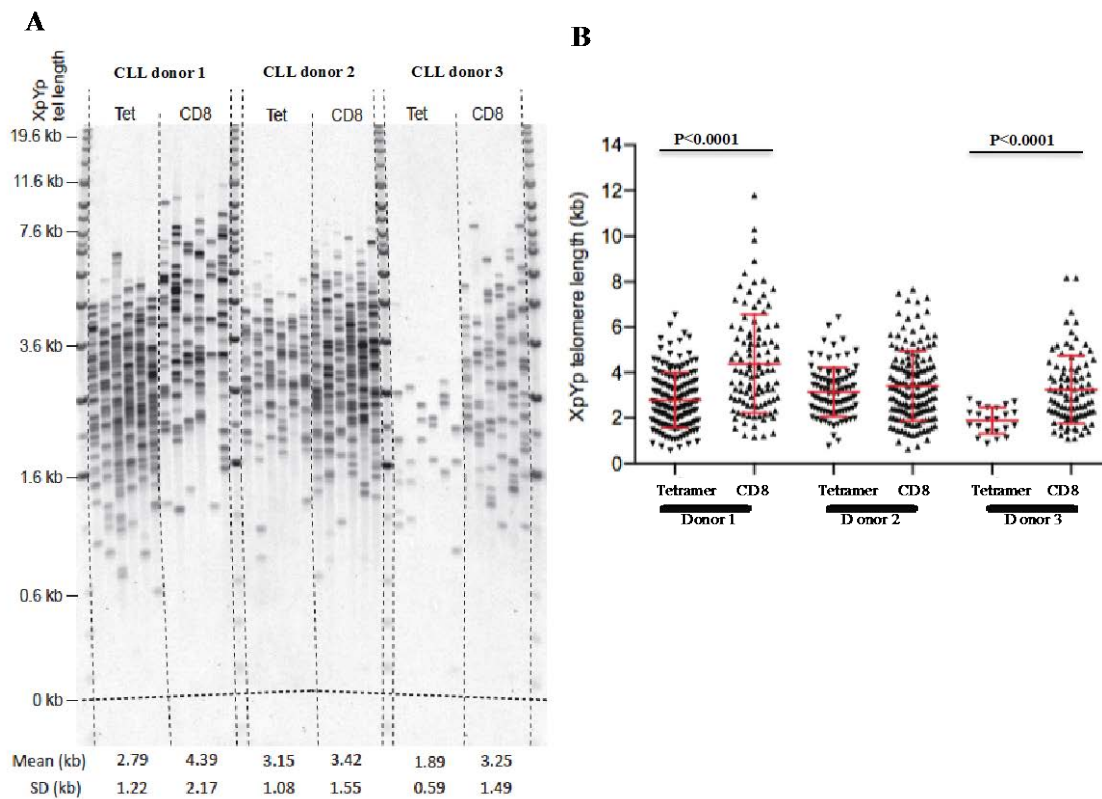
Figure 3.7

Telomere lengths of CD8+ CMV-specific T cells are significantly shorter than CD8+ T cells.

A) A comparison of the telomere lengths from a tetramer population (tet) and the tetramer negative CD8+ T cell population (CD8) of 3 patients with CLL is shown on the gel image. Each vertical line represents a PCR reaction (with a total of 6 PCR reactions per donor) and within these reactions, each band represents the molecular weight of a single telomere from a single cell. The mean and standard deviation is then calculated from the molecular weight of the bands detected. B) graphical representation of the significant difference in molecular weight of the CD8+ CMV-specific cell telomere lengths compared to the total CD8+ T cell population (Student's T test).

3.3.10 CD4:8 ratio

The shortened telomere lengths and effector memory phenotype described in CMV-specific T cells in patients with CLL suggest that CMV-specific T cells have frequently encountered antigen, and contribute to the oligoclonal populations of CD8+ T cells observed. In health, CMV is attributed to reversing the CD4:8 ratio. In order to confirm whether CMV leads to an inverted ratio in patients with CLL, I have examined the CD4:CD8 ratio in an untreated patient cohort. Firstly, no difference was observed in CD4:8 ratio when comparing HD to patients with CLL in these cohorts (HD ratio: 1.16 vs CLL ratio: 1.205; p=0.696) (data not shown). When CLL patients were analysed by CMV status, the CD4:8 ratio did not differ between those CMV positive (1.22) and those CMV negative (1.20) (p=0.534).

Next, the CD4:8 ratio was examined by stage of disease. As chemotherapy can alter the distribution of CD4+ and CD8+ T cells, untreated patients were analysed. Due to low numbers of Binet stage B and C patients who were untreated, these two groups were combined and compared to the CD4:8 ratio of Binet stage A patients. This analysis revealed a significant decrease in the CD4:8 ratio in those untreated patients with more advanced stage disease (Stage A ratio: 1.7 vs Stage B/C ratio: 0.8; p=0.009) (figure 3.8A). Consistent with this, a significant increase in the percentage of CD3+CD8+ T cells were observed with advanced disease compared to patients with Binet stage A CLL (Stage A CD8%: 31.85% vs Stage B/C: 41.90%; p=0.01). However, no difference was observed with CD3+CD4+ T cell percentages and disease stage (stage A: 53.75% vs stage B/C: 44.4%; p=0.113) (figure 3.8B). Finally, the ratio was examined in relation to those patients with CLL who had received treatment and those who were untreated. Interestingly, amongst patients who had been treated, a lower CD4:8 ratio was observed compared to those untreated (untreated ratio 1.43 vs treated 0.77; p=0.0003) (figure 3.8C).
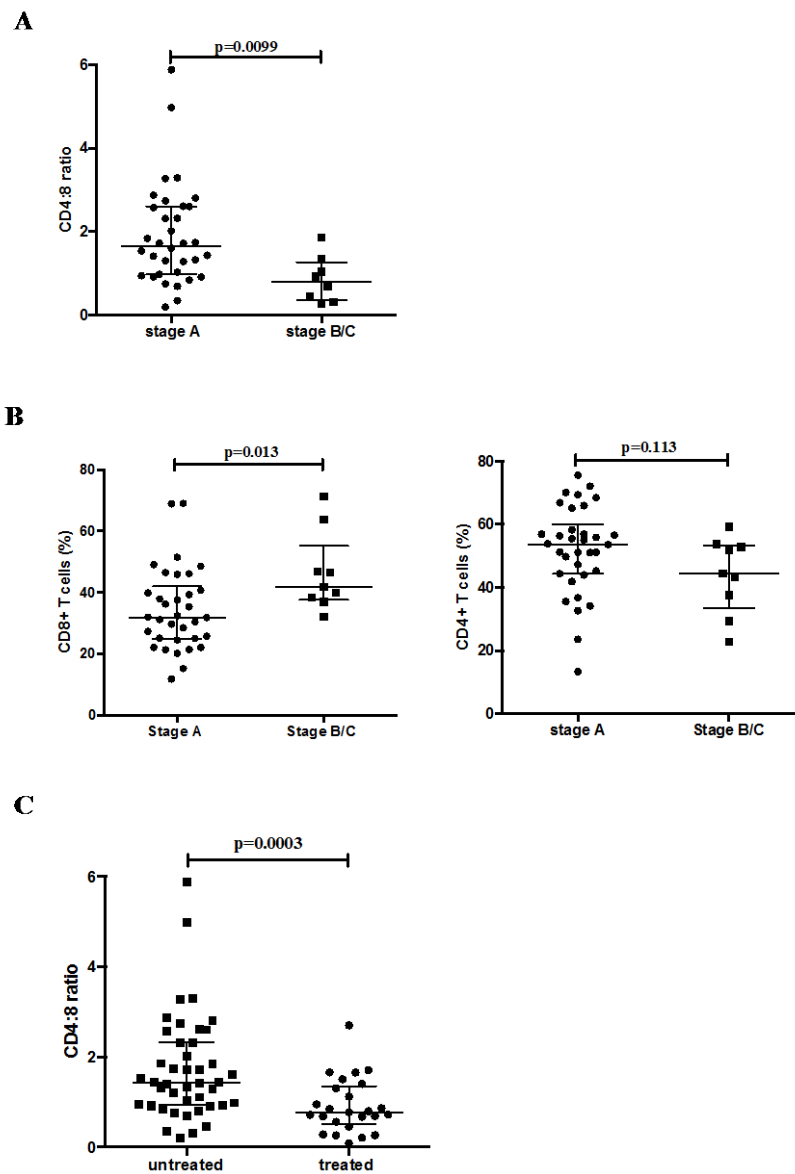
Figure 3.8

CD4:8 ratio analysis

A) demonstrates a dot-plot graph of the CD4:8 ratio by stage of disease. Patients with more

stage B/C disease had a significantly lower CD4:8 ratio than stage A patients. B) An increase

in the percentage of CD8+ T cells was observed in those with more advanced stage disease

and the reverse was true of CD4+ T cells, although this did not reach statistical significance.

C) Patients who had received prior chemotherapy had a significantly lower CD4:8 ratio than

patients who were untreated (Mann-Whitney testing used).

3.4 Discussion

In this chapter, the contribution of the CMV-specific immune response to the total CD8+ T cell repertoire of patients with CLL has been examined in a large cohort. 60 CMV-specific CD8+ tetramer responses (the largest reported to date) were identified in patients with CLL and were used to ascertain the phenotypic contribution of these virus-specific cells to the total CD8+ T cell population.

The term memory inflation is used to describe the expansion of CD8+ CMV-specific T cells, which is observed with ageing (Karrer et al., 2003). This work demonstrates 'memory inflation', with an increased frequency of CMV-specific T cells observed with increasing age when examined both continuously (by correlation with age) and also categorically (in those below and above the age of 80). It has previously been reported that expanded clonal populations of CMV-specific T cells occur in greater frequency than in healthy controls (Mackus et al., 2003). However, no difference in the magnitude of CMV-specific CD8+ T cell responses was found amongst this very large cohort of patients with CLL. It is possible that in this study, the 7 year age difference observed between healthy donors and patients with CLL may account for the lack of difference observed. Despite finding no difference in the frequency of CMV-specific responses amongst patients with CLL and HD, 5 of the CD8+ CMV-specific T cell responses seen in this study were of a greater magnitude than has previously been described in the literature (29.6% of CD8+ T cells being the highest, compared to 14% previously reported) (Mackus et al., 2003).

When CMV-specific T cell responses were analysed by stage of disease, an overall trend was observed for increased frequencies in patients with more advanced stage disease, and a significant difference was observed between stage A and C patients. CD4+ CMV-specific T cell responses have similarly been shown to increase in frequency with progressive disease

(Pourgheysari et al., 2007). Paradoxically, these findings suggest that the gradual impairment in humoral and T cell immunity that arises in patients with CLL, actually contributes to the expansion of CMV-specific CD8+ T cell populations observed. It also suggests that perhaps the expanded populations of CD8+ CMV-specific T cells arise in response to more frequent subclinical CMV reactivation as the host immunity declines with disease progression. In support of this, the immunosenescence observed in healthy elderly people is also thought to lead to an increased frequency of CMV viral reactivation (Stowe et al., 2007).

In agreement with others, this work has shown an expansion of effector memory cells that are re-expressing CD45RA in patients with CLL who are CMV positive (Tonino et al., 2012, Riches et al., 2013). A restriction in the naïve CD8+ T cell pool amongst patients with CLL who are CMV positive was also noted. A similar trend was also observed in healthy donors but due to small sample number of healthy donors did not reach the statistical significance previously reported (Pawelec et al., 2010, Derhovanessian et al., 2011). The expansion of CD8+ T_{EMRA} cells and diminishing T_{naive} populations is thought to lead to immunosenescence and poor response to new antigens observed in those over 50 years of age (Weltevrede et al., 2016) and may also contribute to the poor response to infectious agents seen in patients with CLL and discussed further in chapter 6.

This study has shown that the memory phenotype of CMV-specific CD8+ T cells is split between T_{EM} and T_{EMRA}, with a slightly greater proportion of T_{EMRA} cells observed. This contrasts with that observed in HD, whose CMV-specific CD8+ T cells were over 80% T_{EMRA} in phenotype. Re-expression of CD45RA is generally thought to represent quiescent cells, which have not encountered antigen for a prolonged period (Carrasco et al., 2006). It is of interest therefore that less CD45RA expression was observed in patients with CLL compared

to HD and may suggest more frequent viral reactivation or antigen exposure than that observed in HD.

This work has also confirmed the findings of others that the exhaustion marker PD-1 is increased on the total CD8+ T cell population in patients with CLL (Nunes et al., 2012, Brusa et al., 2013, Riches et al., 2013). It also finds PD-1 expression to be unrelated to stage of disease and therefore is unlikely to be in response to overall tumour burden. Riches *et al* have previously reported this increase in PD-1 on CD8+ T cells in patients with CLL to be attributed to CMV negative donors, and showed that patients that were CMV positive had lower frequencies of PD-1 positive CD8+ T cells. Having now compared CMV positive patients to CMV negative patients in a larger cohort, this work reports no difference in the expression of PD-1 at the level of the CD8+ T cell population.

However, I have shown that CMV-specific CD8+ T cells do have lower frequencies of PD1 expression than the total CD8+ T cell population and also, that this is of a lower frequency than that observed on CMV-specific CD8+ T cells from healthy donors. This corroborates the findings by Te raa *et al,* who used class I tetramers to show that CMV-specific CD8+ T cells have decreased PD1 expression (te Raa et al., 2014).

Given there is low expression of PD-1 on CMV-specific CD8+ T cells, it is surprising overall no difference was observed between CMV positive and negative patients on the expression of PD-1 on the total CD8+ T cell pool. However, the magnitude of CMV CD8+ T cell responses varies greatly between donors and may account for the lack of a difference in PD-1 expression seen amongst the total CD8+ T cell population compared to the low level observed when CMV-specific CD8+ T cells are examined in isolation. Importantly, healthy donors were shown to have higher expression of PD-1 on their CMV-specific CD8+ T cells, a finding previously reported, yet HD also had greater IFN-γ and TNF-α responses to peptide

stimulation (te Raa et al., 2014). This suggests that PD-1 is not present as an exhaustion marker on CD8+ CMV-specific T cells.

The shortened telomere lengths observed in CD8+ CMV-specific T cells suggest these clonally expanded cells with their effector memory phenotype have frequently encountered antigen in patients with CLL. In fact, the telomere lengths recorded for the CMV-specific CD8+ T cells were of such low molecular weight that multiple rounds of cell division had to have occurred for this result. These findings also suggest that CMV-specific CD8+ T cells contribute substantially to the reduced telomere lengths that are reported in patients with CLL compared to healthy age matched controls (Röth et al., 2008).

Interestingly, CMV-specific CD8+ T cells from patients with CLL produced less IFN-γ and TNF-α compared to healthy donors following peptide stimulation. These experiments used unadultered PBMCs to investigate this, in order to reflect likely in-vivo findings. Te Raa *et al*, also described reduced cytokine production in patients with CLL where peptide is presented via CLL cells (te Raa et al., 2014). This finding is unsurprising, as CD8+ T cell function has previously been described to be impaired in patients with CLL and is thought to be due to poor synapse formation and a lack of appropriate vesicle trafficking (Riches et al., 2013). In an attempt to circumnavigate the TCR for stimulation and thus avoid the need for synapse formation, Riches *et al* stimulated CD8+ T cells from patients with CLL with PMA and ionomycin and reported no difference in the total amount of IFN-γ and TNF-α produced in CMV positive patients compared to CMV negative patients and concluded that the capacity for cytokine production was not impaired (Riches et al., 2013). In support of this finding, te Raa *et al* showed that when CMV peptide was presented via LCLs, cytokine production was comparable to that of healthy donor CD8+ CMV-specific T cells and further supports the notion that these CD8+ T cells are not functionally impaired or exhausted in patients with

CLL (te Raa et al., 2014). Despite these published findings, without the modification of B:T cell ratios and aided professional antigen presentation, the in vivo response to CMV peptide is still likely to be impaired based on the experimental data presented here and by others (te Raa et al., 2014) This impaired cytokine production may compromise host immunity towards the virus, potentially increasing the chance of reactivation and contributing to the expanded T cell populations of CMV-specific T cells reported.

Although CMV has been attributed to an inverted CD4:8 ratio, this work found no association with CMV serology and CD4:8 ratio in patients with CLL. However, this work did find that patients with stage B and C disease had lower CD4:8 ratios compared to stage A patients and furthermore found this to be the result of an increased frequency of CD8+ T cells with advancing disease. This supports the work of Nunes *et al*, who found early stage patients were more likely to progress if the CD4:8 ratio was reversed (Nunes et al., 2012). In addition, I have also found that treatment influences the CD4:8 ratio, with those who have received chemotherapy, demonstrating a lower ratio that those who have not. This suggests that following chemotherapy, normalization of the CD4:8 ratio does not occur.

3.5 Conclusion

In conclusion, CMV-specific CD8+ T cells in patients with CLL contribute to the effector memory populations which are greatly expanded in patients with CLL but do not contribute to the increased expression of PD-1 observed on CD8+ T cells in patients with CLL. The impaired cytokine production following peptide stimulation of CMV-specific CD8+ T cells, is likely to be representative of in-vivo findings given the published evidence for poor immunological synapse formation and antigen presentation that exists in patients with CLL and may give rise to more frequent or more protracted episodes of viral reactivation that

further contribute to the memory inflation and shortening of telomere lengths of CMV-

specific CD8+ T cells described here in patients with CLL.

4 CD4+ CMV-SPECIFIC IMMUNE RESPONSE

4.1 Introduction

Acquired defects in T cell function are well described in CLL and contribute to the increased risk of infection and autoimmune phenomena observed in patients with CLL (Scrivener et al., 2001, Hamblin, 2006, Hamblin and Hamblin, 2008). The abnormalities include an inverted CD4:8 ratio, expansions of oligoclonal and terminally differentiated effector CD4+ and CD8+ T cells, changes in gene expression profiles and increased markers of T cell exhaustion and poor immunological synapse formation (Nunes et al., 2012, Frolova et al., 1995, Ramsay et al., 2008, Hamblin and Hamblin, 2008).

Despite an imbalance in the normal CD4:8 ratio, patients with CLL actually have increased CD4+ T cell numbers compared to healthy donors (HD) (Pourgheysari et al., 2010, Riches et al., 2013). Amongst CD4+ T cells, expanded populations of T regulatory cells (Tregs) are observed (Jadidi-Niaragh et al., 2013, Piper et al., 2011), whilst T helper-17 populations are thought to be reduced, particularly in advanced stages of the disease (Piper et al., 2011, Jadidi-Niaragh et al., 2013, Beyer et al., 2005). These findings imply a shift in the balance of CD4+ T cells towards an immunoregulatory phenotype and away from a pro-inflammatory phenotype, but a significant proportion of CD4+ T cells exist that are highly cytotoxic and are capable of producing IFN-γ (Pourgheysari et al., 2010). These cytotoxic cells have been estimated to account for 50% of the CD4+ T cell population in CLL (Porakishvili et al., 2004). Functionally, CD4+ T cells in patients with CLL display a range of abnormalities, including elevated levels of intracellular IL-4 and TNF-alpha (Mu et al., 1997, Mainou-Fowler et al., 2001), but perhaps the most striking abnormality amongst the CD4+ T cell pool is the oligoclonality and expansion of CD4+, CD57+, CD28- cells (Porakishvili et al., 2004). Oligoclonality has been shown through restricted T cell receptor V beta gene usage and is more prominent amongst CD4+ T cells compared to CD8+ T cells (Rezvany et al., 1999).

Furthermore, these expanded oligoclonal populations of CD4+ T cells are also less responsive to stimulation *in vitro* with autologous leukaemic B cells compared to CD8+ T cells (Rezvany et al., 2003). T cells expand clonally in response to recognition of cognate antigen and although this has led to speculation that tumour-derived antigens may be driving these oligoclonal CD4+ populations, little evidence currently exists to support this notion.

As described in chapter 1, CLL affects predominantly people in their later decades, at an age where thymic involution has occurred and reactivation of latent viruses such as varicella zoster becomes increasingly common. It is also an age at which approximately 70% of patients within Western Europe and USA would be latent carriers of CMV (Parry et al., 2016a). As such, chronic viral infection may represent one factor that acts to drive the accumulation of the increased populations of CD4+ T cells seen in patients with CLL. Previous work investigating the CMV-specific CD4+ T cell response in patients with CLL have indeed found it to be 4 fold higher than that of healthy age matched individuals and this response is further increased with disease progression and in response to chemotherapy (Pourgheysari et al., 2010). These expanded populations of CMV-specific CD4+ T cells are known to be of a differentiated memory subtype, predominantly effector memory in phenotype and express high levels of perforin (Walton et al., 2010). Little is known about their transcriptional profile or exhaustion marker status (Pourgheysari et al., 2010, Walton et al., 2010, Fletcher et al., 2005).

T cell 'exhaustion' is a term used to describe T cell dysfunction that results from persistent antigen exposure and was first described in the context of chronic viral infections but more recently has been described in relation to the immune response to malignancies, including melanoma, lung cancer and leukaemias (Turnis et al., 2015). Exhausted T cells are characterized by expression of multiple inhibitory receptors, low CD57 expression and NK

cell markers and poor functionality (Wherry and Kurachi, 2015). One important inhibitory receptor is programmed cell death one (PD-1), which is increased on CD4+ T cells in the context of both chronic viral infections and CLL (Riches et al., 2013, Wenjin et al., 2012, Rusak et al., 2015). Following engagement with its ligand (PDL1 or PDL2), PD-1 activation serves to inhibit T cell activation and effector function, and may facilitate tumour or viral escape from immunosurveillance (Wherry, 2011). In patients with CLL, both CD4+ and CD8+ T cells have increased PD-1 expression compared to healthy age matched controls (Brusa et al., 2013, Rusak et al., 2015) and recently, the use of PD-1-blocking antibodies has shown therapeutic potential (McClanahan et al., 2015, Gassner et al., 2015), with recruitment into a phase 2 trial underway for patients with CLL (clinicaltrials.gov, 2016).

Given the high prevalence of latent CMV infection and its dominant impact on the immune repertoire, CMV remains a significant confounding factor in any T cell immunophenotyping results for patients with CLL and in particular for characterizing its contribution towards CD4+PD-1+ cell populations. As discussed in chapter 3, CD8+ T cell abnormalities have been the focus of attention in patients with CLL and the use of class I tetramers to identify CMV-specific CD8+ T cells in patients with CLL has found preserved functionality and low levels of PD-1 expression, such that CMV-specific CD8+ T cells appear relatively unaffected considering the dysfunctional CD8+ T cell abnormalities described in patients with CLL (te Raa et al., 2014, Riches et al., 2013, McClanahan et al., 2015).

In comparison, the CD4+ CMV-specific immune response in CLL is relatively understudied. Using novel class II CMV tetramers, this work aims to identify, for the first time, populations of CMV specific CD4+ T cells in both patients with CLL and healthy donors and assesses their phenotype and transcription factor profile. Information derived from these studies will

evaluate the impact of CMV-specific CD4+ T cells on the global CD4+ T cell dysfunction seen in patients with CLL compared to healthy donors.

4.2   Methods

4.2.1   Patient recruitment.

146 patients with CLL attending outpatient clinics at either University Hospital Birmingham or Birmingham Heartlands Hospital were recruited for study. All patients were either untreated or at least six months post treatment. A maximum of 50ml of blood was taken at each outpatient visit in lithium heparin.

For healthy donor recruitment, 61 participants aged 65 or above were enrolled. Samples were prepared using ficoll-paque gradient centrifugation and cryopreserved as described in chapter 2. Samples were then defrosted prior to use.

4.2.2   CMV ELISA and HLA typing

CMV status was confirmed in HD and patients using the ELISA technique described in chapter 2. DNA was extracted from pelleted whole PBMC and HLA class II typing performed by PCR.

4.2.3   CD4 CMV specific T cell immunophenotyping

PBMCs were defrosted and stained as described in chapter 2. The following antibody panels were then used in this chapter: panel 2 for analysis of memory phenotype, panel 3 for analysis of activation markers and panel 8 for analysis of cytotoxicity. For statistical analysis, results are expressed as the median and Mann-Whitney testing was performed for comparative analysis of non-parametric data, unless indicated in the figure legends.

4.2.4   Peptide stimulation assay

Following identification of a DR7 or DR52B tetramer response, $2x10^6$ cells were incubated at

37^oC for 6 hours with either 0.5μg/μl DYSNTHSTRYV (DYS) peptide or

AGILARNLVPMVATV (AGI) 0.5μg/μl respectively. Tetramer and antibodies (both surface

and intracellular) were then applied, as described in chapter 2. For analysis of cytokine

responses, SPICE version 5.3 (downloaded from http://exon.niaid.nih.gov/spice 28th January

2016) was performed.

4.2.5   Intracellular staining for transcription factors

1×10^6 PBMCs were defrosted, washed and stained with tetramer, followed by live/dead stain

as above. Antibody panels 9 and 10 were applied.

4.2.6   Telomere length analysis

$20x10^6$ PBMCS were defrosted. CD4+ T cell and tetramer positive cells were enriched as

described in chapter 2 on the MoFlo™ sorter. A purity of 97% tetramer positive cells and

98% CD4+ tetramer negative cells was obtained. Samples were then shipped on dry ice as a

semi-dry pellet to the University of Cardiff for further analysis. STELA telomere length

analysis with single cell PCR amplification of the XpYp telomere region was obtained as

previously described (Baird et al., 2003). Student's T test was then used to compare the

molecular weight of the PCR product between tetramer positive and background CD4+ T

cells.

4.3   Results

4.3.1   Donor characteristics

Of the 146 patients recruited, 47 were found to be both CMV seropositive and to have the

appropriate HLA alleles that permit examination of the CMV specific CD4+ T cell response

with the two novel tetramers available. A total of 15 responses were found, 10 from patients recognising the peptide DYS derived from glycoprotein B and 5 recognising the peptide AGI from pp65. The median age of the patients with CLL with identifiable tetramer responses was 77 years (IQR 54-93). Using the same tetramers and for comparative analysis, 11 healthy donors (HD) were also identified to have CD4+ CMV specific T cell responses (8 gB and 3 pp65 responses), with a median age of 61 years (IQR 51-86). The characteristics are shown in table 4.1.

4.3.2 The magnitude of the CMV specific-CD4 T cell responses in patients with CLL is greater than in healthy donors

The CMV specific CD4+ T cell response was identified and compared between patients with CLL and healthy donors (HD). Figure 4.1A shows an example of staining for both DYS and AGI tetramers. A true tetramer response was verified through the lack of background staining by gating all CD3+ T cells, against CD4+ T cells. The magnitude of CD4+ CMV-specific T cell responses was found to be greater in patients with CLL compared to HD (HD 0.7% (range: (0.01-8.6%) vs CLL 4% (range: 0.1-37.7%); p=0.049) Figure 4.1B.

Table 4.1

Clinical characteristics of CLL patients with CMV-specific CD4+ T cell responses

Median age at diagnosis (years)	77 (IQR 54-93)
Male sex	11/15 (73%)
Follow up time at the point of sample collection (years)	7.75 (IQR 3.8-14)
Previous history of treatment	7 (47%)
Time since treatment finished (years)	1.1 (IQR 0.5-11)
Binet stage A	6 (40%)
Binet stage B	1 (7%)
Binet stage C	8 (53%)
Missing	0
Number of deaths	6 (40%)
CD38 positive	4 (27%)
CD38 negative	5 (33%)
CD38 missing	6 (40%)
IGHV mutated	1 (7%)
IGHV unmutated	6 (40%)
IGHV missing	8 (53%)
FISH	
Normal	8 (53%)
13q-	2 (13%)
12+	1 (7%)
11q-	1 (7%)
17p-	1 (7%)
Other	
Missing	3 (20%)

Figure 4.1

An example of AGI and DYS tetramer staining and the magnitude of the CMV-specific CD4+ T cell response in patients with CLL and HD.

A) An example of tetramer staining is shown for both AGI (left) and DYS (right). Cells displayed are CD3+ T cells. B) The frequency of CMV-specific CD4+ T cells are increased in patients with CLL compared to HD (HD: 0.7% Vs CLL: 4%; p=0.049) (Mann-Whitney).
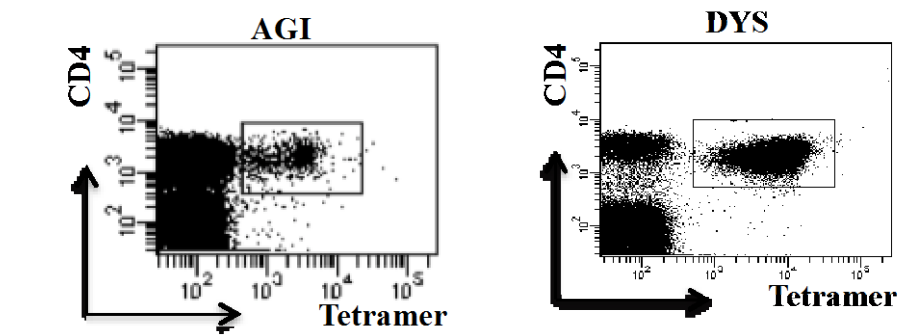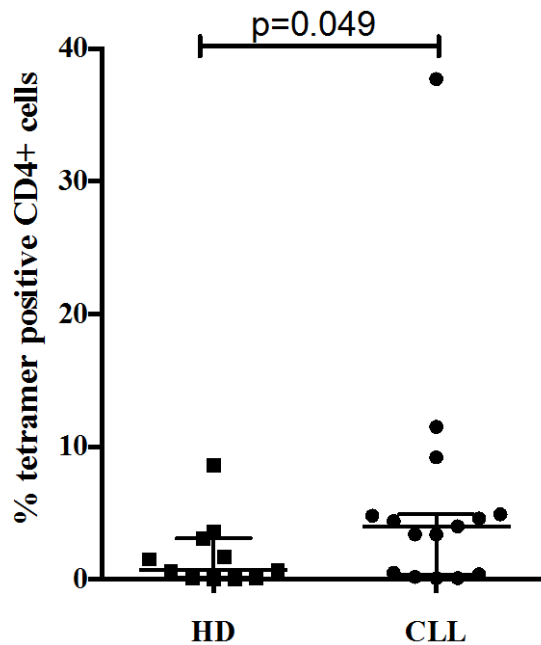
4.3.3 CMV specific CD4+ T cells have an effector memory phenotype

Next, the phenotype of the CMV specific CD4+ T cells was investigated in HD and patients with CLL. Using CCR7 and CD45RA, memory subsets were defined as naïve (CCR7+, CD45RA+), central memory (T_{CM}) (CCR7+, CD45RA-), effector memory (T_{EM}) (CCR7-, CD45RA-) and terminally differentiated effector memory cells (T_{EMRA}) (CCR7-, CD45RA+). An example of the staining can be seen in Figure 4.2A. Firstly, CD4+ T cell memory subsets were compared between CMV positive (n=68) and CMV negative (n=27) patients with CLL and in HD (CMV positive n=20; CMV negative n=10). A trend towards a greater frequency of T_{EM} cells was seen in patients with CLL who were CMV positive compared to those who were CMV negative, although this did not reach statistical significance (CMV negative T_{EM}: 50.3% Vs CMV positive T_{EM} 53.6%; p=0.210). No difference was observed in T_{naive} or T_{CM} phenotypes when examined by serostatus in both HD and patients with CLL. The only significant difference was observed between cells of T_{EMRA} phenotype, which were increased in both HD and patients with CLL in CMV positive subjects compared to CMV negative individuals (HD: 0.95% Vs 2%; p= 0.042 and CLL: 1.1% Vs 3.3%; p=0.0009 (figure 4.2B +4.2C). However, the percentage of CD4+ T cells of T_{EMRA} phenotype overall (regardless of CMV serostatus) was much lower in both HD and patients with CLL compared to that seen in CD8+ T cells (as reported in chapter 3) (HD CD4+:1.35 vs CD8+ 29.5% and CLL CD4+ 2.6% vs CD8+ 35.10%) (data not shown).

Next, tetramer responses in both HD and patients with CLL were examined and found to predominantly be T_{EM} in phenotype compared to T_{EMRA} (HD: T_{EM} vs T_{EMRA} 91.2 vs 0.6%; p<0.0001 and CLL: T_{EM} 94.4% VS T_{EMRA} 1.4%; p=0.0003 (figure 4.3D + 4.3E). This contrasts with CD8+ CMV-specific T cell populations, whose frequency was described in chapter 3 and was found to be equally distributed between a T_{EM} and T_{EMRA} phenotype.

Interestingly, no difference was observed between the CD4+ T cell population and CMV specific CD4+ cells in patients with CLL but this may in part be due to the fact that patients with CLL already have expanded populations of T_{EM} cells compared to HD, regardless of CMV status (HD 29.70% T_{EM} Vs CLL 52.9% T_{EM}; p=0.0003) (data not shown). Amongst HD, an increased proportion of CMV-specific CD4+ T cells with T_{EM} phenotype were observed compared to the background CD4+ T cell population (CD4+ T_{EM} cells: 35.2% Vs HD tetramer+ T_{EM} cells 91.2%; p=0.001 Figure 4.3E).

Figure 4.2

CMV specific CD4+ T cells predominantly have an effector memory phenotype

(A) Example gating for memory subset phenotyping of CD4+ T cells (red) and CMV-specific CD4+ T cells (black) is shown (B) The memory phenotype of CD4+ T cells in patients with CLL did not differ greatly between CMV seropositive (n=69) and seronegative donors (n=27), other than a significant but overall small increase in the percentage of cells with a T_{EMRA} phenotype in CMV positive patients. (C) Demonstrates the memory phenotype in CD4+ T cells in healthy donors who were CMV positive and CMV negative by serology, with a greater proportion of T_{EMRA} cells seen in those who were CMV positive. In both healthy donors and in patients with CLL, a comparison of CMV specific CD4+ T cells with the total CD4+ T cell population, revealed an increase in cells with a T_{EM} phenotype amongst tetramer positive cells. This did not reach statistical significance in patients with CLL, which is most likely due to the fact that patients with CLL already have a CD4+ T cell repertoire that is enriched with cells of T_{EM} phenotype (D) but did reach significance in healthy donors (p=0.001) (E) (Mann-Whitney).
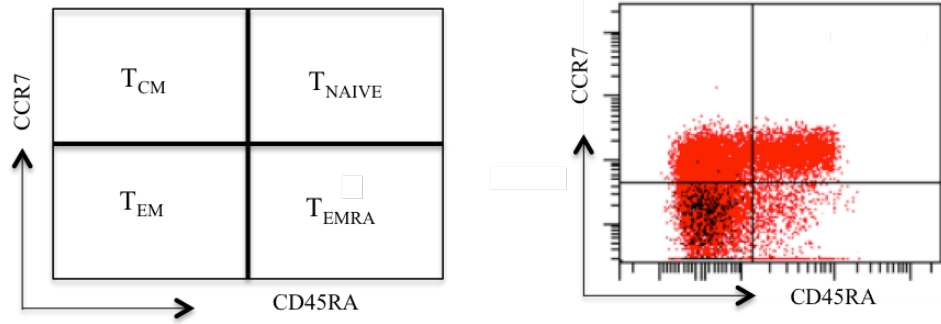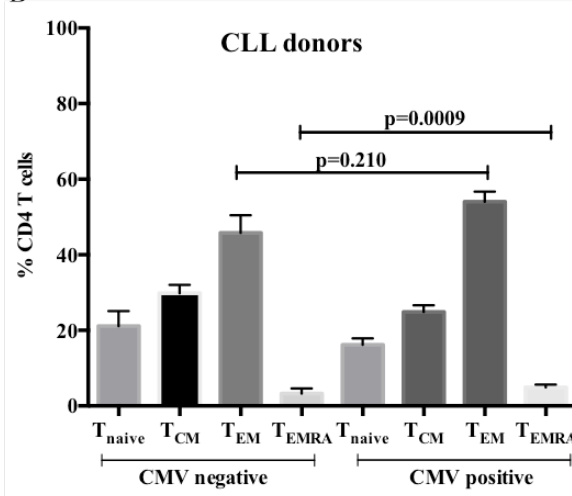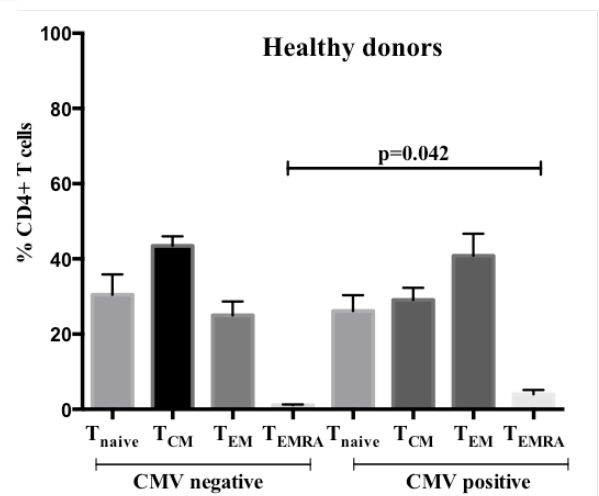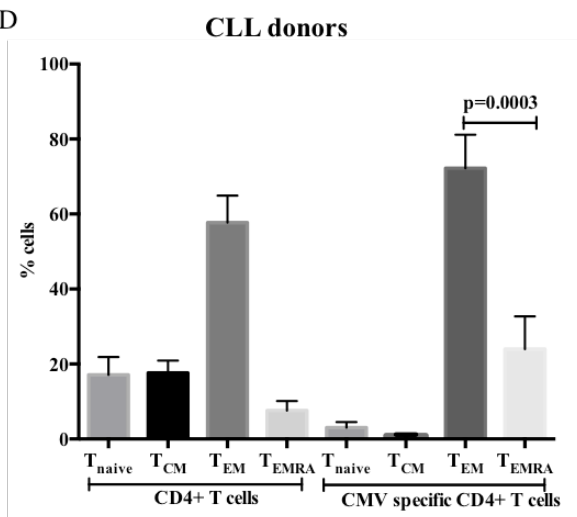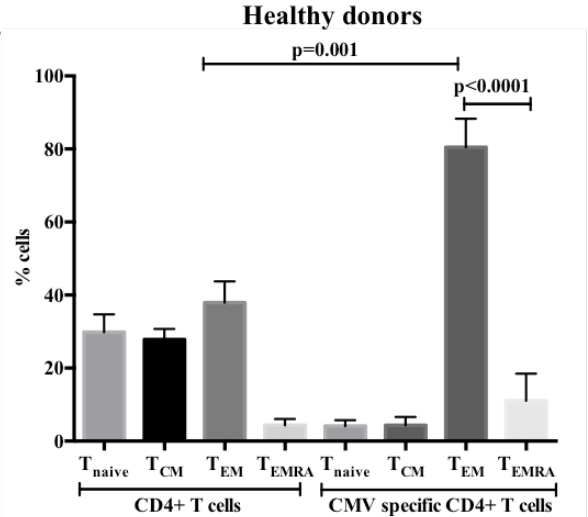
4.3.4 CMV-specific CD4+ T cells demonstrate increased expression of CD38 in patients with CLL.

I next went on to examine the expression of activation markers on CMV-specific T cells in both HD and patients with CLL. CD69 is a C-type lectin receptor, which is amongst the first protein to be expressed following T cell activation and is important for lymphocyte proliferation following antigen recognition. It is only transiently expressed on activated CD4+ T cells. In contrast, CD38 is another transmembrane glycoprotein receptor induced through T cell activation, but has a maximum expression 14-17 days following T cell activation (Chadburn et al., 1992). Immunophenotyping for CD69 and CD38 on CD4+ T cells was performed on 10 HD and 10 patients with CLL. No difference was observed in CD69 expression on CD4+ T cells in HD or patients with CLL (HD: 2.6% Vs 4.3; p=0.197). A low frequency of CD69 was also observed on CMV-specific CD4+ T cells in both HD and patients with CLL (HD median 3.8% Vs 4.2% in CLL; p=0.54). This was further confirmed by MFI (HD 734 Vs CLL 733; p=0.58). No difference was observed between CMV-specific cells and CD4 T cells in HD (p=0.255) or patients with CLL (p=0.565).

A trend towards a higher frequency of CD38 expression was found on CD4+ T cells from patients with CLL (HD: 13.7 Vs CLL: 18.2; p=0.342) but was even more pronounced on CMV-specific CD4+ T cells from patients with CLL (HD: 13.6% Vs. CLL: 36.6%, p=0.014). This was further confirmed by MFI (HD 422 Vs. CLL 858, p=0.052). Interestingly, one donor with CLL demonstrated a CMV-specific CD4+ T cell response that comprised 37.7% of the CD4+ T cell repertoire and 75% of this population were found to be CD38 positive. This profile was observed in the whole cohort where CMV-specific CD4+ T cells had greater CD38 expression than the total CD4+ T cell population (p=0.006).
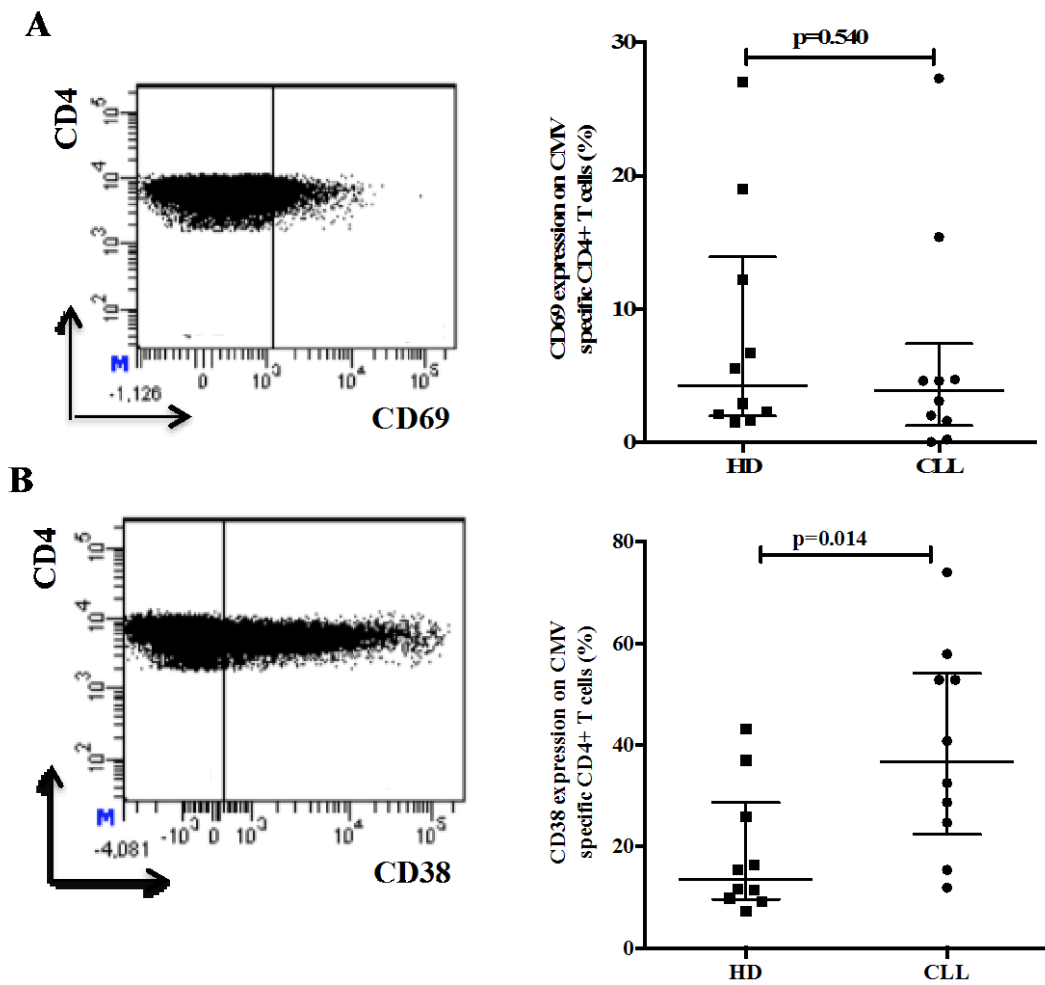
Figure 4.3

CD38 expression is increased on CMV-specific T cells, particularly in patients with CLL.

CD69 expression was examined on CMV-specific T cells and found to be of low frequency on CMV-specific T cells in both HD and patients with CLL (A). CD38 expression was also examined as a marker of activation and was found to be higher on CMV- specific CD4+ T cells in patients with CLL compared to HD (B) (Mann-Whitney).

4.3.5 Functional response of tetramer-specific T cells following CMV peptide stimulation.

In order to examine the cytokine profile of CD4+ CMV-specific T cells, experiments were performed firstly to ascertain the optimal stimulation conditions. Initial attempts to stimulate the cells using PMA and ionomycin lead to reductions in the frequency of tetramer positive staining, even at low concentrations. Te Raa *et al* had recently published their method for studying CD8 CMV-specific T cell functionality using peptide stimulation (te Raa et al., 2014). This method was adopted but optimized for CD4+T cell work. Variables that I investigated included the optimal peptide concentration, whether peptide or lysate stimulation of CD4+ T cells could produce superior cytokine production, the use of dasatinib during incubation and whether the order with which tetramer and peptide were added produced different results. Aside from titrating peptide concentrations, all optimisation experiments were carried out using a young HD with a known DYS response. The results of the experiments used to assess these variables are as follows:

4.3.5.1 Peptide concentration

In order to ascertain the optimal concentration of peptide at which surface tetramer staining was observed alongside maximum cytokine production, a single healthy donor was identified with either DYS or AGI tetramer response and used for each titration experiment. PBMCs were stimulated using DYS or AGI peptide at concentrations shown in figure 4.4 for 6 hours at 37°C prior to tetramer staining. Cytokine response was measured as the percentage of double positive CMV-specific CD4+ T cells producing IFN-γ and TNF-α. For both tetramers, the optimal concentration was determined as shown in figure 4.4. With an increasing peptide concentration greater than 0.5µg/ml, the percentage of surface tetramer staining was reduced, resulting in an overall decrease in the percentage of cytokine producing CMV-specific T cells.

In contrast, peptide concentrations less than 0.5μg/ml preserved the frequency of positive tetramer staining but resulted in poor stimulation and a suboptimal cytokine response. For each peptide a concentration of 0.5μg/ml was confirmed to be optimal and used in all subsequent experiments (figure 4.4).
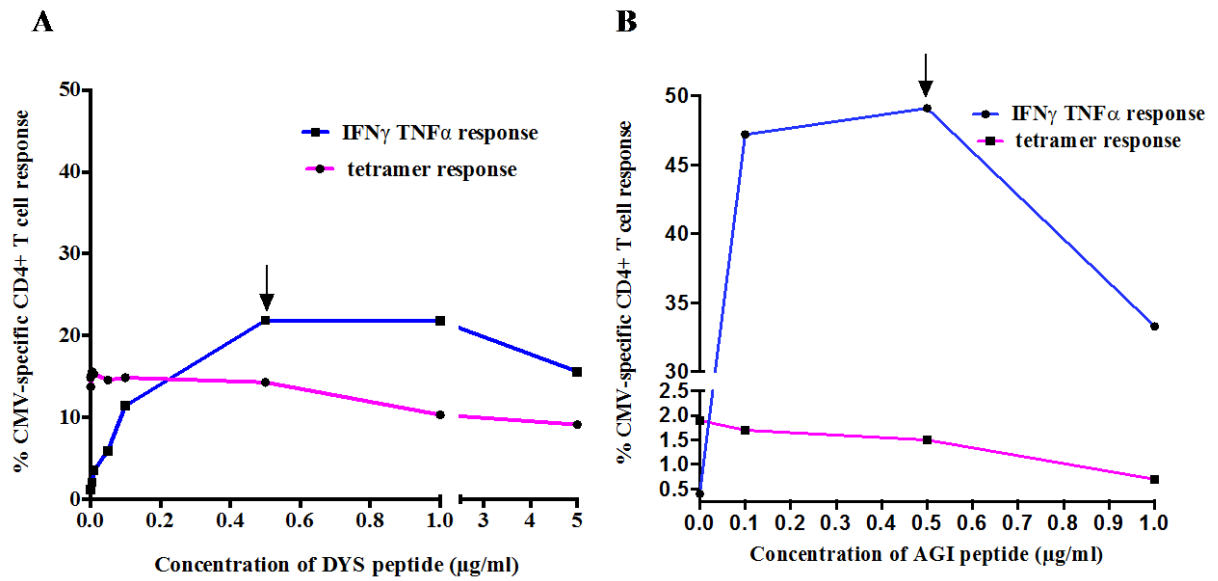
Figure 4.4

DYS and AGI peptide concentrations were titrated for optimal stimulation response.

Using a range of DYS peptide concentrations, CMV-specific CD4+ T cells were identified

from the same donor and their CMV response assessed. The optimal peptide concentration

was found to be 0.5µg/ml, which produced a maximal cytokine response of 22%, whilst

maintaining a tetramer response of 14% (the same as that found in an unstimulated sample).

Similarly, AGI was also titrated and again, 0.5µg/ml was found to be optimal. This produced

a maximum cytokine response of 49% of the tetramer positive cells, whilst maintaining the

tetramer population at 1.5% (79% of CMV-specific T cells were still identifiable, as the

unstimulated sample demonstrated a 1.9% tetramer response).

4.3.5.2 Lysate or peptide stimulation

Next, a comparison of lysate stimulation compared to peptide stimulation for tetramer specific assessment of cytokine release was performed. Using fresh HD cells, a 6 hour 0.5μg/ml DYS peptide or lysate stimulation was compared to unstimulated cells. A similar percentage of tetramer positive cells were identified using either method (peptide stimulation: 1.09% Vs. 1.04% lysate stimulation). Peptide stimulation was found to be slightly superior, with 79.9% of tetramer+ cells producing IFN-γ and TNF-α compared to 78.6% in lysate stimulation (table 4.2). Peptide stimulation rather than lysate stimulation was then used for future experiments.

4.3.5.3 The addition of tetramer before or after peptide stimulation

Using the same HD, but with previously cryopreserved cells, a comparison of the order with which tetramer was added to the stimulation assay was compared with unstimulated cells. Unstimulated cells, with the tetramer added just prior to surface staining resulted in a tetramer response of 2.07% and no IFN-γ TNF-α production. In contrast, cells incubated first with tetramer for 1 hour and then washed and rested for 6 hours (without further peptide stimulation) resulted in only 1.6% of tetramer cells being identified but of these, 10.25 % produced TNF-α but no IFN-γ, This demonstrated that the tetramer binding to the TCR was able to stimulate a small but significant amount of cytokine production in the absence of peptide stimulation. Adding tetramer first for an hour at 37 °C followed by 6 hour incubation with 0.5μg/ml DYS peptide resulted in 77.6% of tetramer+ cells producing IFN-γ and TNF-α with 1.06% tetramer cells detectable. In comparison, performing the 6 hour peptide stimulation before adding tetramer produced 72.3% IFN-γ and TNF-α, but with 1.8% tetramer positive cells identified (table 4.2). These results suggested that the addition of tetramer first produced a greater stimulation period but this resulted in greater downregulation of the TCR.

Adding the tetramer after the 6 hour stimulation, improved the percentage of tetramer cells detectable but also provided no detection of cytokines in the unstimulated control sample. It was therefore decided to incubate with peptide prior to surface staining with tetramer and fixation.

4.3.5.4 The use of Dasatinib

Dasatanib is a reversible protein kinase inhibitor that has been shown to reduce the affinity threshold at which tetramer binds to the TCR and inhibit tetramer-induced lymphocyte cell death, whilst improving the intensity of staining (Lissina et al., 2009). To investigate if dasatinib could enhance the ability to capture all tetramer positive cells during a peptide stimulation assay, cells from a HD were incubated with or without 1μM of dasatinib for 30 minutes at 37°C, followed by tetramer staining and then a 6 hour stimulation with 0.5 μg/ml of DYS peptide at 37°C. Results were compared with an unstimulated sample. Dasatanib was found to maintain the frequency of tetramer positive cells to that seen in the unstimulated sample (2% for unstimulated, 2% for stimulated with dasatanib and 1.2% for stimulated without dasatanib). However, a distinct reduction in the production of IFN-γ and TNF-α was found compared to cells stimulated in the absence of dasatanib (70% IFN-γ when stimulated without dasatanib vs 11% in the presence of dasatanib). Therefore, although tetramer staining was preserved, the addition of dasatanib was not found to enhance the experimental data due to the poor cytokine staining and was therefore omitted from further experiments.

4.3.5.5 The conclusions from optimisation experiments

Minimal difference was noted between lysate and peptide stimulation for cytokine production using fresh cells. This, together with the need for fresh cells for lysate stimulation compared to cryopreserved cells for peptide stimulation resulted in a decision to use peptide stimulation

for future stimulation experiments. The use of defrosted cells was also shown to be comparable to fresh cells for cytokine responses using peptide stimulation (although of note, this comparison was made from two separate experiments carried out on different days.) Adding tetramer first prior to peptide stimulation was found to initiate cytokine production and also diminish tetramer responses further, so a decision to add tetramer after peptide stimulation was made. The addition of dasatanib did not enhance the experiment and in fact impaired cytokine responses, so was discarded from further experiments. Finally, a peptide stimulation with 0.5μg/ml was found to be optimal for both types of CD4+ CMV-specific T cells.

Table 4.2

Optimisation of CD4+ CMV-specific cell staining following stimulation.

Experiment	Cell type	Tetramer %	Tetramer IFN-γ TNFα %
Unstimulated	fresh	1.87	0
Lysate Stimulation	fresh	1.04	78.6
Peptide stimulation	fresh	1.09	79.9
Unstimulated. Tetramer added just before surface staining	defrosted	2.07	0
Tetramer added then 6 hours incubation without peptide	defrosted	1.6	10.25
Tetramer added then 6 hr incubation with peptide	defrosted	1.06	77.6
6 hour incubation with peptide then tetramer added	defrosted	1.8	72.3
Unstimulated with dasatanib	defrosted	2	0
Peptide stimulation without dasatanib	defrosted	1.2	70
Peptide stimulation with dasatanib	defrosted	2	11

4.3.6 CMV-specific CD4+ T cells have a Th1-skewed cytokine profile

Following on from the optimization experiments, and to assess the functionality of CMV-specific CD4+ T cells in patients with CLL and HD, a 6 hour peptide stimulation at 37°C was performed prior to tetramer incubation and antibody staining. CMV-specific T cells are described in the literature to have a Th1 phenotype and make TNF-α and IFN-γ (Edwards et al., 2014) Therefore these cytokines were chosen to identify any Th1 response. T cell production of Il-4 and serum levels of Il-10 are increased in patients with CLL, so these cytokines were chosen in order to evaluate if CMV-specific CD4+ T cells could be Th2 skewed and contribute to this finding (Mainou-Fowler et al., 2001, Mu et al., 1997, Fayad et al., 2001). The Th2 cytokine, Il-5 was also chosen, as a recent article had detected low-level production amongst CD4+ T cells responding to CMV peptide in healthy donors (Mason et al., 2013). For each experiment, an unstimulated control was incubated for 6 hours at 37°C in the absence of any peptide stimulation. Tetramer staining was added to all samples prior to surface staining. Minimal expression of cytokines was seen in unstimulated tetramer-positive cells but, if this was detected, this percentage was subtracted from the percentage expression found in stimulated cells. This subtraction allowed only the inducible cytokine production following peptide stimulation to be ascertained, which is presented in Figure 4.5. Overall, the proportion of CD4+ CMV tetramer+ T cells producing both IFN-γ and TNFα was decreased in patients with CLL (n=9) compared to HD (n=8) (21% vs 47.6%; p=0.0037. Figure 4.5) suggesting CLL cells are suboptimal at producing cytotoxic cytokines following induced stimulation. Most CMV-specific cells that produced IFN-γ also produced TNF-α, only 0.2% of CMV-specific cells from patients with CLL and 2.2% of HD CMV-specific cells made IFN-γ in the absence of TNFα. In contrast to this finding, TNFα was frequently produced in the absence of IFN-γ and this was most notable in patients with CLL. In HD, on average 2 %

of CMV-specific cells were seen to make TNF-α only, whilst amongst patients with CLL, 11.5% produced TNF-α alone (p=0.056). A small percentage of CMV-specific cells in healthy donors (0.7%) and in patients with CLL (3.7%) were found to also produce Il-4, with no difference observed between HD and patients with CLL (p=0.595).
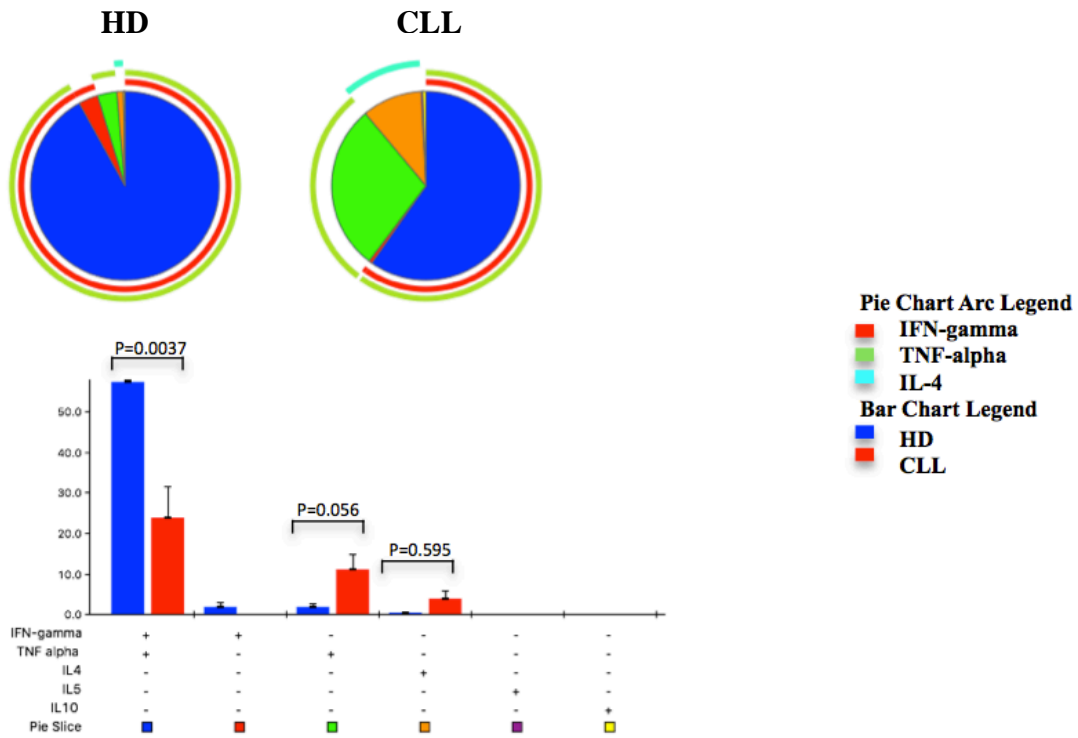
Figure 4.5

The cytokine profile of peptide stimulated CMV-specific CD4+ T cells

Using Spice software, plots of HD (left) and CLL donors (right), consisting of pie charts representing the percentage of cytokine expression of all subjects studied and by graphically representation underneath where coding for the pie slice colours can also be found. The arcs around the pie charts represent the total percentage production of each individual cytokine (see coding within figure). Within the bar chart HD are shown in blue and patients with CLL in red. Production of both IFN-γ and TNF-α by CMV-specific CD4+ T cells was greater in HD than patients with CLL, whilst patients with CLL had more CMV-specific cells producing just TNF-α (Mann-Whitney). A small amount of IL-4 from CMV-specific cells was made by both HD and patients with CLL.

4.3.7 CMV-specific CD4+ T cells have a cytotoxic phenotype with a high frequency of perforin and granzyme B.

The expression of perforin and granzyme B was then examined in CD4+ T cells and CMV-specific CD4+ T cells. Cells were analysed for single positive granzyme or perforin staining, as well as a dual positive expression pattern. Figure 4.6A shows an example of the staining observed using flow cytometry and Table 4.3 shows the median frequency of cells positive for these cytotoxic granules in both HD and patients with CLL.

The frequency of dual positive staining for perforin and granzyme B was found to be greatest in CMV-specific CD4+ T cells. Amongst the total CD4+ T cell populations, the frequency of dual positive staining was less, with no difference being observed in the proportions of granzyme B and perforin positive cells in patients with CLL compared to HD (median 21.2% in HD and 17.5% in CLL donors, p=0.618). Comparatively, the majority of CMV-specific CD4+ T cells displayed a significantly higher proportion of both granzyme B and perforin compared to the total CD4+ T cell population (84.75% in HD (p=0.0001) and 79.05% in patients with CLL (p=0.009). No difference was observed between HD and CLL CMV-specific CD4+ T cells (p=0.362). These findings highlight the pronounced cytotoxic capacity and potential for cell lysis by CMV-specific CD4+ T cells in both HD and patients with CLL (Figure 4.6B).

Table 4.3 The percentage expression of perforin and granzyme

	HD CD4+ T cell	CLL CD4+ T cells	HD CMV-specific CD4+ T cells	CLL CMV specific CD4+ T cells
Perforin +	1.1 (0.18- 3.45)	0.3 (0.08-1.30)	0.95 (0.07-3.05)	0.1 (0.00-3.05)
Perforin and granzyme +	20.15 (6.27- 31.93)	17.9 (7.50-52.03)	79.05 (27.33-94.08)	84.75 (69.98- 92.38)
Granzyme +	5 (0.98-38.63)	7.50 (3.98-27.80)	5.4 (2.65-9.20)	6.25 (2.78-34.68)
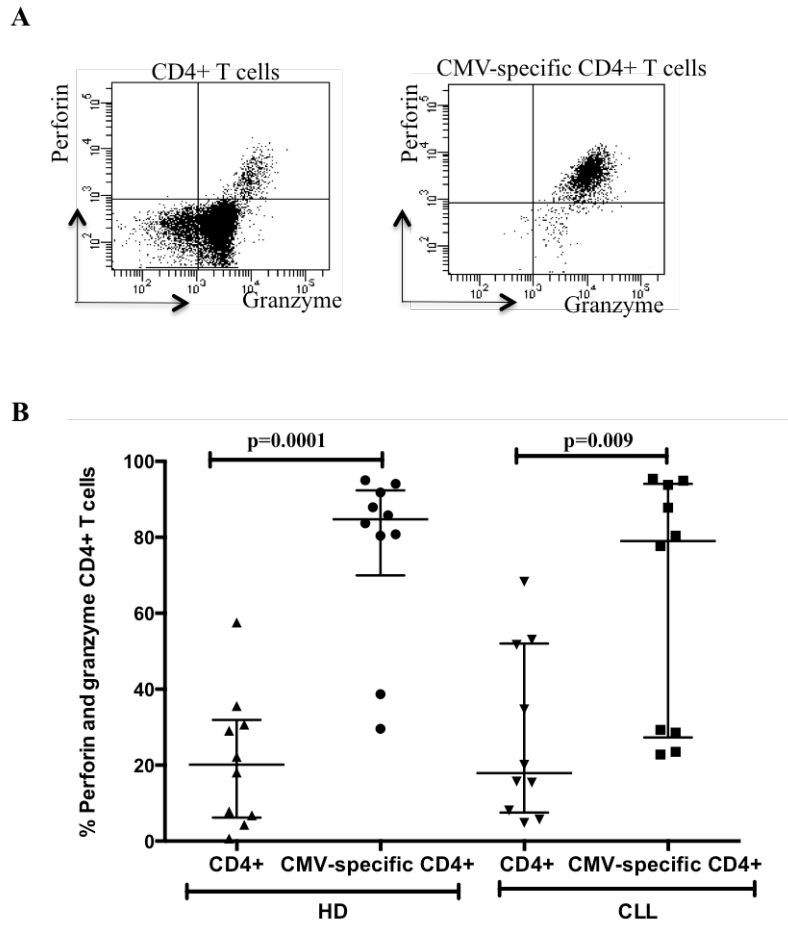
Figure 4.6

CMV-specific CD4+ T cells display great cytotoxic capacity with perforin and granzyme B expression.

A) CD4+ T cells and CMV-specific CD4+ T cells were fixed and permeabilised with PFA and saponin prior to intracellular staining for perforin and granzyme B. The flow plot on the left demonstrates a typical example of CD4+ T cell staining, whilst the flow plot on the right, demonstrates the high frequency of perforin and granzyme B positive CMV-specific CD4+ T cells in the same patient. B) A dot plot graph representing the frequency of dual positive perforin and granzyme B CD4+ T cells. A significant increase in dual expression amongst CMV-specific CD4+ T cells is seen in both HD and patients with CLL (Mann-Whitney).

4.3.8 CMV-specific CD4+ T cells from patients with CLL have increased PD-1 expression compared to HD.

Given the interest in PD-1 as a marker of T cell exhaustion in patients with CLL, we next went on to assess expression of this inhibitory T cell marker on CD4+ T cells with particular focus on CMV-specific CD4+ T cells. Figure 4.7A shows an example of PD-1 staining of CD4+ T cells and CMV-specific CD4+ T cells by gating and MFI. As described by others, CLL patients have a greater proportion of CD4+ T cells expressing PD-1 than healthy age-matched donors (HD: 9.1% Vs. CLL 15.6%; p=0.0007) (Riches et al., 2013, Brusa et al., 2013).

In contrast to reports investigating CMV-specific CD8+ T cells (te Raa et al., 2014), the proportion of CMV-specific CD4+ T cells expressing PD-1 was increased in both patients with CLL and HD compared to the whole CD4+ T cell population (HD: CD4+ 6.4% Vs. CMV-specific CD4+ 26.8% p= 0.004 and CLL: CD4+ 22.2% Vs. CMV-specific CD4+ 52.4% p=0.017). Overall, the frequency of PD-1+ CMV-specific cells was greatest in patients with CLL compared to HD (HD 26.8% Vs. CLL 52.4%; p=0.028) (Figure 4.7B). This finding was also observed by MFI (HD CMV specific CD4+ T cells: 584 Vs. CLL: 988; p=0.031. (Figure 4.7C).

4.3.9 Tim-3 expression is not increased on CD4+ CMV-specific T cells

Tim-3, another inhibitory T cell receptor associated with T cell exhaustion, was also investigated, Tim-3 expression was low on CD4+ T cells in HD and no increase was observed amongst CD4+ T cells in patients with CLL. Furthermore, no difference was observed between CMV-specific and the total CD4+ T cell population in HD or patients with CLL (HD CD4+: 1% vs CMV-specific CD4+: 1.2%; p=0.573 and CLL: CD4+ 0.3% vs CMV-specific CD4+: 0.7% p=0.733).

Figure 4.7

PD-1 expression is increased on CMV-specific CD4+ T cells and this is more pronounced in patients with CLL compared to HD.

(A) An example flow plot of CD4+ (left) and CMV-specific CD4+ (right) T cells and their expression of PD-1. PD-1 is seen to be greater on CMV-specific T cells. (B) This was also demonstrated by MFI. The grey histogram shows unstained cells, the dark solid line demonstrates CD4+ T cells and the dashed line shows CMV-specific CD4+ T cells. (C) A comparison of PD-1 expression by percentage of cells is shown. PD-1 expression is greater on CD4+ T cells in patients with CLL compared to HD. Whilst CMV-specific T cells have greater PD-1 expression in both HD and patient with CLL compared to the total CD4+ T cell population (Mann-Whitney). (D) These findings were also confirmed to be significant by MFI (Mann-Whitney).
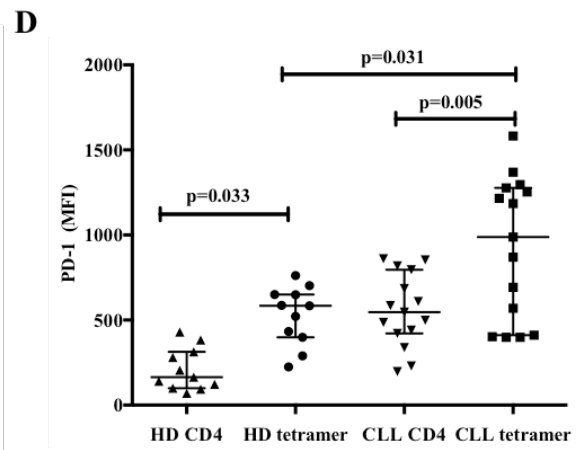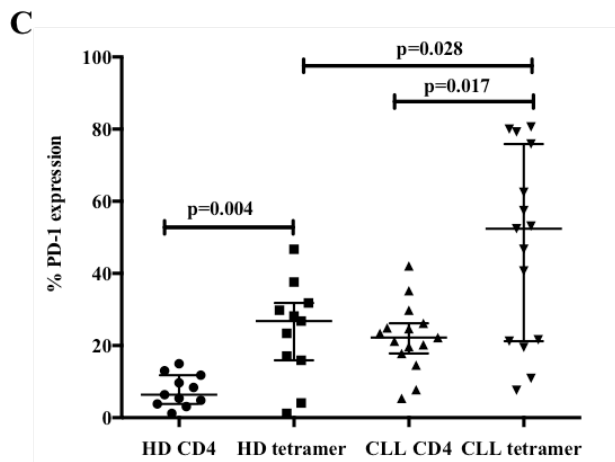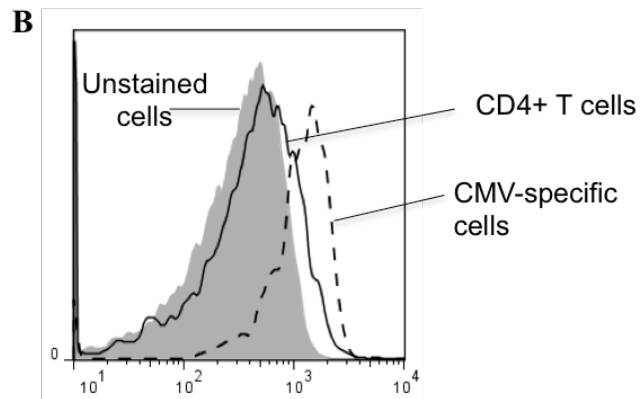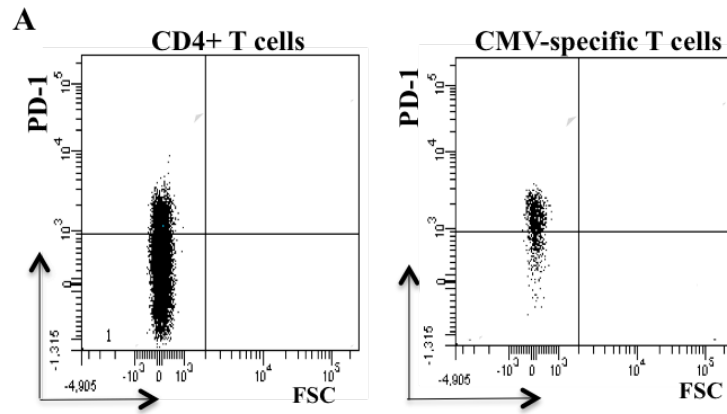
4.3.10 PD-1 expression on CMV-specific T cells is not associated with recent activation

PD-1 is known to be expressed on the surface of activated T cells (Keir et al., 2008) and it was therefore important to investigate if PD-1 expression on CMV-specific CD4+ T cells is a reflection of recent activation, particularly given the finding of increased CD38 expression in section 1.3.4. Combining data from 10 HD and 10 patients with CLL, I initially examined the percentage of PD-1+ cells that expressed a CD38+ or CD38- phenotype. No increase in PD-1 expression was observed on CD38+ CMV-specific T cells. In fact, the opposite was observed, with increased PD1+ seen on CMV-specific T cells that were CD38 negative (median CD38 negative PD-1%: 38.7 Vs CD38 positive PD-1%: 8.05%; p=0.004). To investigate this further, I analysed the total CMV-specific tetramer population for potential correlation between PD-1 expression and CD38 expression, this found no significant correlation and an R^2 value of 0.0099 (figure 4.8).

Figure 4.8

PD-1 positive CMV-specific CD4+ T cells are not recently activated

(A) CD38 negative CMV-specific CD4+ T cells were found to have a greater percentage PD-1 expression than CD38 positive cells (38.7% vs 8.05%) (Mann-Whitney) and when correlating PD-1 expression against CD38 expression on CMV-specific CD4+ T cells, no correlation was observed (Pearson's correlation co-efficient) (B). These data suggest that PD-1 is not expressed on CMV-specific CD4+ T cells as a result of recent T cell activation.
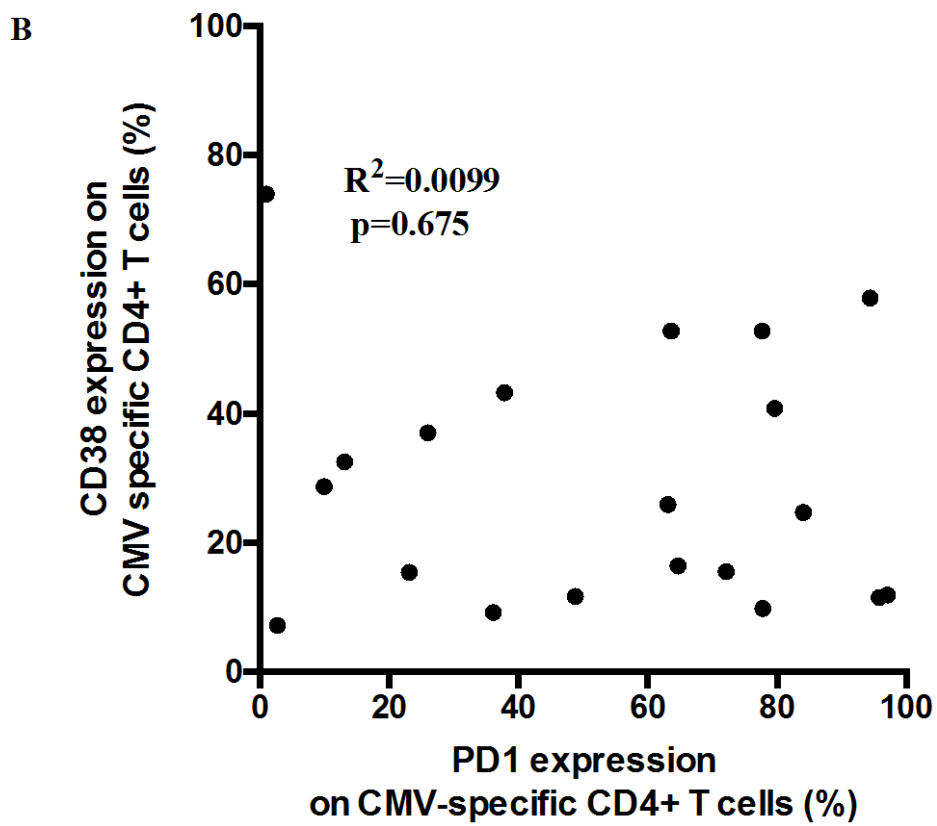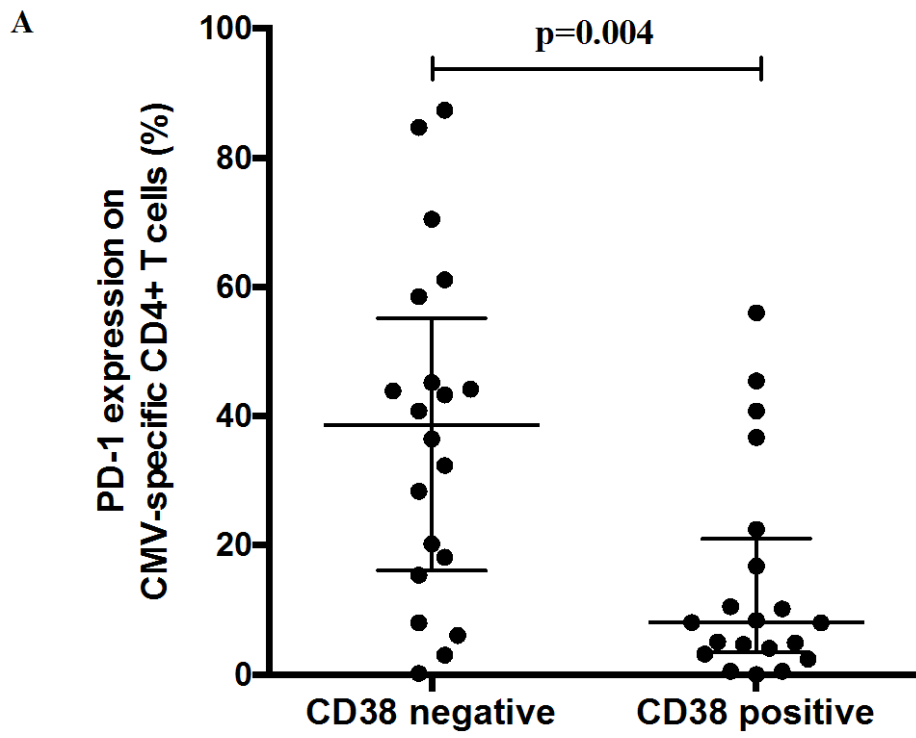
4.3.11 PD-1 expression remains constant over time.

Over a year period, repeat bleeding of both healthy donors and patients with CLL revealed PD-1 expression remained constant over time on CMV-specific CD4+ T cells. Details of the percentage PD-1 expression at specified time points of 6 donors are shown in table 4.4 and the data is graphically represented in figure 4.9. No significant difference was found between PD-1 expression over time (p=0.688). This finding again supports the notion that PD-1 expression on CMV-specific CD4+ T cells is not in response to recent activation but that a set level of expression arises at some point during the course of infection.

4.3.12 PD-1-positive CMV-specific CD4+ T cells produce less IFN-γ and TNF-α

9 samples from patients with CLL and 8 HD with known tetramer responses were stimulated with peptide for 6 hours prior to tetramer staining as described in section 1.3.6. Surface staining for PD-1 was followed by intracellular staining for IFN-γ and TNF-α. CMV-specific CD4+ T cell populations were identified and the amount of cytokine production from PD-1 positive and negative cells was assessed. PD-1+ CMV-specific CD4+ T cells showed reduced production of both cytokines compared to PD-1- CMV-specific CD4+ T cells (Figure 4.10A) (IFN- γ produced from CMV-specific PD-1- cells 25.35% vs CMV-specific PD-1+ cells 13.4%; a 11.95% reduction; p=0.037 and TNF-α produced from CMV-specific PD-1- cells 40.1% vs CMV-specific PD-1+ cell 14.8%; a 25.3% reduction; p=0.0007). No difference was observed amongst the percentage of PD-1+ cells and PD-1- cells able to produce Il-4 (p=0.306). An example of cytokine staining for CMV-specific CD4+ T cells with PD-1 expression can be seen in Figure 4.10B. Overall, inducible cytokine production of IFN-γ and TNF-α is impaired in PD-1+CMV-specific CD4+ T cells.

Table 4.4

PD-1 expression is relatively constant over time

Sample	Date bled	% PD1 on CD4-specific T cells	Date bled	% PD1 on CD4-specific T cells
HD1	12/06/13	25.5	08/01/15	22.4
HD2	17/08/12	4.1	16/01/15	2.7
HD3	16/08/12	27.7	12/01/15	25.6
CLL1	13/09/12	80.6	18/12/13	86
CLL2	21/06/12	10.3	08/07/14	9.9
CLL3	12/04/12	62.6	08/01/15	63.6
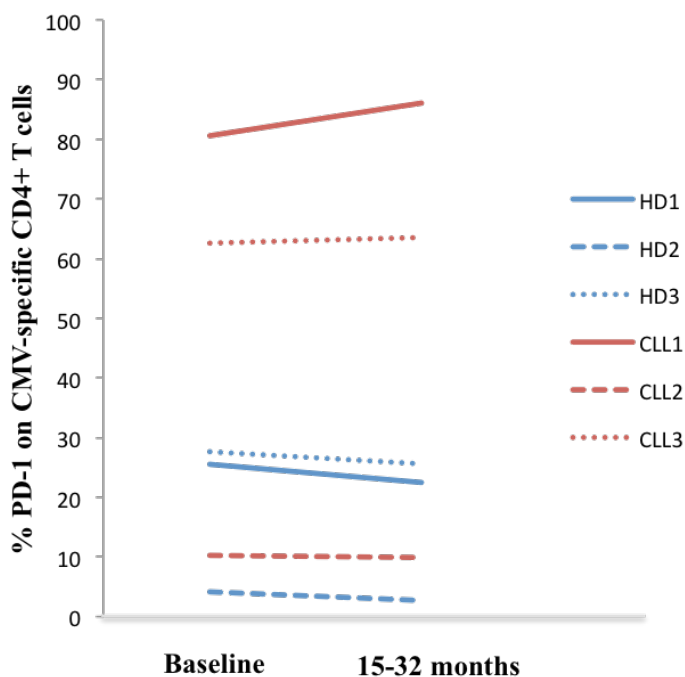
Figure 4.9

PD-1 expression on CMV-specific CD4+ T cells is stable up to 32 months later.

PD-1 expression on CMV-specific T cells is shown in 3 HD and 3 patients with CLL and was found to be stable over time (p=0.688; Wilcoxon matched-pairs rank testing).
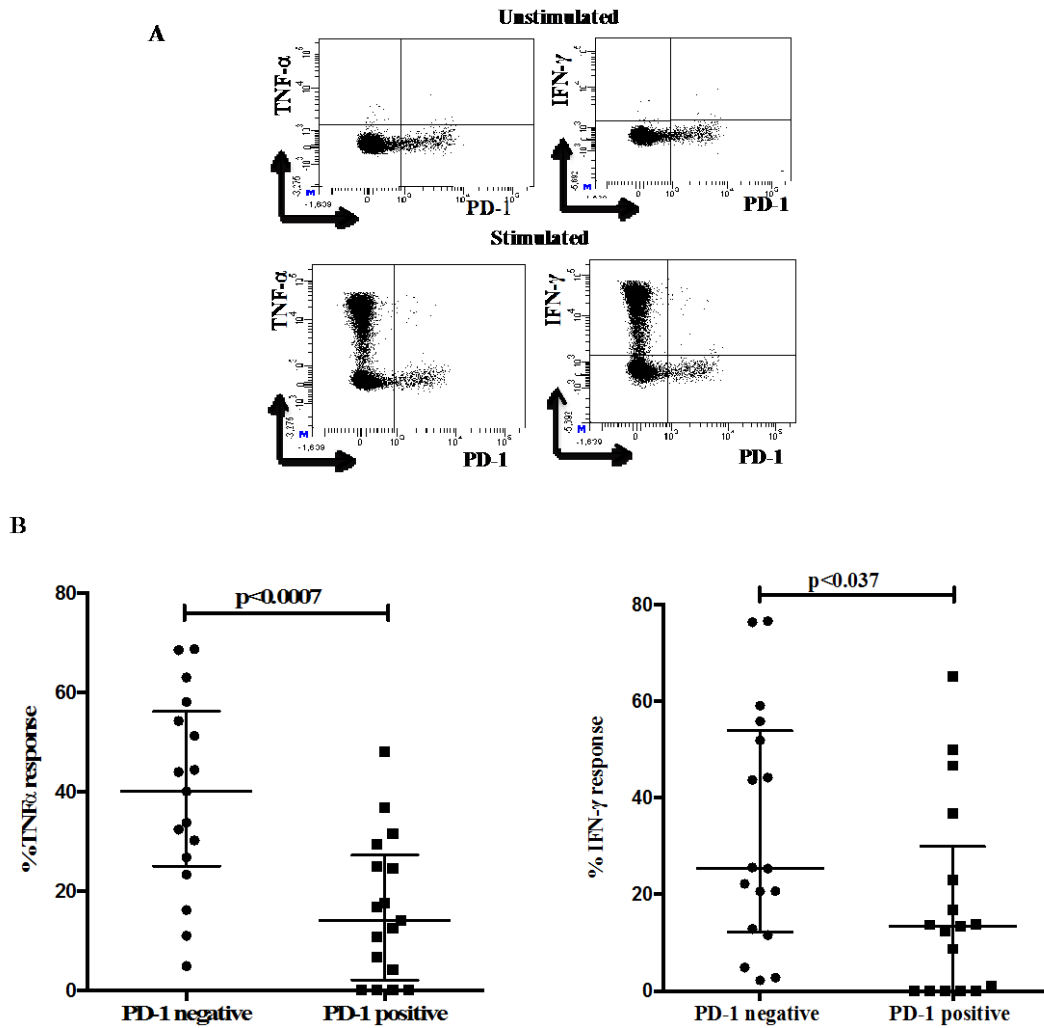
Figure 4.10

PD-1 positivity is associated with reduced cytokine production from CD4+ CMV-specific T cells

(A) Example plots of unstimulated CMV-specific T cells demonstrated no TNF-α (left plot) or IFN-γ (right plot) production but positive PD-1 expression. Following stimulation, both IFN-γ and TNF-α production was observed but not by PD-1 positive cells. (B) Both TNF-α and IFN-γ production was greater in PD-1 negative CMV-specific CD4+ T cells compared to PD-1 positive CMV-specific CD4+ T cells (p<0.0007 and p<0.037 respectively) (Mann-Whitney).

4.3.13 Transcription factor expression of CMV-specific CD4+ T cells

4.3.13.1   Tbet and eomesodermin

The expression of the transcription factors Tbet, Eomesodermin and Foxp3 was next

investigated within the CMV-specific T cells. Eomesodermin (Eomes) and Tbet play a crucial

role in T cell development and are widely expressed in CD8+ T cells where they have been

most widely studied and regulate effector cell differentiation (Cruz-Guilloty et al., 2009,

Knox et al., 2014). Expression of Tbet is attributed to the upregulation of perforin and

granzyme B in CD8+ effector T cells and is known to increase within 24 hours of T cell

activation (McLane et al., 2013, Knox et al., 2014). In CD4+ T cells, Tbet is thought to be the

masterregulator of Th1 CD4+ T cell development and inhibits Th2 and Th17 CD4+ T cell

formation (Szabo et al., 2000). In contrast to Tbet, Eomes expression does not increase

following T cell activation but appears the more differentiated an effector memory cell

becomes and its absence leads to a deficiency in effector memory T cells (McLane et al.,

2013, Pearce et al., 2003). Both Tbet and Eomes are thought to influence the production of

IFN-γ (Knox et al., 2014). In comparison to CD8+ T cells, expression of Tbet and Eomes in

CD4+ T cells is far less frequent and has not been studied to such an extent (Knox et al.,

2014, Pearce et al., 2003). Given the Th1 profile of CMV-specific CD4+ T cells that I had

observed, I next investigated this transcription factor profile and its relationship with

functional status in 10 CLL and 10 HD CMV-specific CD4+ T cells. Cells were firstly

incubated at 37°C with tetramer, prior to surface staining and fixation using the transcription

factor method. After permeabilising the cells, antibodies for Eomes and Tbet were added.

Figure 4.11A gives an example plot of the Tbet and Eomes staining observed. Virtually all

CD4+ CMV-specific cells in HD and patients with CLL expressed Tbet, whilst a much lower

expression was observed in CD4+ T cells (HD: 86.2% Vs 26.5% respectively; p=0.0007 and

CLL: 90.85% Vs.38.65%; p<0.0001). No difference in Tbet expression within CMV-specific CD4+ T cells was found between patients with CLL and HD (p=0.215) (Figure 5.11B, left panel).

Eomes expression was also similar between patients with CLL and HD CD4+ T cells (18.75% HD Vs. 22.75% CLL, p=0.542) and again, expression was of much greater magnitude in CMV specific CD4+ T cells (median HD 60% Vs 57.70% in CLL). Remarkably, the positive Eomes staining on CMV-specific CD4+ T cells in many of the donors accounted for the total Eomes positive staining in the whole CD4 population, suggesting CMV impacts greatly on the presence of this transcription factor amongst CD4+ T cells of CMV positive donors. Notably, no single positive Eomes staining was observed amongst CMV-specific CD4+ T cells. Furthermore, combined analysis of HD and CLL patient data confirmed CMV CD4+ T cells to express more Eomes than the total CD4+ T cell population (CMV specific CD4+ T cell 57.7% Vs. CD4+ T cells 18.75%, p=0.0005).

Next, using the combined HD and CLL dataset, expression of Tbet and Eomes was correlated with the expression of PD-1. Interestingly, neither Tbet nor Eomes expression were found to correlate with PD-1 MFI (r=-0.219 and r=-0.13 respectively) (Figure 4.11C).

Next, the functional responses to peptide stimulation observed in section 1.3.6 were correlated with the individuals' transcription factor profile. No correlation was observed between Tbet+ expression and dual TNF-α+ IFN-γ + cytokine production. However, the frequency of Tbet+ Eomes+ CMV-specific CD4+ T cells positively correlated with an increase in production of TNF-α and IFN-γ (r=0.4489; p=0.050) Figure 4.11D. Together, these findings suggest that Eomes positivity is important for CD4+ T cell differentiation and positively influences the production of cytotoxic cytokines, but not PD-1 expression.

4.3.13.2 FoxP3

PD-1 is expressed on many regulatory cells and regulatory cells may produce IL-4. To address the question of whether or not CMV-specific CD4+ T cells may also include conventional Tregs, expression of the transcription factor FoxP3 and CD25 was next assessed on tetramer-positive cells in 5 HD and 5 patients with CLL. Following incubation with tetramer, surface staining including CD25 was performed before cells were fixed and permeabilised using the transcription factor kit, with subsequent incubation with anti-FoxP3 antibody. Amongst CD4+ T cell populations, CD25+FoxP3 populations were identified at a low level (HD:1.8% (IQR: 1.4-3.3); CLL: 3% (1.4-6.4) p=0.547). However, no FoxP3+CD25+ phenotype was found amongst CMV-specific CD4+ T cell populations in patients with CLL or HD, confirming the CMV-specific CD4+ T cells identified do not have any FoxP3+ T regulatory cell phenotype to account for the expression of IL-4 or PD-1 (Figure 4.12).

Figure 4.11

The T-bet and eomesodermin profile of CMV-specific CD4+ T cells

(A) An example plot is shown of CD4+ T cells (left) and CMV-specific CD4+ T cells (right) for T-bet and Eomesodermin staining. Both transcription factors were more commonly expressed in CMV-specific CD4+ T cells compared to the whole CD4+ T cell population in both HD and patients with CLL as demonstrated in the dot plots shown in part (B) (Mann-Whitney). Virtually all CMV-specific CD4+ T cells were T-bet positive (HD: 86.2% and CLL: 90.85%) and CMV-specific CD4+ T cells also demonstrated positive Eomes staining but at a lower magnitude (HD: 60% HD vs CLL: 57.70%). No CMV-specific cells were Eomes positive without co-expression of T-bet in either patients with CLL or HD. No correlation was observed between the MFI of PD-1 and T-bet (Spearman's rank correlation) or Eomes (Pearson's correlation co-efficient) (C). Combining the HD and CLL donor results, no correlation was observed with T-bet expression and TNFα+IFN-γ+ production (Spearman's rank correlation). However, a positive correlation was observed between Eomes expression and cytokine production (p=0.05) (Pearson's correlation co-efficient).

A

B

C

D
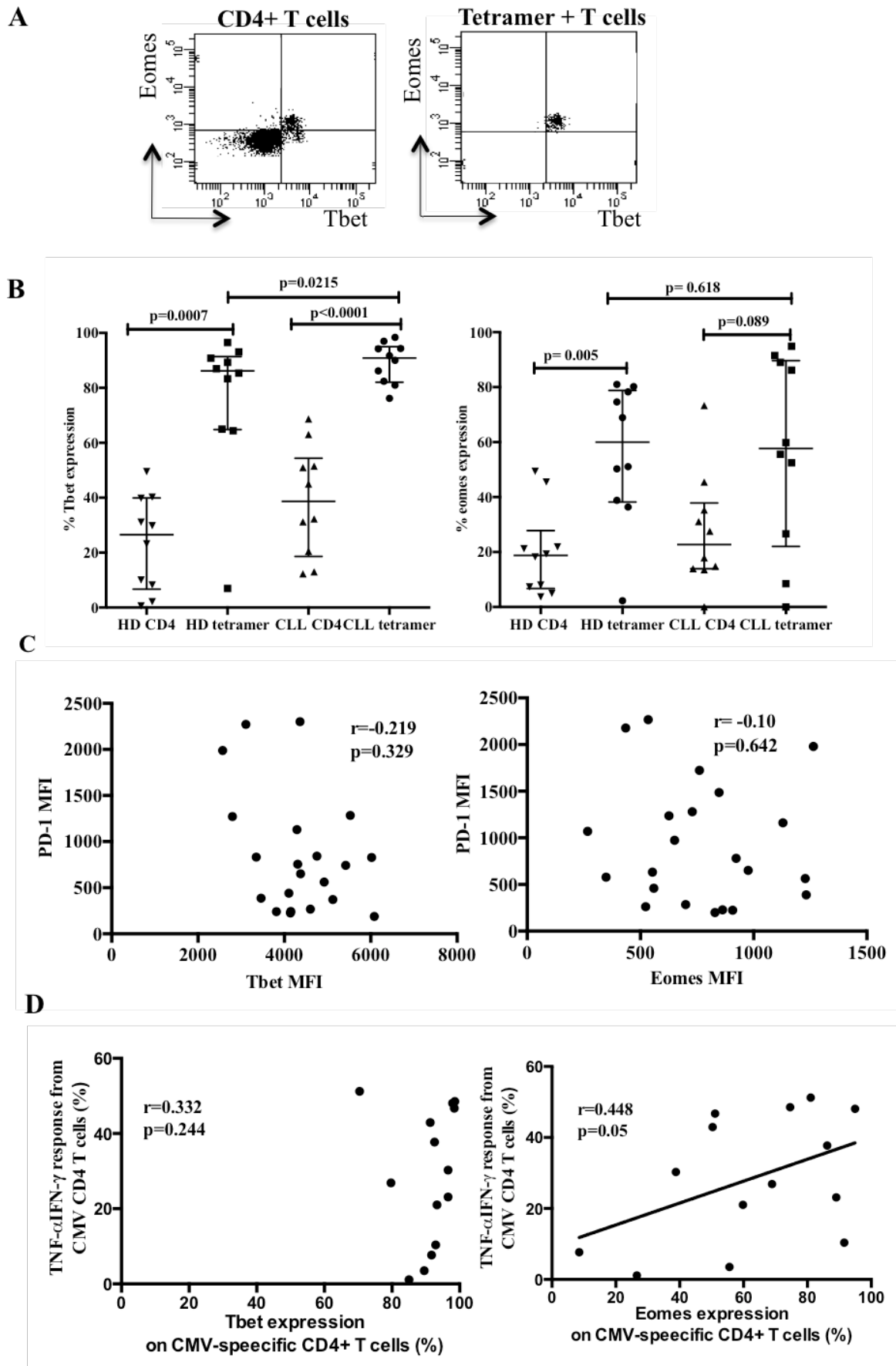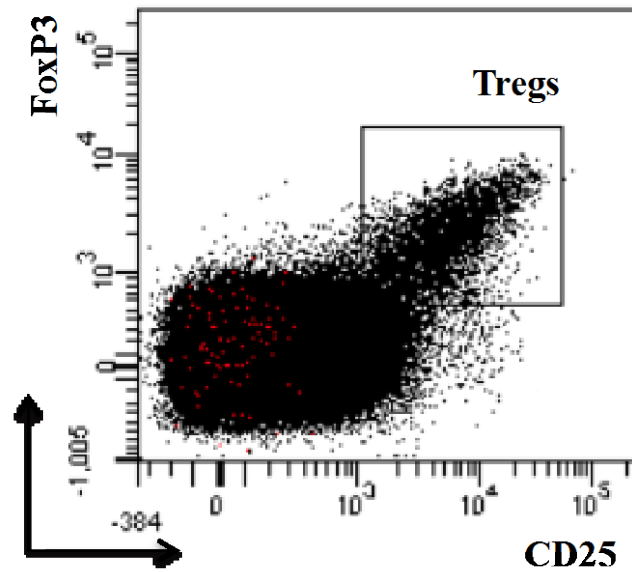
Figure 4.12

CMV-specific CD4+ T cells are not conventional Foxp3+ T regulatory cells.

Identification of conventional T regulatory cells (Tregs) by CD25+FoxP3+ staining of CD4+

T cells (black) is shown. CMV-specific cells (red) are both CD25- and FoxP3-.

4.3.14 Telomere lengths of CMV-specific CD4+ T cells are significantly shortened.

Single cell length analysis of the XpYp telomere end was performed for CD4+ T cells and CMV-specific CD4+ T cells from 4 patients with CLL, as described in chapter 3 for CD8+ T cells. Multiple single cell telomere lengths from a cell population were used to determine the mean telomere length of the cells of interest. For this work, CMV-specific CD4+ T cells and non-tetramer positive CD4+ T cells were sorted at the University of Birmingham and then transported and analysed at the University of Cardiff by Professor Duncan Baird. CMV-specific CD4+ T cell populations were found to have reduced telomere lengths compared to the background CD4+ T cell populations in patients with CLL, with the mean difference of 0.36 kb in those with shortened telomere lengths (maximum 1.6 kb) (Donor 1 CD4: 3.33 kb vs tetramer 2.03 kb; p= <0.0001. Donor 2 CD4: 5.36 kb vs tetramer 3.53 kb; p=<0.0001. Donor 3 CD4: 3.37 kb vs tetramer 2.39 kb; p=<0.009 and donor 4 CD4: 3.45 kb vs tetramer 3.34 kb; p= 0.87). These telomere length differentials are consistent with large differences in the proliferative history of the cell populations and suggests CMV-specific CD4+ T cells contribute to the reduced telomere lengths seen in patients with CLL and also confirms CMV-specific CD4+ T cells have a substantial mitotic history, likely in response to both primary and reactivated CMV viral load (Figure 5.12).

Figure 4.13

CMV-specific CD4+ T cells show significantly shortened telomere lengths

Two examples of single cell telomere length analysis of CMV-specific CD4+ T cells are shown (tet) alongside the total CD4+ T cell telomere length (CD4) (A). Below the gel is the mean telomere length and standard deviation for each sample. To the left of each example is the reference ladder. B) The mean telomere lengths are significantly shorter in CMV-specific T cells compared to non-tetramer CD4+ T cells in 3 of the 4 donors tested (Student's T test).

4.4 Discussion

CD4+ T cell responses are crucial for the effective control of CMV infection. Patients with advanced and untreated HIV infection exemplify the deleterious impact of diminished CD4+ T cell control and exhibit frequent episodes of CMV reactivation. In contrast, patients with CLL rarely develop overt CMV reactivation, despite most suffering from a subtle but global immunosuppression that includes T cell dysfunction (Duraiswamy et al., 2011). Paradoxically, CMV seropositive patients with CLL actually have an expansion of CMV-specific T cells compared to healthy donors. Previously, the study of virus-specific CD4+ T cell responses has relied on lysate or peptide based stimulation to identify CMV recognising CD4+ T cells or to cells expressing CD28-CD57+. At the time of writing, this work represents one of the first uses of class II tetramers to identify CD4+ CMV-specific T cells and has permitted analysis of responses recognising just one individual epitope, rather than a global CD4+ T cell response to CMV. It has also allowed characterization of these cells in the absence of any prior stimulation. Using two different tetramers, this work has confirmed the unparalleled magnitude of CD4+ CMV-specific T cell responses in healthy donors and consistent with published data found that patients with CLL have an even greater frequency of circulating CD4+ CMV-specific T cells which contribute substantially to the CD4+ T cell immune repertoire in CMV seropositive patients (Pourgheysari et al., 2010, Walton et al., 2010). This work has also confirmed the highly cytotoxic profile of CMV-specific CD4+ T cells with great proportions of perforin and granzyme B positive cells observed (Walton et al., 2010, Porakishvili et al., 2004). Furthermore, these virus-specific cells had substantial expression of PD-1 in health and to an even greater extent in patients with CLL. PD-1 expression is known to be increased in patients with CLL but also correlates with disease progression on CD4+ T cells (Rusak et al., 2015). The great frequencies with which CMV-

specific T cells were observed and also the extent to which they express PD-1 highlights the importance of determining the CMV profile of donors (be it healthy or patients with disease) for any PD-1 studies investigating the global CD4+ T cell repertoire in future studies.

Over the 3 year study period, the percentage of CD4+ CMV-specific T cell responses was remarkably stable, as was expression of PD-1 on CMV-specific cells. One exception to this was noted in a 93 year old patient who was hospitalised for 5 months following community acquired pneumonia. In this case, the CMV-specific CD4+ T cell response towards a single epitope increased to more than half of all CD4+ T cells (from 11% to 51%) without any symptoms or signs of overt CMV reactivation. This suggests dynamic change to the CMV T cell repertoire may arise in the context of immunosuppression or psychological stress. This finding also suggests that subclinical viral reactivation may account for the greater frequency of CMV specific T cell responses observed in patients with CLL and supports the hypothesis of a "silent war" against CMV, previously proposed in 2011 (Akbar, 2010). Incidentally, the percentage of PD-1 positive cells in the CMV-specific T cell population did not change dramatically in this donor (21% on initial sample compared to 9.9% on subsequent sample) and over the study period. PD-1 expression was found to be stable amongst CMV-specific CD4+ T cells, this may be reflective of the poor proliferative capacity but extended lifespan observed amongst CMV-specific T cells (Wallace et al., 2004). In a mouse model investigating CD8+ T cells in the context of chronic infection, the percentage of PD-1 positive cells recognizing the gp-33 epitope of LCMV-13 was also found to remain stable even following antigen withdrawal, suggesting this stable phenotype is imprinted early following primary infection (Utzschneider et al., 2013). This contrasts with acute viral infections such as hepatitis B, whereby PD-1 positive cells are upregulated during the inflammatory stage but then decrease as inflammation subsides (Wenjin et al., 2012).

Following primary infection with CMV, PD-1 is known to upregulate on CD4+ T cells and inhibit the production of IL-2, TNF-α and IFN-γ production (Antoine et al., 2012). Interestingly, work by Utzschneider et al, also found that stable populations of PD-1+ CD8+ T cells in the setting of chronic viral infection were able to re-expand when required and daughter cells demonstrated the same PD-1 expression and cytokine capacity. These findings suggest that proportions of PD-1+ T cells exist in the setting of chronic infection, not as exhaustive T cells but as cells that prevent overwhelming host mediated immune damage (Utzschneider et al., 2013). Given the cytotoxic profile observed amongst these virus specific T cells and their Th1 skewed phenotype, it would seem appropriate for some inhibitory checkpoint blockade to be in place for averting any damage to self in the event of viral reactivation. Interestingly, PD-L1, one of the two ligands recognized by PD-1 is known to be expressed on CLL cells but is also found together with PD-L2 on activated macrophages which present viral antigen via MHC II to PD-1 expressing CD4+ T cells (Brusa et al., 2013, Rodriguez-Garcia et al., 2011).

As previously reported, CMV positive individuals have expanded populations of CD8+ T_{EMRA} cells in both health and in patients with CLL (Riches et al., 2013). This work has also found this to be true of CD4+ T cells too, although to a much lesser extent (CD4+ T_{EMRA} cells are 19 fold less common than CD8+ T_{EMRA} cells in HD compared to 13 fold in patients with CLL CLL). The expanded populations of T_{EMRA} CD8+ T cells have been shown to have low PD-1 expression and this is thought to account for the lower frequency of PD-1+ CD8+ T cells in CMV positive patients with CLL (Riches et al., 2013). In contrast, PD-1 expression on CD4+ T cells in patients with CLL is known to be similar in both CMV positive and negative individuals (Riches et al., 2013). This work investigated this association further and has found the CMV-specific CD4+ T cells contribute to the increased PD-1+ CD4+ T cell pool

described in patients with CLL (Riches et al., 2013, Rusak et al., 2015) and that these cells are predominantly T_{EM} in phenotype. Amongst both HD and patients with CLL, the proportion of CMV-specific CD4+ T cells with a T_{EM} phenotype exceeded that of the total CD4+ T cell population, although this only reached significance in HD. This work reports that patients with CLL already have greatly expanded T_{EM} CD4+ T cells regardless of their CMV serostatus (p=0.0003) and provides an explanation as to why no significant difference was observed in T_{EM} status between CMV-specific T cells and the total CD4+ population in patients with CLL, despite 94% of CMV-specific cells being T_{EM} in phenotype. Importantly, this predominant T_{EM} phenotype of CD4+ CMV-specific T cells contrasts with the memory profile of CD8+ CMV-specific T cells, where almost half of identified CMV T cell populations were T_{EMRA} in phenotype. Given the reported differences in PD1 expression by T cell differentiation status, this may account for the difference in PD1 expression observed amongst CD4+ and CD8+ CMV-specific T cells (Legat et al., 2013, Baitsch et al., 2012, Duraiswamy et al., 2011, Riches et al., 2013).

Another important factor in determining the expression of inhibitory receptors, including PD-1, is the activation status of T cells (Keir et al., 2008) In the case of CD4+ CMV-specific T cells, expression of CD69, a short-lived activation marker was negligible, whilst expression of CD69 in the total CD4+ T cell population was consistent with previous publications (3.8% in HD and 4.2% in patients with CLL (Waldrop et al., 1997). An increase in CD38 expression was observed amongst CD4+ T cells in patients with CLL compared to HD but was increased further on CMV-specific CD4+ T cells. This may reflect recent subclinical viral reactivation given the ages of the donors studied. However, there was no suggestion that recent activation was the cause for PD-1 expression amongst these virus-specific cells and indeed, despite an

overall increase in CD38+ expression being observed on CMV-specific CD4+ T cells, those cells which expressed more CD38 were actually found to have less PD-1 expression. Following optimisation of the peptide stimulation assay using a younger donor, CMV-specific CD4+ T cell cytokine responses were assessed and found to include predominantly the production of IFN-γ and TNF-α but also small amounts of Il-4. No Il-5 or IL-10 was observed, although the lack of prolonged incubation with peptide (used in order to preserve tetramer staining) meant optimal stimulation periods of >10 hours could not be reached for this to be conclusive. However, there is no evidence from the literature of IL-5 or IL-10 production from CMV-specific CD4+ T cells, suggesting the Th1 skewed phenotype observed is likely to be a representative. Interestingly, IL-4 production has been reported in the context of CD4+ CMV T cell responses previously (Waldrop et al., 1997, Kallas et al., 1998) and is also seen amongst Th1 dominant responses to other pathogens such as tuberculosis, where a small but definite population of pathogen specific CD4+ T cells are postulated to dampen the cytotoxic response and prevent damage to host (Prabha et al., 2007). An alternative explanation would be the presence of conventional Tregs, as these are expanded in patients with CLL and produce Il-4 amongst other cytokines (Tiemessen et al., 2007). However, no evidence for a FoxP3+CD25+ phenotype was found amongst identified populations of CMV-specific CD4+ T cells to account for the Il-4 observed.

Notably, a reduced functional response was observed in CMV-specific CD4+ T cells of patients with CLL. T cell function is known to be diminished in patients with CLL but this does not necessarily relate to the functional potential of the cell being investigated. Poor synapse formation and impaired antigen presentation are also contributing factors (Ramsay et al., 2012, Scrivener et al., 2003). Indeed, Te Raa *et al* recently demonstrated that, using CMV peptide-loaded LCLs and a controlled B:T cell ratio, cytokine production in CD8+ CMV-

specific T cells from CLL patients was similar to that of HD and concluded poor antigen presentation by CLL cells to contribute to the reduced cytokine responses observed in CMV-specific CD8+ T cells from patients with CLL (te Raa et al., 2014). In my work, unadulterated PBMC cells have been used in patients and donors in order to simulate the presentation of peptide to CMV-specific T cells as it would be *in vivo*. Using this approach I have observed that, at an individual epitope level, functionality amongst CMV-specific CD4+ T cells appears to be reduced in patients with CLL. This impaired functionality may be one reason why such large expansions of CMV-specific T cells are required to control viral load. Furthermore, it has previously been reported that the global proportion of CD4+ T cells producing IFN-γ in response to lysate stimulation is actually increased in patients with CLL (Pourgheysari et al., 2010). This again provides insight into how great the overall magnitude of CD4+ T cells dedicated to recognizing CMV can actually be in this disease.

The expression of PD-1 was also shown to influence the diminished functional response to the exogenous cognate peptide in this work. Similar findings have been shown in renal transplant recipients, where high PD-1+ expression on CMV-specific CD4+ T cells correlated with poorer IFN-γ production and even loss of viral control (Sester et al., 2008). The CD4+ PD-1+ FoxP3- phenotype of CD4+ CMV-specific cells have previously been described in patients with CLL but in this work, I have shown that the same phenotype is also found in health on CMV-specific CD4+ T cells. Although PD-1 expression is one characteristic feature described on exhausted T cells, this work has not found any other features that suggest these CMV-specific CD4+ T cells are indeed truly exhausted (Brusa et al., 2013).

Amongst both healthy donor samples and patients with CLL, PD-1+ CD4-specific T cells producing IFN-γ and TNF-α were still observed. Work is now required to identify if other additional inhibitory markers or features of exhaustion are present on CMV-specific CD4+ T

cells and to interrogate the impact of PD-1 expression in the context of an intact immunological synapse formation. Furthermore, in patients with CLL, a state of "pseudoexhaustion" has been suggested for such PD-1+ T cells, which are capable of some cytokine production, and may be a reflection of the antigen affinity dictating the responsiveness seen amongst CMV-specific T cell populations (Riches et al., 2013). Tbet and Eomes are important for memory cell differentiation and are the 'master regulators' of CD8+ T cell function. Comparatively little is known of their expression and impact on CD4+ T cell plasticity and cellular cytotoxicity (Knox et al., 2014). My work reports that both Tbet and Eomes are co-expressed in most CMV-specific CD4+ T cells recognising gB and pp65 proteins, along with expression of perforin and granzyme, and that this occurs in the context of a T_{EM} memory phenotype. Eomes expression is not frequent amongst CD4+ T cells in comparison to CD8+ T cells, yet it was observed amongst CMV-specific CD4+ T cells. Furthermore, this expression was seen to consist of the entire Eomes+ CD4+ T cell population amongst certain donors and at the time of writing, this appears to be the first report highlighting the impact of CMV on the total CD4+ T cell Eomes frequency.

Eomes expression has also been implicated in PD-1 expression and more recently the production of IFN-γ (Edwards et al., 2014, Buggert et al., 2014). In support of this, Eomes expression correlated positively with TNF-α IFN-γ production. However, no correlation was observed between Eomes and PD-1 expression within CMV-specific CD4+ T cells. One reason to explain this difference is that PD-1 positive cells have been described to have single positive expression and are usually negative for Tbet (Buggert et al., 2014). Amongst the CD4+ CMV-specific T cell populations investigated here, all cells with positive expression of Eomes were also positive for Tbet.

4.5 Conclusion

Collectively, this work has shown that CMV-specific CD4+ T cells have increased PD-1 expression and contribute to the PD-1+ phenotype observed in CD4+ T cells from both healthy donors and to a greater extent, patients with CLL. Although impaired in their ability to produce cytokines, it is not clear if these cells are truly exhausted. Their effector memory phenotype, Eomes expression and shortened telomere length, suggest CMV-specific T cells have undergone multiple rounds of cell division and the stable expression of PD-1 is most likely representative of a means by which an overwhelming CD4+ T cell response can be inhibited to prevent host damage, whilst permitting effective control of viral replication. The CMV-specific CD4+ T cell response in patients with CLL is increased and associated with increased expression of CD38 and PD-1 but reduced IFN-γ and TNF-α production. In conclusion, this work describes a proportion of CMV-specific CD4+ T cells with a PD-1+ CD25- phenotype which most likely function to maintain homeostasis through an, as yet uncharacterised, immuneregulatory T cell role.

5 DIGITAL PCR TO ASCERTAIN LATENT CMV VIRAL LOAD

5.1 Introduction

CMV like other herpesviruses, maintains a state of lifelong latency following primary

infection. The cellular and humoral CMV-specific immune response controls viral replication

and this immunity must be maintained throughout life in order to prevent episodes of

clinically significant viral reactivation (Moss and Khan, 2004b). As described in chapter 3

and 4, the magnitude of the CMV-specific immune response is higher than has been recorded

against other pathogens and increases with age, a phenomenon termed 'memory inflation'

(Chidrawar et al., 2009, Karrer et al., 2003). The resulting impact of CMV on the human

immune system is evident through the inverted CD4:8 ratio and accumulation of large

numbers of late-differentiated memory cells (Strindhall et al., 2012). As described earlier,

CMV infection has been suggested to accelerate immune senescence with ageing and is

associated with a variety of clinical problems and increased risk of mortality (Savva et al.,

2013, Roberts et al., 2010, Strandberg et al., 2009, Schmaltz et al., 2005).

Subclinical episodes of CMV reactivation are thought to frequently occur during a lifetime

but are rapidly controlled by the host immune response, thus averting any clinically apparent

disease (Stowe et al., 2007). In order to understand more about the mechanisms by which

CMV infection may impact on the health of elderly donors it is important to improve

understanding of the level of CMV load within the blood and how this is related to specific

features of the CMV-specific immune response (Parry et al., 2016b).

In addition to the immunosenescence of ageing, latent CMV is also attributed to a continued

level of systemic chronic inflammation in health. This appears to be a perpetual cycle, as

cytokines such as prostaglandin and TNF-α are released during inflammation and have been

shown in turn to prompt viral reactivation (Kline et al., 1998, Docke et al., 1994). In diseases

such as CLL, vasculitis and some auto-immune conditions, where a state of relative but not

overt immunosuppression exists, CMV specific T cells have been shown to accumulate and are thought to arise in response to more frequent episodes of viral reactivation. Despite this, amongst studies investigating this relationship, the opportune sampling of patient's blood or tissue, ordinarily fails to detect any appreciable viral load by PCR (Pourgheysari et al., 2010, Morgan et al., 2011, Mackus et al., 2003). This is unsurprising, as it is well documented that the level of CMV within whole blood is very low and conventional PCR assays are almost invariably negative in healthy donors (Parry et al., 2016b, Slobedman and Mocarski, 1999, Stanier et al., 1992, Jordan, 1983).

An alternative means of assessing CMV viral load would be to assess its presence amongst latently infected cells. The sites of CMV latency include haemopoietic stem cells and monocytes and purification of discrete cell subsets that harbor the virus, followed by PCR amplification, is one approach that has been used to increase the sensitivity of viral detection (Leng et al., 2011b). As discussed in chapter 1, viral latency is maintained during monocytic carriage (Sinclair, 2008). In contrast, lytic viral replication is thought to only arise following differentiation of monocytes into macrophages (Reeves et al., 2005, Bain et al., 2003, Stevenson et al., 2014).

The use of highly sensitive PCR assays increases the frequency of CMV detection. Nested PCR offers the advantage of substantial sensitivity but is poorly quantitative (Leng et al., 2011b), whereas droplet digital PCR (ddPCR) is a new approach that provides both a sensitive and direct method for detecting target DNA without the need for a 'standard curve'. ddPCR emulsifies an oil-based PCR reaction into thousands of droplets, each of which then acts as a PCR micro-reaction and increases the chances of a rare event being detected. Using Poisson's distribution, a direct measurement of the target DNA can then be determined. ddPCR does not therefore rely on any interpretation of rate-based data, as is the case with Q-

PCR (Parry et al., 2016b). The versatility of ddPCR for detection of low copy number events is now being recognised and gradually becoming part of clinical practice for monitoring mutations levels in disease (Watanabe et al., 2015, Kinz et al., 2015).

This work, using purified monocyte DNA combined with droplet digital PCR, demonstrates an absolute quantification of CMV viral load is obtainable in the blood of healthy donors and patients with CLL. Furthermore, absolute quantification of latent viral load in healthy donors, according to age is presented, as well as a comparison of the CMV load obtained in CLL patients.

5.2   Methods

5.2.1   Recruitment of healthy donors

Forty-four healthy donors were confirmed to be CMV positive using the CMV ELISA described in chapter 2. Healthy donors were between the ages of 25 and 86 (median 50 years; IQR 41.5-62.75). Following a 50 ml blood donation, plasma and PBMCs were extracted over a Ficoll-density gradient and cryopreserved, as described in chapter 2. PBMCs were used for monocyte extraction whilst plasma was used for CMV ELISA testing.

5.2.2   Recruitment of peripheral blood stem cell donors

5 CMV positive peripheral blood stem cell donors who had received G-CSF mobilization were also recruited and aliquots of PBMC were cryopreserved prior to defrosting and extraction of myeloid cell subsets.

5.2.3   Recruitment of CLL patients.

25 patients with CLL with a median age of 66 were recruited from outpatient haematology clinics at BHH and QEHB and clinical characteristics were obtained from electronic records (Table 5.1). All patients were either untreated or more than 6 months post treatment and all

had a lymphocyte count >1.5x 10^9/L at the time of sample collection. Binet staging was performed at the time of sample collection to reflect the nature of the current sample rather than previous staging which may have been influenced by therapy. PBMCs and plasma were isolated using a ficoll-density gradient described in chapter 2 and then cryopreserved under sterile conditions.

5.2.4 Extraction of CD14, CD16 and CD34 cells from stem cell donations

Enrichment of CD14, CD16, CD34 and dual positive CD14/16 cells from stem cell donations were sorted by flow cytometry using panel 12 of table 2.3, as described in chapter 2 (MoFLow sorter, BD Biosciences).

5.2.5 DNA extraction of healthy donor and CLL CD14+ cells from PBMC

Positive selection of CD14+ monocytes was undertaken using magnetically labeled beads as described in chapter 2. The average enrichment of 4 samples was found to be 98.73% (SD 0.39) by flow cytometry (Figure 5.1). DNA extraction was then performed using the GenElute Mammalian Genomic DNA miniprep kit described in chapter 2.

5.2.6 Q-PCR and ddPCR of glycoprotein B in enriched cell populations

50ng of DNA was extracted from isolated CD14+, CD34+, CD16+ and CD14+/CD16+ populations in each PCR reaction. The methodology for Q-PCR and ddPCR are described in detail in chapter 2.

5.2.7 Statistical Analysis

Kruskal-Wallis test and post hoc Dunn's testing was used to compare CMV viral load with each decade of age. A growth exponential curve was used to assess the CMV load doubling time and linear regression used to assess the relationship between CMV copies (log_{10}) and the age of participants. For the standard dilution series ran in triplicate, the results were log

transformed $\log_{10}$ and Pearson's correlation co-efficient was used after confirming normality, to compare the quantitative agreement between ct value and plasmid copy number. Linear regression was used to examine the relationship between ddPCR copy number reading and that of the plasmid dilution and also to compare Q-PCR copy number with that of ddPCR in healthy donor samples. Mann-Whitney testing was used to compare detectable viral load in HD compared to patients with CLL. Comparison of ages within CLL groups and to healthy donors was performed using Student's T-test after normality was confirmed using D'Agostino & Pearson normality testing.
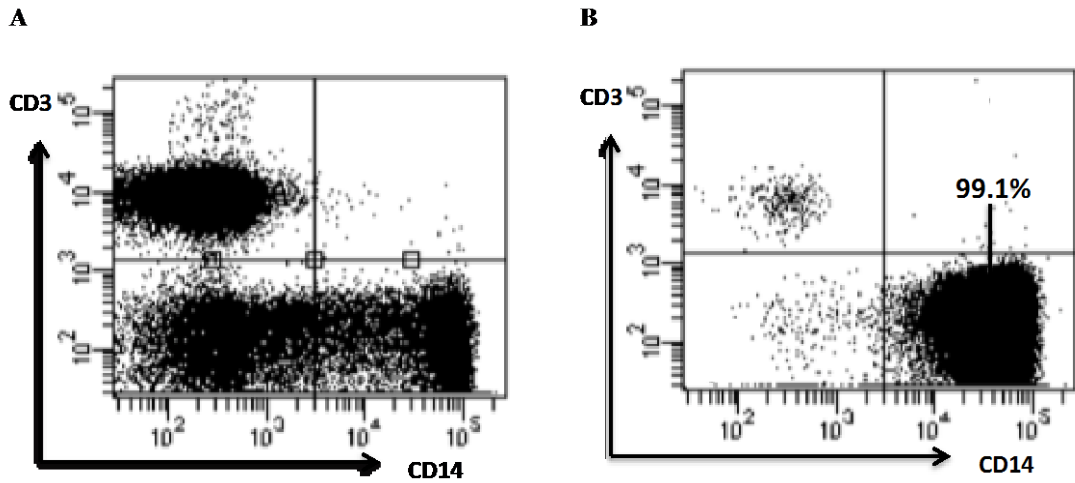
Figure 5.1

Monocyte enrichment using CD14+ positive selection magnetic bead kit

Flow plots demonstrating before (A) and after (B) CD14+ magnetic bead sorting of peripheral blood mononuclear cells. The average enrichment was found to be 98.73% (SD 0.39).

Table 5.1

Clinical data of patients with CLL

	Digital PCR cohort
Median age at diagnosis (years)	66 (58.5-69.5)
Male sex	17/25 (68%)
Follow up time at the point of sample collection (years)	8.5 (4.3-11)
Previous history of treatment	18 (72%)
Time since treatment finished (years)	2.8 (1.5-8.8)
Binet stage A	16
Binet stage B	1
Binet stage C	8
Missing	0
CD38 positive	7
CD38 negative	9
CD38 missing	9
IGHV mutated	5
IGHV unmutated	5
IGHV missing	15
FISH	
Normal	8
13q-	5
12+	3
11q-	1
17p-	2
Other	0
Missing	6

5.3   Results

5.3.1   Healthy donors

5.3.1.1 Digital PCR detection of CMV within the monocytes of healthy donors

CD14+ monocytes were purified using magnetic beads from 44 healthy donors samples and DNA was extracted using a mini-prep kit. Into a well of the ddPCR cartridge, 50ng of sample DNA was added together with the PCR reaction mixture. 70µl of droplet generation oil was then used to emulsify the sample and generate the droplets (Figure 5.2). After transferring the formed droplets to a 96 well plate, amplification using standard PCR was the performed. Assessment of the number and proportion of positive and negative droplets was determined using Quantasoft software and an absolute quantification based on poisson distribution was produced for each sample. Figure 5.3 gives an example of the typical positive and negative droplets ascertained. The threshold for positive and negative droplets was verified by a Biorad scientific advisor and was the same between independent runs. A positive and negative control was used on each 96 well plate used. Detectable viral load was found in 16 donors (36%) and was confirmed in each case by 3 independent runs (Parry et al., 2016b).

5.3.1.2 CMV viral load within monocytes increases markedly above the age of 70 years

When these results were assessed in relation to donor age it was clear that the proportion of donors in which CMV was detectable increased markedly with age. Specifically, CMV was detected in 9 of 37 (24%) of donors aged below 70 years, whereas a positive test was seen in each of the 7 donors aged over 70 (Figure 5.4A). In those aged 20-30, 1 out of 6 (16.7%) had a detectable load; 1 out of 4 (25%) in 30-40 year olds; 1 out of 13 (7.7%) in 40-50 year olds; 3

out of 8 (37.5%) in 50-60 year olds and 3 out of 6 (50%) in 60-70 year olds (Parry et al., 2016b).

The absolute quantification of CMV viral load per μl was firstly determined by QuantaSoft software and was also found to increase with age, with a marked increase in donors aged over 70 years (kruskal-wallis p<0.0004). Post hoc Dunn testing showing significant differences between 20-30 and 70-80 year olds (p=0.019) and 40-50 compared to both 70-80 (p=0.001) and 80-90 year olds (p=0.023). Viral load varied from 0.144 copies per l to 9.14 copies per μl, with a mean copy number per μl of 0.756 (SD 1.395) in those under the age of 70, compared to 5.006 copies per μl (SD 2.13) in those over the age of 70 (Figure 5.4B) (total reaction volume 20μl with 50ng of DNA).
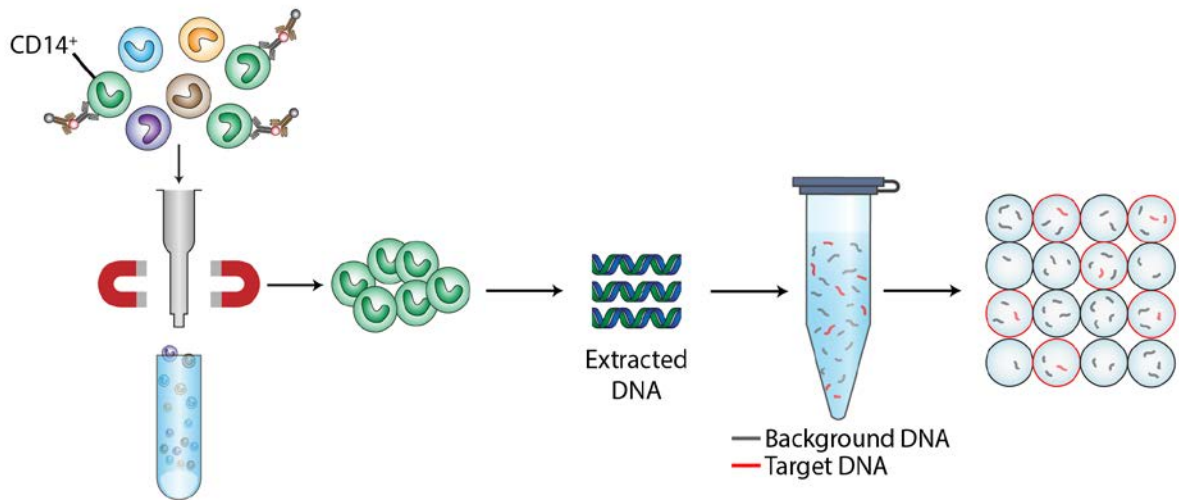
Figure 5.2

A schematic of the method used to generate the viral load.

Monocytes were firstly labeled with magnetic CD14 beads and then passed through a column

for purification. DNA was then extracted and used for ddPCR reaction, whereby the starting

DNA is emulsified into thousands of tiny droplets to increase the chance of a rare event being

detected by PCR (Illustration edited by Tyler Lieberthal, Imperial College London).
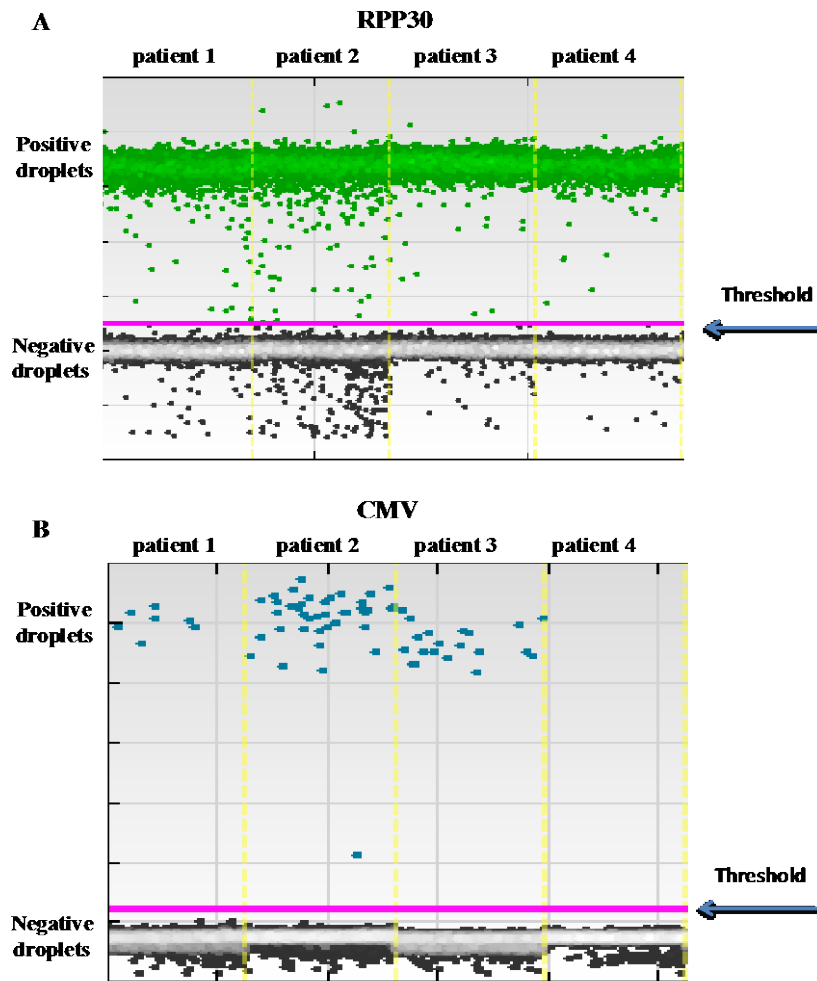
Figure 5.3

Droplet digital PCR examples of positive and negative droplet detection.

Examples of the read outs produced with QuantaSoft software are given. The internal copy

number variation assay measuring RPP30 is shown in (A) and CMV detection in (B). The

threshold set is marked with an arrow and was consistent in all experiments. Patients 1-3 have

a detectable CMV viral load, whilst patient 4 has no detectable CMV viral load but clearly

has genomic DNA present by the positive droplet detection in the RPP30 assay seen in (A).
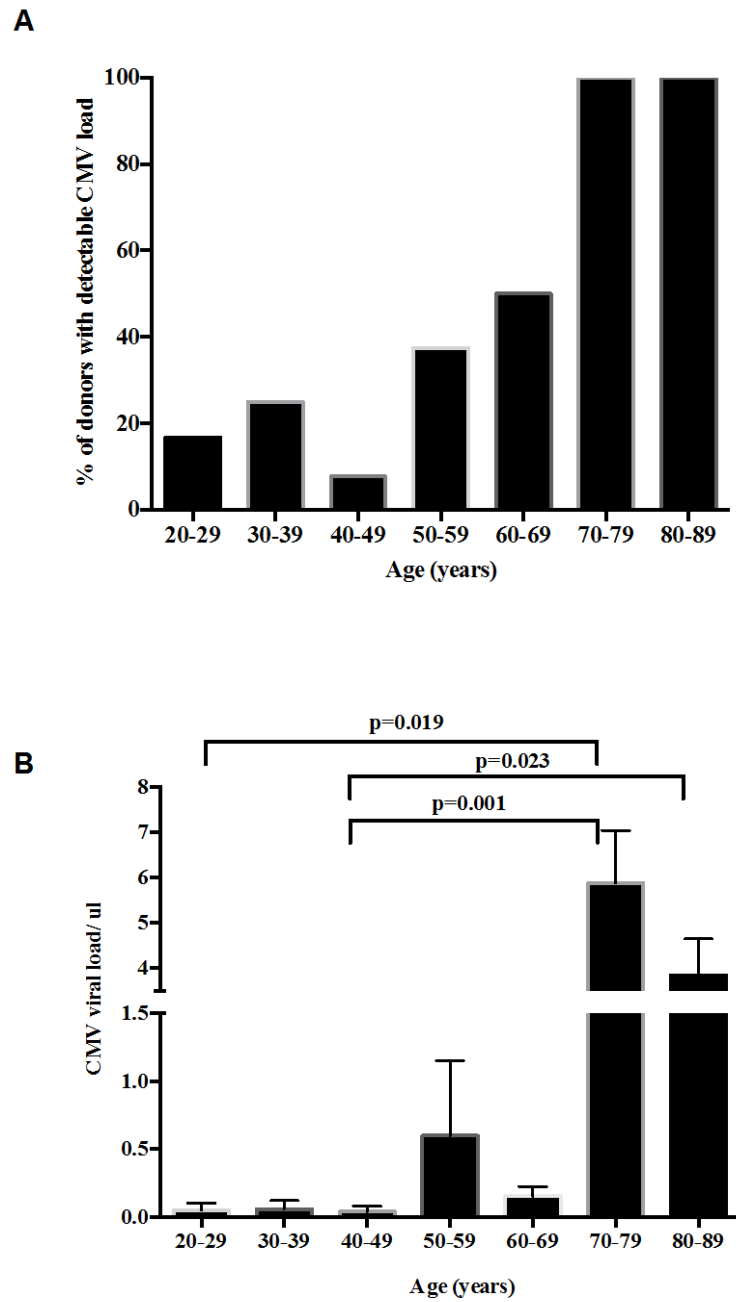
Figure 5.4

CMV viral load is detectable with increasing frequency and quantity throughout life

(A)The percentage of donors with detectable CMV DNA increased with age, such that everyone tested above the age of 70 had a detectable viral load. (B) The CMV viral load also increased with increasing age (range: 0.144 copies per ul to 9.14 copies per ul; p<0.0004).

5.3.1.3 Viral load quantified per monocyte

Next, the number of CD14 positive monocytes in each ddPCR reaction was measured by ddPCR using the copy number variation assay RPP30. RPP30 is an endogenous gene, which can be used to normalise copy number. An example of the positive and negative RPP30 droplets can be seen in Figure 5.3. When incorporating RPP30 into the reaction, once again, viral load was seen to increase substantially with increasing age. The absolute viral load varied markedly from 3 copies per 10,000 monocytes to 353 copies per 10,000 monocytes, a range of 117 fold. This value was also found to increase with age, again with a marked increase observed in donors aged over 70 years (kruskal-wallis p=0.0005). The mean viral load in donors aged below 70 years was 8.6 copies per 10,000 monocytes (SD 38), with a 29-fold increase in those over the age of 70, where the mean viral load was 249 copies per 10,000 monocytes (SD 59) (Figure 5.5A). Modelling of the data with an exponential growth curve showed that the CMV viral load doubling time was 9.6 years (R^2 =0.64) (Figure 5.5B) (Parry et al., 2016b).

5.3.1.4 The increase in CMV viral load with age is confirmed through the use of quantitative PCR

A second method was then used to confirm the observation of an increase in CMV load within monocytes in relation to age. At first, quantitative PCR (QPCR) was calibrated using an average plasmid series dilution with 50000, 10000, 2500, 500, 250, 100, 50,10, 5 and 1 copies per reaction. This produced an R^2 value of 0.983 (p<0.0001) (Figure 5.6A). Using triplicate runs, QPCR was sensitive down to a single copy of plasmid CMV per reaction. This same series of diluted plasmids was then verified using ddPCR and the absolute copy number of CMV was calculated through QuantaSoft® software. ddPCR was again capable of

detecting 1 copy of CMV per reaction indicating that results generated from ddPCR and QPCR correlate very strongly (R^2=0.995; p=<0.0001) (Fig 5.6B).

We then used QPCR to assess CMV load within the 44 healthy donor monocytes. 13 of the 16 samples positive by digital PCR were also found to be positive by QPCR (81%). 27 out of the 28 samples that were negative by ddPCR were also negative by QPCR, although one was reported positive by QPCR. The relationship between the two techniques using 13 results that were positive by both methods had a co-efficient of determination of R^2=0.626 (p=0.0013). The QPCR technique also revealed a pronounced increase in viral load in association with age (Parry et al., 2016b) (Fig 5.6C).

5.3.1.5 CMV viral load is focused within CD34+ haemopoietic cells and CD14+ monocytes

As CMV latency and peripheral carriage is thought to predominantly take place within the myeloid series, the CMV load in different cell types was next assessed. PBMCs from 5 G-CSF mobilized CMV positive donors were defrosted and washed before applying the antibodies listed in section 5.2. Using flow cytometry 6 x 10^7 cells were sorted into 4 different cell populations; these were CD34+ haemopoietic stem cells and 3 monocyte subsets CD14+, CD14+CD16+ and CD16+ (an example of gating strategy can be found in figure 5.7). A further fraction of whole PBMC was also assessed. A detectable CMV load was found in 3 of these 5 samples through the use of ddPCR and qPCR. The average viral load found in the CD34+ stem cell fraction was 0.266 copies per μl. Within the monocyte subsets, CMV was found only within the CD14+ and CD14+CD16+ subsets. For CD14+ cells, an average of 0.137 copies per μl were recorded whilst for CD14+CD16+ the average copy number was 0.352 per μl. No detectable load was found in any of the CD16+ populations (Parry et al., 2016b)
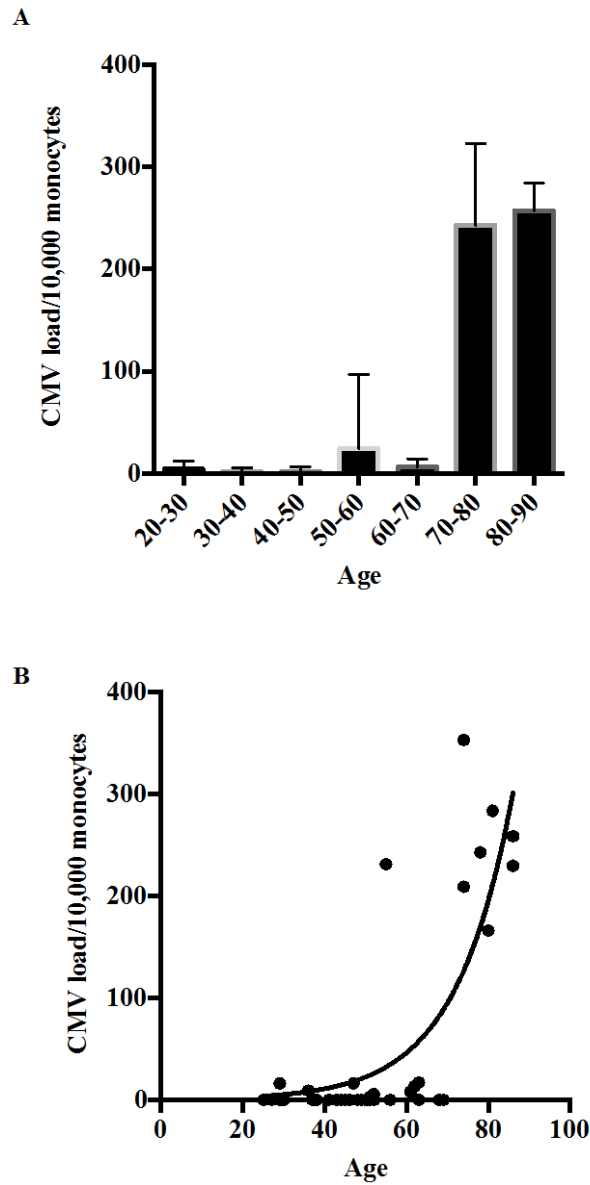
Figure 5.5

CMV viral load when quantified per 10,000 monocytes, increases with age.

A) The CMV viral load per 10,000 monocytes increases with age and is most notable over 70 years of age (kruskal-wallis p=0.0005). B) Using an exponential growth equation, CMV doubling time was found to be 9.6 years.

Figure 5.6

Q-PCR for testing CMV load in monocytes.

(A) The CMV plasmid was diluted to produce the following copies per reaction: 50000, 10000, 2500, 500, 250, 100, 50,10, 5 and 1 and ran in triplicate. Results were then logged and the co-efficient of determination was found to be 0.983 using linear regression (B) Next, the copy number of each plasmid dilution was explored using ddPCR. Again, using a log scale, the co-efficient of determination was found to be 0.995. (C) Finally, a comparison of ddPCR and QPCR values for the 13 samples found to be positive by both techniques revealed a co-efficient of determination of 0.626.

A

B

C
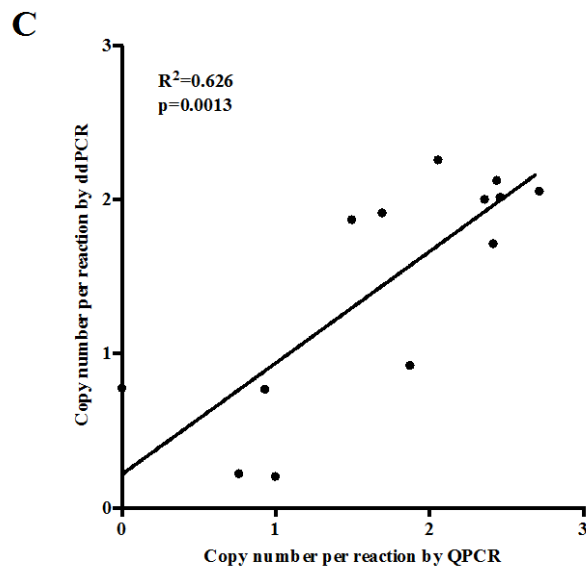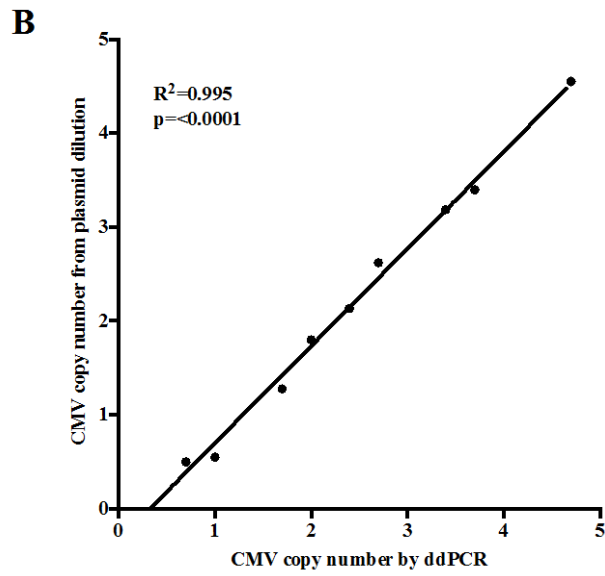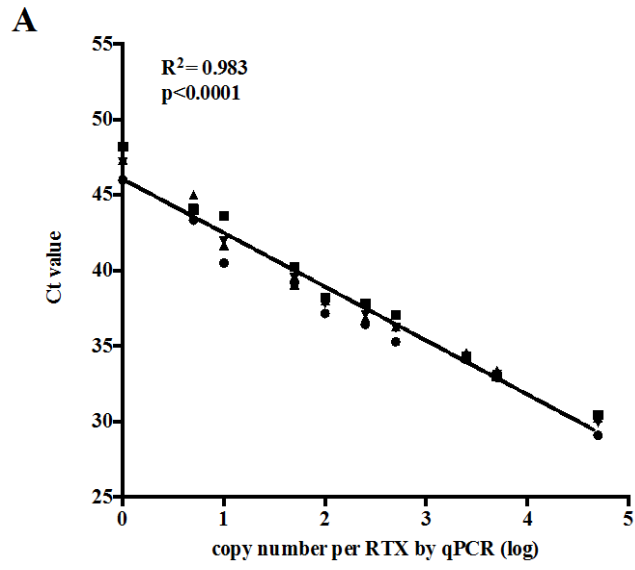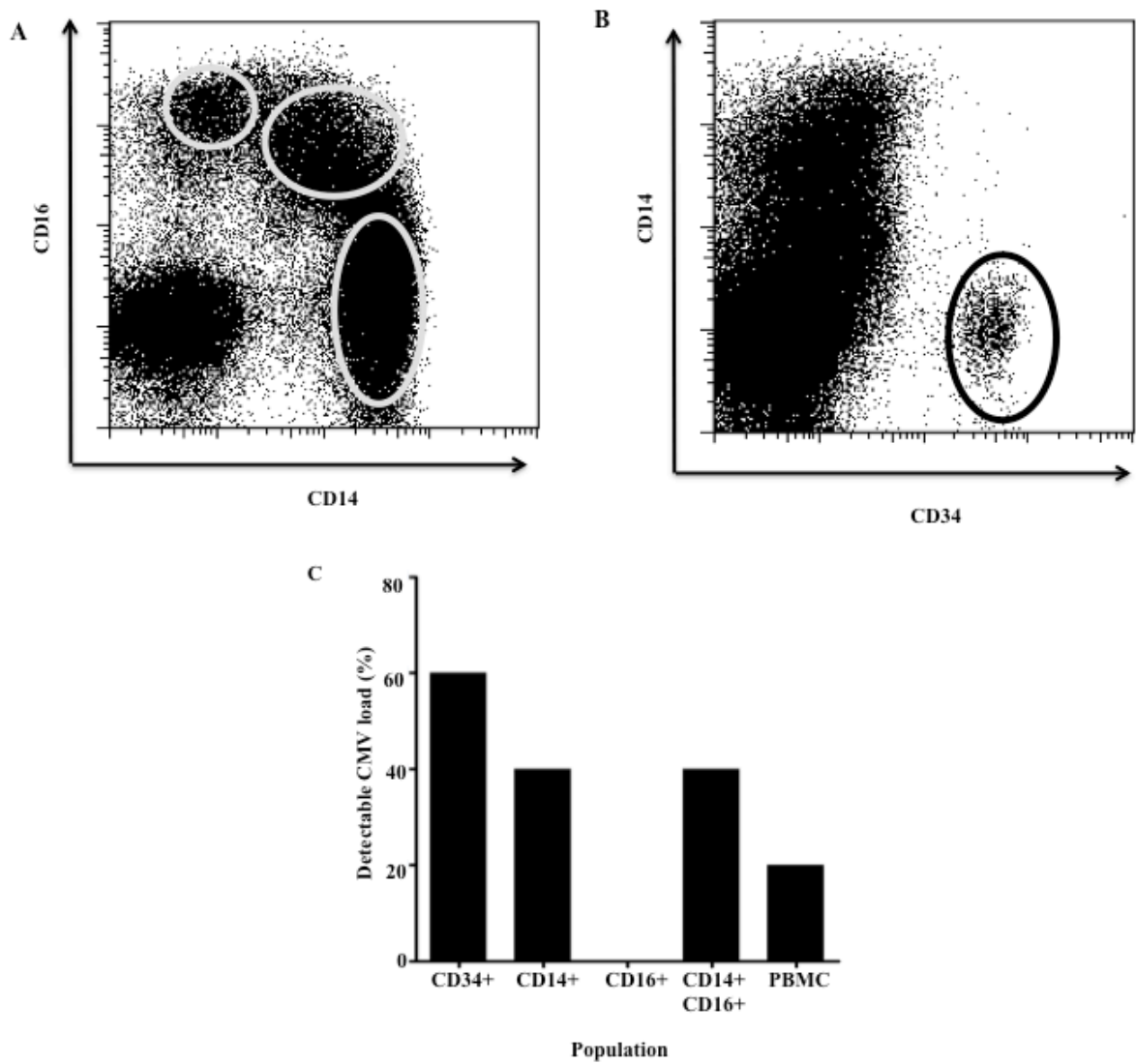
Figure 5.7

Gating strategy for cell purification by flow cytometry and the detection of CMV amongst cell subsets.

A) After excluding CD56+ PBMCs, cells were then gated for CD16+, CD14+ and CD14+CD16+ as shown. B) CD34+ cells were extracted from the whole PBMC population. C) CMV viral load was detectable amongst all subsets tested, except CD16+.

5.3.2   Patients with CLL

5.3.2.1 CMV load increases amongst patients with more advanced disease.

Next, CMV viral load was investigated amongst patients with CLL using CD14+ monocyte

DNA as described above. 25 patients (16 stage A, 1 stage B and 8 stage C) were used to

firstly assess if viral load was detectable. 24 out of 25 patients tested had a viral load

detectable. These were then compared to the 13 healthy donors aged between 60 and 90,

tested in section 1.3.1. Despite attempting to obtain samples from similar ages of healthy

donors and patients with CLL, the mean age was found to be significantly different between

HD donors tested and patients with CLL (HD: mean±SEM 72.7±2.51 Vs CLL: 65.04±2.10;

p=0.03). No difference was found between the ages of stage A patients and stage B/C (Stage

A: 64.31±2.51 Vs stage B/C 66.33±3.91; p=0.654) and similarly, no difference was observed

between HD age and stage B/C patients (p=0.166) However, a significant difference was

observed in age between stage A patients and HD, with HD being on average 8.39 years older

than the recruited stage A patients (p=0.027).

Droplet digital PCR analysis found no difference between the CMV viral load in patients with

CLL and HD (HD:166.3 IQR. 4.118-251.2 Vs. CLL 73.04 IQR. 18.49-484.3; p=0.3646).

Similarly, no difference in stage A patients with CLL and HD was also observed (HD: 166.3;

IQR 4.118-251.2 Vs. CLL: 29.18; IQR 12.45-196.7 Vs. p=0.991). However, analysis of more

advanced stage patients (stage B and C) revealed a substantial increase in CMV viral load,

with significant differences observed when compared to both stage A patients (p=0.003) and

also HD (p=0.043) (Figure 5.8). Given no significant difference in age was observed between

stage A and stage B/C patients, this suggests that the increased viral load detected is as a

result of advanced stage disease rather than an age effect.

5.3.2.2 CMV viral load is dynamic amongst patients with CLL

As the magnitude of CMV load was greater amongst patients with more advanced stage CLL, 10 patients were next investigated over a period of time (median 12 months; range 0-25 months). Using cryopreserved PBMC samples, which had been collected over a period of time, CD14+ monocytes were first enriched using magnetic beads and then DNA extracted. ddPCR was then performed and CMV viral loads compared. Interestingly, viral loads were found to be dynamic in all patients tested, with fluctuations in viral load observed in the same donors at different time points. The most striking change in CMV viral load resulted in a 214 fold difference from the lowest load detected to the highest viral load per 10,000 monocytes. Figure 5.9 illustrates the fluctuations in CMV viral load amongst 5 of the donors investigated. These fluctuations are most likely the result of episodes of intermittent subclinical viral reactivation, which may arise more frequently as disease progresses in the context of increasing immunosuppression.

5.3.2.3 CMV viral load is influenced by disease progression

During the study period, 5 patients with evidence of disease progression were investigated over a median period of 8 months (IQR. 5-17). Progression was defined as an increasing white cell count or the development of lymph nodes or splenomegaly or pancytopenia. Interestingly, patients with evidence of disease progression also showed dynamic changes in their viral load but in particular it was noted that an increase in viral load coincided with documented disease progression. Amongst the 5 patients, an average 25 fold increase in CMV load was found comparing the initial sample viral load (when disease was stable), to the last sample at which point patients showed definitive signs of disease progression (median viral load 26.01 copies/10,000 monocytes on initial sample to 660.9 copies/10,000 monocytes on the final sample; p=0.016). Figure 5.10 shows 3 examples of viral load changes and their

corresponding clinical features. These examples suggest that loss of viral control occurs more frequently with disease progression (Figure 5.10).

5.3.2.4 No viraemia was detectable on plasma sampling.

To assess if the presence of detectable viral load amongst monocytes represented reactivation of latent virus, 9 CMV positive patient plasma samples were sent to Heart of England NHS Foundation trust, together with the corresponding monocyte enriched DNA retrieved on the same day. Samples of monocyte enriched DNA were already identified to have a detectable load by ddPCR prior to sending. Samples were treated according to the standard operating procedures for PCR CMV testing at the hospital. 7 out of the 9 CD14+ monocyte samples had a detectable load by PCR, whilst none of the serum samples had any detectable CMV viral load. This suggests that the detection of CMV within its CD14+ monocytes is of latent form and not coinciding with reactivation. It also confirms results from section 1.3.1.4 that ddPCR is more sensitive than standard PCR for the detection of latent CMV viral load.

Figure 5.8

CMV viral load is greatest in patients with advanced stage disease.

No difference was observed in CMV viral load in monocyte DNA taken from patients with stage A disease compared to HD. However, stage B/C patients exhibited increased CMV viral loads, which were greater than both HD and early stage CLL patients (Mann-Whitney).

Figure 5.9

CMV load is dynamic in patients with CLL

Figure 5.9 shows a graphic representation of the dynamic changes in CMV viral load over time in patients with CLL.

Figure 5.8
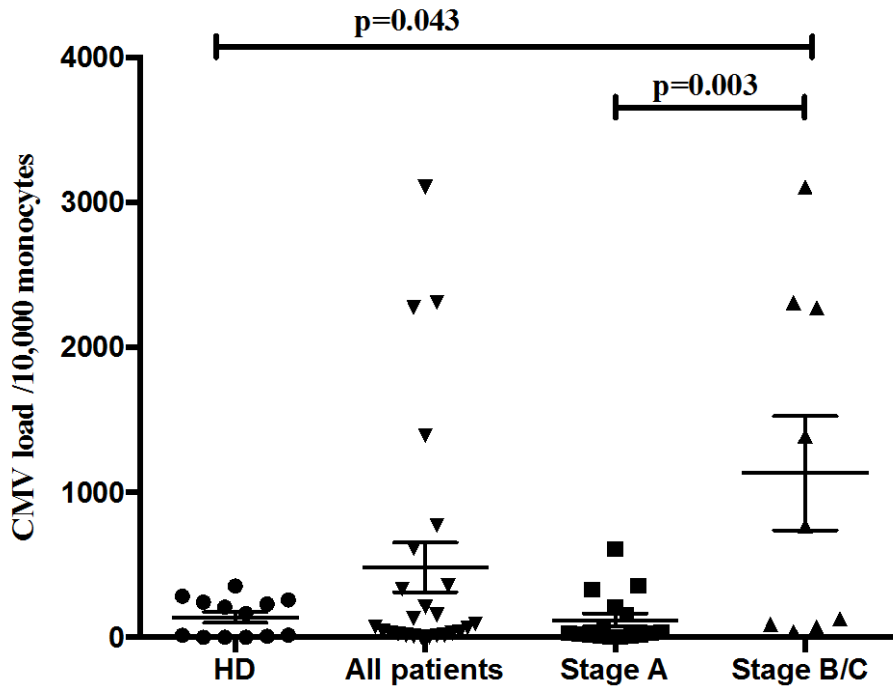
Figure 5.9
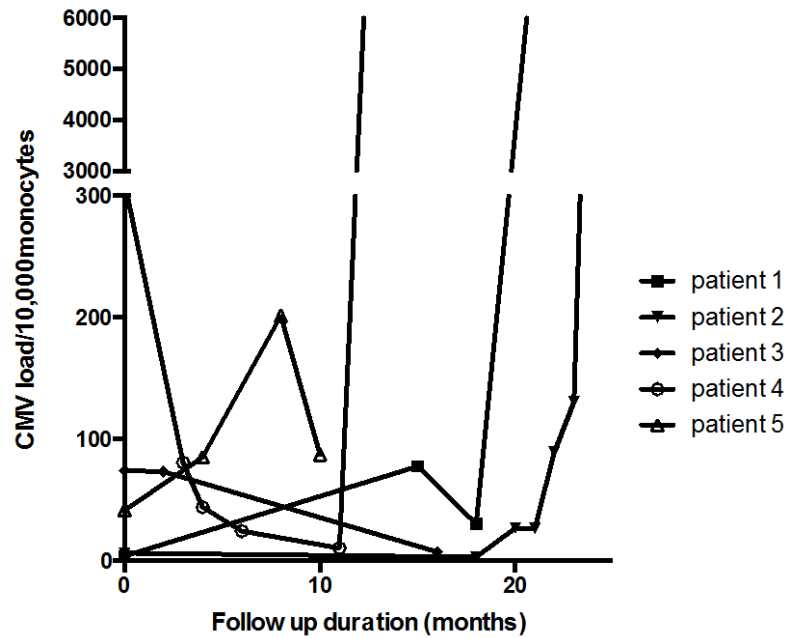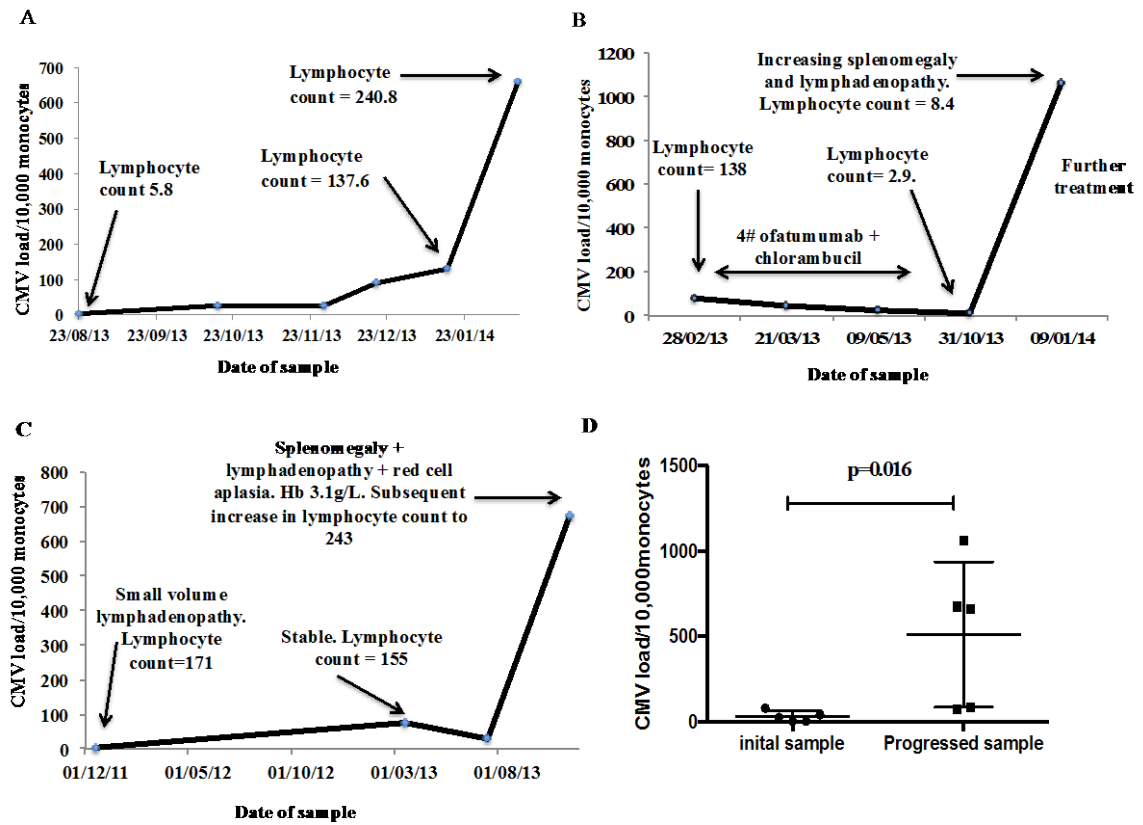
Figure 5.10

CMV load increases with disease progression

A-C) demonstrates the increase in viral load and the corresponding clinical features in 3 individual patients. D) demonstrates the 40 fold difference in viral load between initial samples taken at the point disease was stable, to the last sample where disease progression was evident (Mann-Whitney).

5.4 Discussion

Cytomegalovirus infection has been associated with mortality and morbidity in elderly people. However, the mechanisms that underlie this association are not well established. A key determinant in this regard is a greater understanding of the balance of the viral load and the host immune response during healthy ageing (Parry et al., 2016b). This work, for the first time, found that the level of cytomegalovirus viral load within blood monocytes increased markedly in elderly people but was even more pronounced in patients with advanced stage CLL.

I have described for the first time the use of digital droplet PCR to provide an accurate quantitative measure of latent viral DNA. Previous methods for detection of CMV from monocytic DNA have generally relied on nested PCR techniques, which made quantification challenging and also raised substantial problems with reproducibility (Roback et al., 2003, Parry et al., 2016b). Quantitative PCR is far more accurate but relies on interpretation of the cycle threshold of a sample against a known calibration standard. This is restricted by the lower limit of detection of the standards and the rate of amplification, which can vary between different PCR runs (Parry et al., 2016b). In contrast, digital PCR provides an absolute quantification and avoids these limitations. Our analysis included a direct comparison of ddPCR and Q-PCR and, as expected, we observed an extremely high concordance between the two technologies. However ddPCR was found to offer superior sensitivity and reliability of detection. 17 samples were found to be positive by either ddPCR or Q-PCR, 16 of these by ddPCR and 14 by Q-PCR (Parry et al., 2016b). Simiarly, PCR testing within an NHS hospital laboratory also detected CMV DNA in only 7 out of 9 (78%) CD14+ monocyte samples, whereas all 9 samples were positive by ddPCR.

Monocytes are established as the most important haemopoietic site of viral latency and carriage of CMV throughout the body (Larsson et al., 1998, Soderberg et al., 1993). In a murine model, CMV infected monocytes have also been shown to disseminate viral infection to distal sites such as salivary gland (Daley-Bauer et al., 2014). With this knowledge, Leng et al have previously used monocyte enriched DNA and found high rates of CMV detection using conventional PCR techniques (Leng et al., 2011a).

Using ddPCR, we have shown that amongst healthy donors, CMV was only detectable in a minority, with only 36% of participants having a positive detection of virus despite the presence of CMV-specific IgG antibodies, which confirmed prior infection. Interrogation of this finding revealed detection of CMV in people below the age of 50 years was actually infrequent, and was observed in just over 10% of donors tested. The lower limit of detection provided by ddPCR in our assay was for a single copy of virus within the total reaction volume (20µl) and as such a negative result indicated absent or extremely low levels of virus (Parry et al., 2016b). This low level carriage may reflect a lower intrinsic probability of viral reactivation in younger donors but is perhaps more likely to reflect the consequence of effective immune surveillance of viral replication in younger individuals (Parry et al., 2016b). The frequency of viral detection increased markedly with each decade above the age of 50 years to 37.5% and 50%, whilst all donors over the age of 70 had a detectable level. Interestingly the amount of viral DNA detected within the blood also increased substantially with age with a 29 fold increase observed between donors aged less than 70 and those over this age. This supports the work by Leng *et al* who used a non-quantitative nested PCR method to detect CMV in monocyte populations in 9 out of 16 (56%) elderly healthy donors tested. Importantly, the mean age was 83 years for participants in the study of Leng *et al* (range 72-90), which would account for the higher rate of detection compared to our cohort

(Leng X et al 2011). Overall, it appears that a gradual impairment in the ability to control CMV starts around the age of 50 years but deteriorates markedly over the age of 70.

In support of increasing viral load detection with age, the same scenario is known to arise with other human latent herpes virus infections. For example, VZV DNA by PCR has been shown to increase in PBMCs with ageing (Devlin et al., 1992) and similarly, plasma viral load detection for Epstein Barr Virus (EBV), another human herpes virus, has been shown to increase with age (Stowe et al., 2007). Together, these suggest that cumulatively the chronic burden placed on the immune system by latent infection increases with age, and the frequency of reactivation and viral latent load rises to detectable levels. The difficulty in proving this hypothesis is the inability to detect overt CMV reactivation in healthy individuals and relies on opportune sample collection. However, one means to address this in the future may be to study IgM titres, as an indication of recent reactivation or primary infection (McVoy and Adler, 1989). IgM titres correlated with monocyte viral loads may provide more evidence for a causal link between recent reactivations and the fluctuating latent viral loads observed in this work.

Importantly, this work did not address the number of CMV copies within individual monocytes. This has previously been reported to vary between 2 and 13 copies per cell (Slobedman and Mocarski, 1999). Thus, it remains uncertain if ageing is associated with an increase in the number of viral copies within each infected monocyte or if there is an increase in the proportion of infected cells (Parry et al., 2016b).

The ddPCR assay detects the level of latent viral DNA but does not assess the level of infectious virion. The detection of CMV by PCR in serum is used as a marker of CMV reactivation within a clinical setting. Using standard NHS laboratory procedures, this work did not find any evidence of CMV reactivation amongst serum samples where a

corresponding positive CMV load was detectable by ddPCR of monocyte DNA. In addition, others have previously made attempts to detect CMV virions from whole blood in CLL patients but have failed to identify any evidence of reactivation (Pourgheysari et al., 2010, Mackus et al., 2003). This suggested that viral DNA was retained within cells, with no evidence of extracellular virus and would be consistent with detection of latent virus only (Parry et al., 2016b).

I was also interested to use the sensitivity of ddPCR to examine the presence of CMV within specific subsets of the myelo-monocytic lineage. In humans, differences in carriage of CMV amongst particular monocyte subsets has not been addressed but many groups have confirmed the presence of CMV DNA in CD14 monocyte cells (Larsson et al., 1998, Soderberg et al., 1993). CD14 and CD16 can be used to delineate three major subclasses of monocyte (Ziegler-Heitbrock et al., 2010). Classical monocytes are CD14+CD16- and account for more than 70% of peripheral monocytes. They are known to be important for innate immunity. Intermediate monocytes are CD14+CD16+ and have more phagocytic properties than non-classical monocytes, which are CD14-CD16++ counterparts (Stansfield and Ingram, 2015). In this work, CMV was not detectable within CD14-CD16+ monocyte cells. This finding is in contrast to murine CMV, where CD16+ monocytes have been shown to exhibit higher levels of CMV latency than CD14+ cells (Daley-Bauer et al., 2014).

We were also interested to compare levels of CMV load within CD34+ haemopoietic stem cells from G-CSF mobilized blood donations. CMV was detected in 3 of these 5 samples, which is comparable to a previous report of 8 positive samples out of 12 using an alternative PCR methodology (Slobedman and Mocarski, 1999). This suggests that viral DNA may either pass selectively into cells that differentiate into the monocytic lineage or that some degree of viral replication occurs during myelopoiesis in order to sustain viral loads during the periods

of cellular proliferation prior to monocyte formation (Parry et al., 2016b). Interestingly, 2 of the 3 donors for which a viral load was detectable in our cohort were sibling donors aged 63 and 67 years. Information relating to age for the other donors was not available but unrelated stem cell donation is limited to those under 60 years, suggesting that even amongst G-CSF stimulated PBMCs age may play an important role in influencing the latent viral load and the ability to detect it.

Total immunoglobulin levels are known to fall with age but an increase in CMV-specific antibody has been described to increase with both healthy ageing and in the context of CLL (Ogunjimi et al., 2014, Vanura et al., 2013, McVoy and Adler, 1989, Alonso Arias et al., 2013). Ageing has also been shown to increase Varicella zoster viral (VZV) titres, another herpes virus where clinical reactivation is more overtly apparent and usually accompanied by symptoms in the ageing population (Ogunjimi et al., 2014). This paradoxical accumulation of CMV-specific immune memory may actually suppress the development of heterologous immune responses and may be a contributing factor towards immune senescence.

The steady increase in IgG CMV immunity over a lifetime, suggests that impairment in humoral immunity is not a major contributory factor towards the increase in viral load with ageing. As described in chapters 3 and 4, the T cell immune response to CMV is also markedly increased in the context of ageing and CLL, such that the virus-specific CD8+ T cell response can come to dominate the CD8+ T cell repertoire in some donors (Wikby et al., 2002,Khan et al., 2002). This profile of 'memory inflation' is also seen in murine CMV infection but relatively little is known about the specific profile of CMV proteins that drive CD8+ T cell expansion during ageing, although the importance of structural proteins such as pp65 are well documented (Khan et al., 2002, Moss and Khan, 2004a). In agreement with this, the presence of CMV DNA within monocytic cells has previously been correlated with

an increased pp65-specific T cell immunity in elderly donors (Leng et al., 2011b, Parry et al., 2016b).

One suggestion to explain the rising CMV load that is observed in both ageing and progressive CLL could be that the accumulated T cells are dysfunctional or exhausted or simply not achieving appropriate synapse formation for an adequate immunological response. (te Raa et al., 2014). As described in chapter 4, CMV specific-CD4+ T cells in patients with CLL have elevated levels of PD-1 and impaired release of cytotoxic cytokines. In addition, in healthy elderly individuals, CMV-specific CD4+ T cells have previously been shown to be driven to exhaustion, with restricted replicative capacity (Fletcher et al., 2005) and the importance of CD4+ T cell control of CMV is most evident in the overt reactivation of CMV that is observed amongst HIV patients with low circulating CD4+ cell counts. Together, these findings suggest that functioning CD4+ T cells are a necessity for the control in health of latent CMV. Whether the immunological synapse is impaired or a lifetime of subclinical reactivation are driving T cells towards sensence or exhaustion, the increased latent load observed in both elderly healthy donors and CLL patients is likely the result of some form of T cell dysfunction.

Although a definite increase in viral load was observed amongst patients with advanced stage CLL, no difference was found between healthy donors and stage A patients CLL in this work. This is perhaps surprising given the marked immunosuppression that is described in early stage, untreated patients in chapter 6. One explanation for this difference may be due to the healthy donors being on average 8 years older than the stage A CLL patients (p=0.027) and given ageing has been shown to correlate with increased viral load, this may account for why no difference was observed.

Although episodic reactivation of CMV has been assumed to be the underlying determinant that drives the observed T cell expansion in both elderly individuals and patients with CLL, attempts to amplify CMV viral load using conventional PCR have failed to identify any detectable virus or any causal link (Pourgheysari et al., 2010). For the first time, this work identifies a dynamic latent viral load is present in healthy donors and patients with CLL. The fluctuations in monocyte viral load suggests that subclinical reactivations are likely to be occurring and priming the immune system (reflected in the expansion of differentiated terminal effector memory cells) as well as influencing the latent viral load in the myelo-monocytic lineage.

It has previously been reported that CMV-specific CD4+ T cells are influenced by the stage of CLL and I have shown in chapter 3 that CMV-specific CD8 T cell responses are greater in stage C patients compared to stage A (Pourgheysari et al., 2010). My observed difference in viral load between stage A and stage C patients provides evidence for increased epitope exposure to be driving the expansion of CMV-specific T cells with disease progression. Interestingly, CMV CD4+ T cells have been reported to increase further with treatment. This work did not address CMV viral loads during or after treatment but it is feasible given the immunosuppression entailed with therapy that the frequency of subclinical reactivation would increase alongside the monocytic viral load.

5.5 Conclusion

In conclusion, these data reveal a balance has evolved between chronic CMV infection and the host immune response, which can break down during ageing. A breakdown in this symbiotic relationship appears to accelerate in the presence of advanced CLL, most likely as a result of attritional effects on chronic surveillance, immune senescence and a worsening immune suppression associated with disease progression.

6 THE IMPACT OF CMV INFECTION ON CLINICAL OUTCOME IN CLL

6.1   Introduction

6.1.1   CMV infection in healthy individuals

The prevalence of CMV infection in humans increases with each decade of life and is dramatically influenced by geographical and socio-economic factors. In developing countries up to 100% of people may be infected by the age of one, contrasting with developed countries where the prevalence in adulthood reaches anywhere between 45 and 100% (Cannon et al., 2010). As described in chapters 3 and 4, a considerable proportion of CD4+, and to a greater extent CD8+ CMV specific T cells accumulate over time and are required in order to maintain viral latency and prevent CMV reactivation (Chidrawar et al., 2009). This 'memory inflation" leads to an inversion of the CD4:CD8 T cell ratio (Olsson et al., 2000) and negatively impacts on survival in otherwise healthy CMV positive elderly individuals (Olsson et al., 2000, Wikby et al., 2005).

Increased morbidity associated with CMV infection has also been described, including an increased risk of cognitive defects (Weaver et al., 2002), hypertension (Haarala et al., 2012) and depression (Phillips et al., 2008). Furthermore, higher CMV IgG titres have been found to correlate with increased mortality (Roberts et al., 2010) and the burden of CMV infection has also been shown to impair responses to vaccination (Trzonkowski et al., 2003).

6.1.2   The immunodeficiency associated with CLL

CLL is associated with an increased morbidity and mortality from infection, as a result of a multifactorial secondary immunodeficiency. Patients with CLL demonstrate hypogammaglobulinaemia, T cell dysfunction and treatment related immune suppression (Hamblin and Hamblin, 2008). This increased susceptibility to infection is present even at an

early stage in the disease course and patients often have a history of serious infections at diagnosis (Parry et al., 2015, Moreira et al., 2013).

Hypogammaglobulinaemia is the most recognized immunodeficiency in patients with CLL and it is recommended that serum immunoglobulins level are checked at diagnosis (Oscier et al., 2012). All subclasses of immunoglobulin are affected and the deficiency becomes more apparent as disease progresses, with up to 85% of patients having demonstrable hypogammaglobulinaemia (Hamblin and Hamblin, 2008). Increased mortality is due to a propensity for infections caused by encapsulated bacteria, including *Streptococcus pneumonia,* occurs as a result (Rozman et al., 1988).

In addition to deficiencies in the humoral compartment, changes in the T cell repertoire are also important for overall prognosis in CLL, and an inverted CD4:CD8 T cell ratio is associated with disease progression (Nunes et al., 2012). The CD4:CD8 ratio is known to be lower in CMV seropositive individuals compared to CMV negative (Olsson et al., 2000). In CLL, despite the immunosuppression described, the CMV specific immune response is actually of greater magnitude than in healthy individuals (Mackus et al., 2003). This is thought to be in response to increased levels of subclinical viral reactivation and is discussed further in chapter 5 (Parry et al., 2016b). However, it is not known how the impact of CMV infection influences outcome in CLL. In this chapter, the impact of CMV on overall survival (OS) and time to first treatment (TTT) is investigated for the first time in two independent cohorts (the 'discovery' and 'confirmatory' cohorts) of newly diagnosed patients. A further study into the impact of CMV on the incidence of infections in patients with CLL in the 'vaccination cohort' is also described, alongside the use of functional antibody (Fnabs) testing as a marker for global immune competence and vaccine efficacy in patients attending CLL clinic (Phillips et al., 2006). Functional antibody testing (Fnab) is also referred to as specific

antibody testing and quantifies antibody levels to different bacteria in human serum, including

different serotypes, indicating the efficiency of antibody responses against common bacterial

infections and vaccine responsiveness.

6.2 Methods

6.2.1 Recruitment to the Discovery and Confirmatory Cohorts.

Three hundred and forty-seven patients in the discovery cohort were assessed as part of the

ongoing prospective cohort study of NHL patients from the Molecular Epidemiology

Resource of the University of Iowa/Mayo Clinic Lymphoma Specialized Program of

Research Excellence (SPORE). This study was reviewed and approved by the Human

Subjects Institutional Review Board at the Mayo Clinic and the University of Iowa, and

written informed consent was obtained from all participants in accordance with the

Declaration of Helsinki. Since September 2002, enrollment was offered to consecutive newly

diagnosed patients with CLL, evaluated at Mayo Clinic Rochester or the University of Iowa

within 9 months of diagnosis. All patients were US residents aged 18 years and older.

Exclusion criteria included known HIV infection and unwillingness or inability to provide

written informed consent. Patients fulfilled IWCLL criteria and/or fulfilled the World Health

Organization criteria for the small lymphocytic lymphoma variant (SLL) variant of CLL.

Baseline clinical, laboratory, and treatment data were abstracted from medical records and

participants provided peripheral blood serum samples and were followed every 6 months for

the first 3 years, and then annually thereafter. Time to first treatment and overall survival

were verified through medical record review.

For the confirmation cohort, 236 newly diagnosed patients with CLL were enrolled from

clinics at the University Hospital of Wales and Llandough Hospital. All patients met the

IWCLL criteria for CLL. Samples were taken within 12 months of diagnosis and prior to any

treatment. Data for disease progression and mortality was collected on an annual basis and verified through medical records. For both cohorts, prognostic testing, including immunoglobulin heavy chain variable [IGHV] region gene mutation analysis, ZAP-70 status, CD38 status, CD49d status, and cytogenetic abnormalities assessed by interphase FISH testing, were performed. Adverse FISH results were defined by the presence of 17p deletion or 11q deletion. Serum immunoglobulin measurement was only available at the time of sample collection in the discovery cohort. The clinical characteristics for these two prospective cohorts are shown in table 6.1.

6.2.2   Recruitment to the Vaccination Cohort.

Fifty-six consecutive CLL patients with a median age of 72 were recruited over 3 weeks at BHH and QEHB Hematology clinics in June 2013 and clinical characteristics are shown in table 6.1. Socio-demographic and patient outcome at the most recent point of contact were documented from patient questionnaires and electronic records. None of the patients were receiving immunoglobulin replacement therapy at the time of recruitment. The number of hospital-recorded infections was defined by the presence of a positive microbiology result since the date of diagnosis, or a diagnosis of infection written in a clinical correspondence. Vaccination history was obtained from Primary Care records. The patient questionnaire included health and infection history (Appendix 1). For an aged matched comparison of functional antibodies, results from a control population of 162 unvaccinated healthy elderly patients, with a median age of 74.6 (66.2-83.0), recruited from five medical practices in Birmingham (UK) between September and November 2003, were used (Phillips et al., 2006). Both patients and age matched healthy participants, donated 9 ml of peripheral blood into vacutainer tubes containing clot activator for separation of serum prior to Fnab analysis.

Table 6.1

Characteristics of CLL patients in the discovery, confirmatory and infection cohorts.

	Discovery cohort (n=347)	Confirmatory cohort (n=236)	Infection cohort (n=56)
Median age at diagnosis (years)	62 (37-91)	65.1 (24-99)	72 (65-78)
Male sex	237 (68%)	150 (64%)	30 (54%)
Binet stage A	180	182	49
Binet stage B	155	25	1
Binet stage C	11	27	6
Missing	1	2	0
CD38 positive	82	99	14
CD38 negative	218	117	28
CD38 missing	47	20	14
Zap70 positive	93	82	2
Zap 70 negative	203	134	3
Zap 70 missing	51	20	51
IGHV mutated	176	125	8
IGHV unmutated	104	40	7
IGHV missing	67	71	41
CD49d positive	64	102	0
CD49d negative	131	69	0
CD49d missing	152	65	56
FISH			
Normal	76	61	27
13q-	120	37	9
12+	50	6	4
11q-	22	15	4
17p-	11	5	2
Other	5	2	1
Missing	63	110	13

6.2.3 CMV IgG ELISA

The CMV serostatus of all participants was ascertained using the ELISA technique described in chapter 2.

6.2.4 Functional antibody testing

Alongside immunoglobulin measurements, functional antibody testing was performed on the vaccination cohort as described in Chapter 2. Fnabs were determined against 19 antigens of organisms that are commonly vaccinated against. Thresholds were set as per WHO criteria; $\geq 0.35 \mu g/ml$ in two thirds of 12 pneumococcal serotypes tested for; $>2 \mu g/ml$ for each of meningococcal A, C, W135 and Y; $>0.1 \mu g/ml$ for both tetanus and diphtheria and $>1 \mu g/ml$ for Haemophilus influenzae type B.

6.2.5 Statistical analysis

6.2.5.1 Discovery and confirmatory cohort statistics

Chi-square testing (and Fisher's exact test where appropriate) were used to assess the association between CMV seropositivity, demographic, clinical and prognostic factors in CLL patients. Both the discovery and confirmation cohorts were characterised using descriptive statistics. For compatibility between datasets, Rai staging for the discovery cohort was converted to Binet staging. Rai 0/1 representing Binet A, Rai stage II representing Binet B and Rai stage III/IV representing Binet stage C. TTT was defined as the time from diagnosis to disease progression requiring treatment. OS was defined as the time from diagnosis to death due to any cause. Patients without an event or death were censored at time of last known follow-up. Kaplan-Meier survival curves and Cox proportional hazards regression models were used to assess the association between CMV positivity and the outcomes of interest. Cox models were adjusted for demographic and CLL prognostic factors which were

found to impact significantly on survival and TTT in univariate analysis. An analysis of the continuously distributed CMV IgG ELISA values was also performed. CMV titre was transformed to the base 2 log and entered as a covariate into cox regression analysis.

6.2.5.2 Vaccination cohort statistics

Student's T-test was used to compare data between patient groups when normally distributed and Mann-Whitney testing where normality was not demonstrated. Univariate assessment of the relationships between prognostic factors and time to first infection were carried out using Kaplan-Meier plots, with log rank tests used to evaluate significant differences. Functional antibodies were normally distributed when log transformed and are presented as geometric means.

6.3   Results

6.3.1   Clinical characteristics and demographics by CMV status

6.3.1.1 Discovery cohort

Participant CMV status was obtained by ELISA and associations between HCMV seropositivity and CLL prognostic factors were firstly examined. Of the 347 participants, 198 (57%) were CMV seropositive and 149 (43%) were CMV seronegative. CMV positive patients were significantly older at the time of diagnosis with a median age of 64 years (range 37 to 87) compared to 60 years in CMV seronegative individuals (range 37 to 91) (p<0.0001). There was no association between CMV seropositivity and any other demographic or clinical characteristic (Binet stage, sex, ECOG performance score or absolute lymphocyte count) or prognostic markers (ZAP-70, CD38, IGHV, CD49d, FISH) (Table 6.2).

6.3.1.2 Confirmatory cohort

Of the 236 patients with CLL in this cohort, 179 (76%) were found to be CMV seropositive. Unlike the discovery cohort, CMV serostatus was not associated with age at diagnosis. Nor was it associated with any of the other demographic, clinical or prognostic markers found in the confirmation cohort (Table 6.3).

Table 6.2

Clinical characteristics of the discovery cohort by CMV status.

Variable	Categories	CMV negative (n=149) (%)	CMV positive (n=198) (%)	Total (n=347)	P value
Age at diagnosis (years)	N	149	198	347	p<0.0001
	Median	60.0	64.0	62.0	
	Range	37.0 – 91.0	37.0 – 87.0	37.0 – 91.0	
Gender	Female	43 (28.9)	67 (66.2)	110	p=0.324
	Male	106 (71.1)	131 (33.8)	237	
BINET stage	A	71 (47.7)	109 (55.3)	180	p=0.320
	B	72 (48.4)	83 (42.1)	155	
	C	6 (4.0)	5 (2.5)	11	
	Missing	0	1	1	
CD38	Positive	34 (25.8)	48 (28.6)	82	p=0.587
	Negative	98 (74.2)	120 (71.4)	218	
	Missing	17	30	47	
ZAP-70	Positive	42 (32.8)	51 (30.4)	93	p=0.652
	Negative	86 (67.2)	117 (69.6)	203	
	Missing	21	30	51	
IGHV	Mutated	81 (35.2)	95 (61.3)	176	p=0.546
	Unmutated	44 (64.8)	60 (38.7)	104	
	Missing	24	43	67	
CD49d	Positive	23 (27.7)	41 (36.6)	64	p=0.191
	Negative	60 (72.3)	71 (63.4)	131	
	Missing	66	86	152	
BadFISH	Yes	15 (12.5)	18 (11.4)	33	p=0.777
	No	105 (87.5)	140 (88.6)	245	
	Missing	29	40	69	
FISH	Normal	30 (24.4)	46 (28.6)	76	p=0.211
	13q	50 (40.7)	70 (43.5)	120	
	Trisomy 12	25 (20.3)	25 (15.5)	50	
	11q	13 (10.6)	9 (5.6)	22	
	17p	2 (1.6)	9 (5.6)	11	
	Other	3 (2.4)	2 (1.2)	5	
	Missing	26	37	63	
Titre	N	149	198	347	p<0.0001
	Mean (SD)	1.4 (2.0)	772.1 (1723.5)	441 (1355.4)	
	Median (IQR)	0.4 (0.0 – 2.2)	227.1 (113.8 – 503.4)	72.6 (0.8 – 260.3)	
IgG	N	95	95	232	p=0.632
	Mean (SD)	818.3 (281.3)	854.4 (316.0)	839.6 (302.2)	
	Median	820.0	806.0	814.0	
	Range	190.0 – 1750.0	189.0 – 2220.0	189.0 – 2220.0	
	Missing	54	61	115	

Table 6.3

Clinical characteristics of the confirmatory cohort by CMV status

Variable	Categories	CMV negative (n=57) (%)	CMV positive (n=179) (%)	Total (n=236)	P value
Age at diagnosis (years)	N	57	179	236	p=0.809
	Median	65.0	67.0	65.1	
	Range	24 – 87	28 - 99	24 - 99	
Gender	Female	15 (26.3)	71 (39.7)	86	p=0.068
	Male	42 (70.7)	108 (60.3)	150	
BINET stage	A	47 (82.5)	135 (76.3)	182	p=0.655
	B	5 (8.8)	20 (11.3)	25	
	C	5 (8.8)	22 (12.4)	27	
	Missing	0	2	2	
CD38	Positive	20 (40.0)	79 (47.6)	99	p=0.345
	Negative	30 (60.0)	87 (52.4)	117	
	Missing	7	13	20	
ZAP-70	Positive	21 (41.2)	61 (37.0)	82	p=0.588
	Negative	30 (58.8)	104 (63.0)	134	
	Missing	6	14	20	
IGHV	Mutated	27 (75.0)	98 (76.0)	125	p=0.905
	Unmutated	9 (25.0)	31 (24.0)	40	
	Missing	21	50	71	
CD49d	Positive	23 (60.5)	79 (59.4)	102	p=0.901
	Negative	15 (39.5)	54 (40.6)	69	
	Missing	19	46	65	
BadFISH	Yes	4 (12.9)	16 (16.8)	20	p=0.602
	No	27 (87.1)	79 (83.2)	106	
	Missing	26	84	110	
FISH	Normal	16 (51.6)	45 (47.4)	61	p=0.477
	13q	10 (32.3)	27 (28.4)	37	
	Trisomy 12	0 (0.0)	6 (6.3)	6	
	11q	2 (6.5)	13 (13.7)	15	
	17p	2 (6.5)	3 (3.2)	5	
	Other	1 (3.3)	1 (1.1)	2	
	Missing	26	84	110	
Titre	N	57	179	236	p<0.0001
	Mean (SD)	0.26 (0.85)	497.4 (2060.5)	377.3 (1806.0)	
	Median (IQR)	0.0 (0.0 – 0.0)	202.1 (103.9 – 357.1)	128.3 (24.1 – 256.6)	

6.3.2   CMV infection and its impact on overall survival

6.3.2.1 CMV infection and its impact on overall survival in the discovery cohort.

The median follow up time for the discovery cohort was 3 years (IQR 2.4-4.2) and at the point of last data collection 68 participants (20% (68/347)) had died. 49 deaths (25%) were recorded for the CMV positive group and 19 deaths (13%) were noted in the CMV negative group. Overall survival (OS) was defined as the time from diagnosis to death due to any cause. Patients without an event or death were censored at time of last known follow-up. Kaplan-Meier survival curves and Cox proportional hazards regression models were used to assess the association between HCMV positivity and the outcomes of interest. Overall, patients found to be CMV positive had a significantly inferior OS, with the risk of death at any time point more than twice that observed in CMV negative patients (HR 2.28, 95% CI: 1.34 to 3.88; p=0.0024) (Figure 6.1A). Age, Binet Stage, ZAP-70, FISH, IGHV, CD38, and CD49d were also found to impact significantly on survival in univariate analysis (Table 6.4). These demographic and clinical variables were then incorporated into multivariate modeling where the risk was attenuated, with only age (HR 1.12 (95% CI: 1.06 to 1.19); p<0.0001) and unmutated IGHV (HR 2.78 (95% CI: 1.07 to 7.23); p=0.036) remaining significant as independent risk factors for OS. No difference in survival was seen between CMV positive and negative participants in the multivariate model (HR: 0.61, 95% CI: 0.22 to 1.69; p=0.343).

Table 6.4

The impact of clinical variables on overall survival in CLL in the discovery cohort.

Variable	Cox hazards regression (p value)	Hazard ratio (95% CI)
Age	0.003	1.05 (1.02 to 1.08)
Gender (male)	0.143	1.69 (0.84 to 3.41)
BINET stage A (compared to C)	0.030	0.20 (0.04 to 0.85)
BINET stage B (compared to C)	0.111	0.30 (0.07 to 1.32)
CD38 (positive)	<0.0001	3.74 (1.91 to 7.34)
ZAP 70 (positive)	0.019	2.11 (1.13 to 3.94)
IGHV (unmutated)	0.001	3.28 (1..60 to 6.75)
CD49d (positive)	0.001	4.18 (1.75 to 9.95)
Abnormal FISH	<0.0001	4.35 (1.94 to 9.73)
IgM	0.196	1.00 (0.99 to 1.00)
IgA	0.108	1.00 (0.99 to 1.01)
IgG	0.372	1.00 (0.99 to 1.00)

6.3.2.2 CMV infection and its impact on overall survival in the confirmatory cohort

The median follow up time of the confirmation cohort was 7 years (IQR: 4 to 10.2 years). Of 236 patients in this cohort, 109 (46%) had required treatment at the point of last follow up and 93 had died (39%). As noted in the discovery cohort, a shorter median overall survival of 10.6 years was noted in CMV positive participants (95% CI: 8.4 to 12.5) compared to 15.9 years (95% CI: 6.9 to 25.0) in CMV negative participants although this did not reach statistical significance on either univariate (HR 1.45, 95% CI: 0.86 to 2.43; p=0.158) or multivariate analysis (HR 0.96, 95% CI: 0.57 to 1.63; p=0.882).

6.3.3   CMV status and impact on time to first treatment in CLL

6.3.3.1 CMV infection and its impact on time to first treatment in the discovery cohort

The relationship between CMV infection and the time to first treatment (TTT) was next investigated. TTT was defined as the time from diagnosis to disease progression requiring treatment. This information was available on 322 of the original 349 (92%) patients in the discovery cohort. The median time to first treatment was 7.4 years and at the last follow up, 115 of the 322 patients (36%) were confirmed to have received treatment for CLL. A log rank test for differences between CMV positive and negative participants demonstrated no difference in TTT in either a univariate model (HR=0.90, 95% CI: 0.62 to 1.30; p=0.560) or a multivariate model adjusted for age, Binet Stage and prognostic variables (HR=1.12, 95% CI: 0.68 to 1.84; p=0.651). Binet stage C compared to stage A (HR= 0.33, 95% CI: 0.01 to 0.09; p<0.0001) and stage B (HR= 0.11, 95% CI: 0.04 to 0.28; p<0.0001), as well as expression of

CD49d (HR 2.05, 95% CI: 1.16 to 3.62; p=0.013) remained significant predictors of TTT after adjusting for other variables in the multivariate model (Figure 6.1B).

6.3.3.2 CMV status and impact on time to first treatment in the confirmatory cohort

Binet stage, CD38, CD49d, ZAP-70, IGHV and adverse FISH status were all found to impact significantly on TTT in univariate testing. However, data was complete for prognostic factors in only 68 patients, of which 44 participants required treatment. No association between CMV status and TTT was found once these prognostic variables were adjusted for in multivariate modelling (HR 1.13, 95% CI: 0.50 to 2.55; p=0.766). Only Binet stage C compared to stage A (HR 0.43, 95% CI: 0.12 to 0.14; p<0.0001) and stage B (HR: 0.29 (0.09 to 0.93); p=0.037) were significant independent predictors of time to treatment.

6.3.4   CMV titre and clinical outcome

Using Log2 CMV titre as a continuous variable, the magnitude of the IgG CMV response was next examined for any relationship with OS or TTT. Data were available on 198 CMV seropositive CLL patients within the discovery cohort. 49 deaths had occurred within this cohort. A trend was observed between increasing antibody titre and reduced survival, however this did not reach statistical significance (HR 0.95, 95% CI: 0.82 to 1.09; p=0.460). Of the 75 deaths amongst the 179 CMV positive participants in the confirmation cohort, again no relationship was seen between CMV titre and OS (HR 1.02, 95% CI: 0.88 to 1.19; p=0.793).

Similarly, no significant associations were seen in either cohort between CMV titre and time to treatment (TTT). Given the similar outcome data for CMV seropositivity on OS and TTT in both cohorts, and in an attempt to increase the number of CMV positive individuals

available for statistical analysis, the datasets were pooled and used to assess whether the titre of CMV IgG as a continuous variable impacts on overall survival or TTT. Data from 337 CMV positive individuals were analysed, of which 124 deaths were recorded during follow up, but again no relationship was found between CMV titre and overall survival (HR 1.01, 95% CI: 0.91 to 1.13; p=0.800) or time to treatment (HR 1.00, 95% CI: 0.91 to 1.10; p= 0.972).
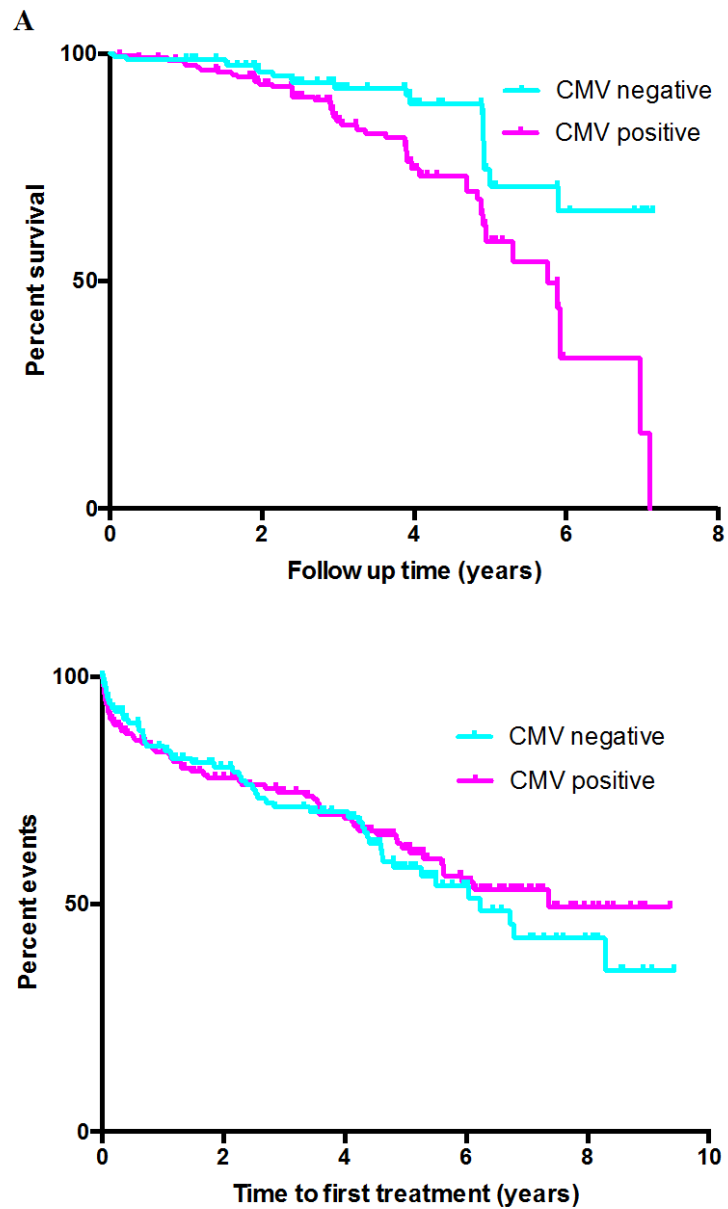
Figure 6.1

The impact of CMV status on overall survival and time to first treatment in the Discovery Cohort.

The overall survival and time to first treatment was investigated in patients with CLL based on seropositivity for CMV. No significant difference was found in overall survival between CMV positive and CMV negative patients (A) (HR: 0.61; p=0.343) or the time to first treatment (B) (HR 1.13; p=0.766). (Proportional hazards ratio)

6.3.5 Investigating infection history and its relationship with clinical characteristics.

6.3.5.1 Total serum IgG predicts infection at diagnosis and respiratory symptoms.

Of the 56 patients recruited to the vaccination cohort, 35 were Binet stage A and untreated at the time of recruitment. The remaining 21 patients had received at least one line of therapy but were at least 1year post any treatment. The median follow up time from diagnosis was 7.8 years (IQR 3.5-9.9). 31 patients (55%) had one or more documented infections, and 15 patients (27%) had required at least one hospital admission due to infection. Serum immunoglobulins were measured at the time of recruitment and were also measured at diagnosis in 36 patients. The median IgG concentration within the cohort at the time of recruitment was 7.6g/l (IQR 5.08-9.01) and patients with a low IgG (<6g/L) had significantly more hospital-recorded infections than those with IgG levels within the normal range (p=0.04) (Figure 6.2A). Even amongst the Binet stage A patients, who had never required treatment for their disease, those with one or more infection(s) had significantly lower IgG concentrations than patients who did not suffer infections (6.3 g/l vs 9.0 g/l, p = 0.037). The median IgG level from the 36 patients who had their immunoglobulins measured at the time of diagnosis was 8.6g/L. 14% had evidence of hypogammaglobulinaemia at this initial time point (IgG<6 g/l). Using Kaplan-Meier plots, with log rank tests to evaluate significant differences, patients with an IgG <6g/l at diagnosis were found to have a shorter time to first infection (44 months vs 108 months; p=0.01) whereas CD38 expression, low IgA/M, stage of disease, gender and age had no such association. Given the lack of other identifiable significant variables for modeling, multivariate analysis was not performed.

Forty-one of the 56 patients (73%) completed the infection questionnaire. 40% (21/56) of the documented infections were respiratory in origin and the total IgG level was strongly correlated with reported symptoms. In particular, patients who reported a cough 'most or every day(s)' had median IgG levels of 6.9 g/l compared to 8.6 g/l in patients reporting a cough as 'never or rare' (p = 0.05) (Figure 6.2B). In relation to sputum production, median IgG was 6.1 g/l in patients reporting sputum production 'most or every day(s)' compared to 8.6 g/l in patients in whom sputum production was 'never or rare' (p = 0.05) (Figure 6.2C). In relation to the specific respiratory infections that were encountered, 14 sputum samples had positive bacterial microbiology and of these 3 were pneumococcal and 3 were *Haemophilus influenzae* infections, serotypes of which were unknown.

Interestingly, treatment for CLL was seen to have a relatively modest impact on antibody responses. An association with total IgG concentration was indeed observed as the median IgG was 7.4 g/l in untreated patients compared to 5.29 g/l in those who had received some form of treatment (p=0.037). However, median IgA and IgM levels were similar between the two groups (Figure 6.3).
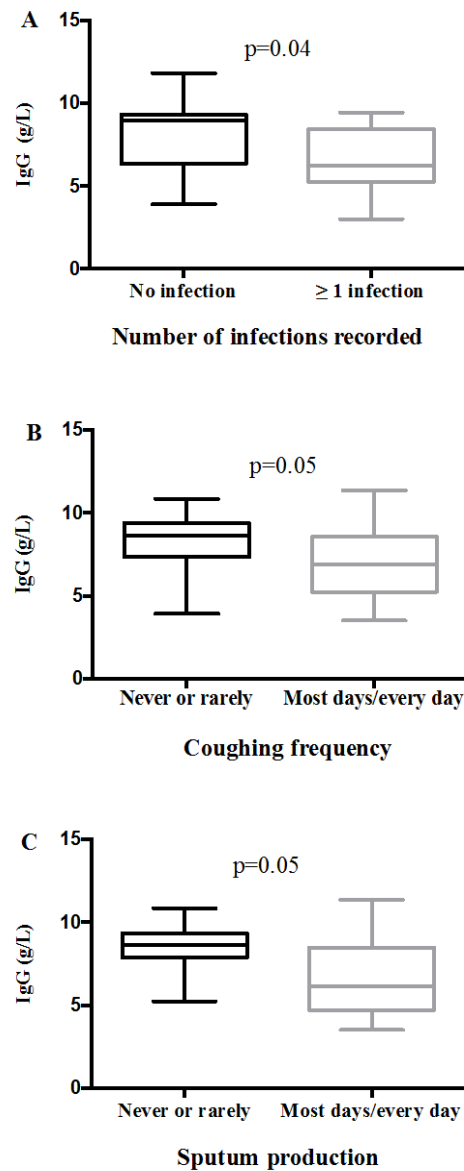
Figure 6.2

The impact of IgG levels on clinical symptoms and infection history.

Box plot diagrams demonstrating; A) those with more than one hospital documented infection had a lower IgG level than those with no infection history (n=56). Of the 41 participants who completed the questionnaire, those with a low IgG reported an increased coughing frequency (B) and sputum production (C) compared to those with low IgG (Mann-Whitney).
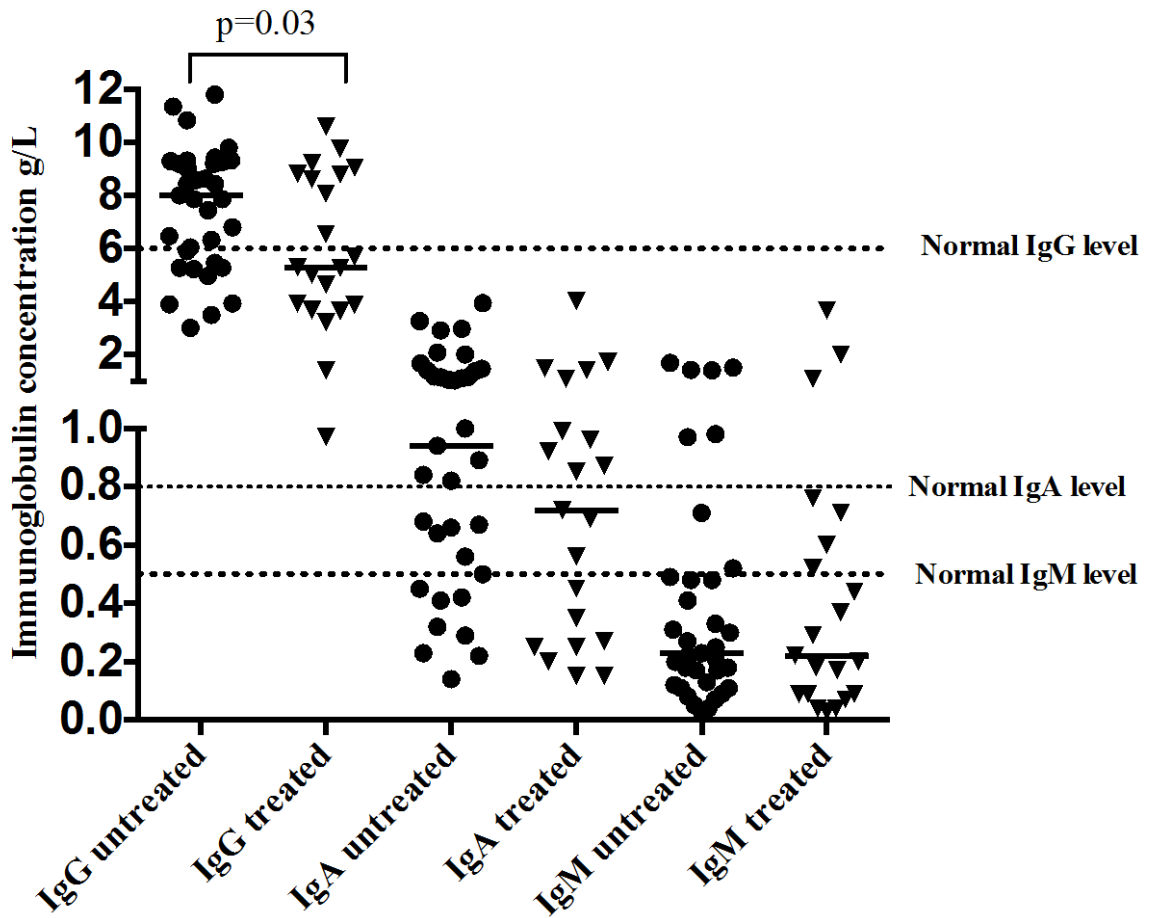
Figure 6.3

The impact of treatment on total immunoglobulins

Total serum immunoglobulins were investigated in patient with CLL and analysed according to treatment history. No difference in the levels of IgA or IgM were observed between patients who had received treatment and those untreated. In contrast, a significant difference was noted in IgG levels, with patients previously treated having a significantly lower serum IgG compared to those untreated (7.93±0.57 g/L vs 6.00± 0.62g/L; p=0.03) (Mann-Whitney).

6.3.5.2 The impact of CMV on infection history

CMV status was ascertained by ELISA in all 56 patients in the cohort. 15 patients were CMV negative (27%), whilst 41 were CMV positive (73%). Firstly, total IgG levels were investigated. No difference was found between CMV positive and CMV negative participants in their total IgG levels at the point of recruitment to the study (CMV negative IgG 5.46 g/l Vs 8 g/l in CMV positive participants; p=0.230). Neither was there any significant difference in CMV titre between those patients deficient in IgG compared to those with normal IgG levels (titres: 72 vs 136; p=0.104). Similarly, there was no difference in the average number of infections CMV positive participants had (1) compared to CMV negative participants (1) (p=0.968). Using log rank testing, no difference was found between CMV positive participants (median 81 months) and CMV negative participants (median 116 months) in their time to first infection, p=0.663 (Figure 6.4).
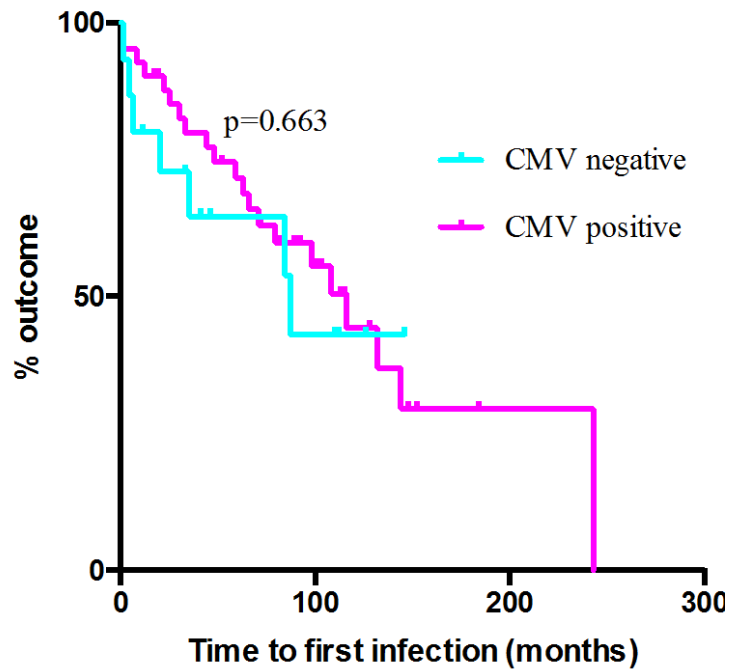
Figure 6.4

CMV does not impact on time to first infection.

No difference in time to first infection was observed between CMV positive and CMV negative patient with CLL (CMV negative 116 months vs 81 months in CMV positive patients; p=0.663).

6.3.6 Functional antibody measurements.

6.3.6.1 Low Fnabs are present in CLL patients with a normal total IgG.

Functional antibodies were next measured on the total cohort. These were then compared to healthy age-matched donors, who were unvaccinated. Overall, functional antibody levels in patients with CLL were very low compared to HD. In particular, significant differences were observed in the titer of antibodies against 16 of the total 19 Fnabs tested (Table 6.4). In relation to specificity for pneumococcal serotypes, for which 12 are tested for, protective antibody levels were seen in only 3 serotypes in patients with CLL, compared to 9 serotypes within HD (Table 6.4). Notably, those with hypogammaglobulinaemia (IgG <6g/l) were particularly at risk and protected against a median of only 2 serotypes compared to 5 in those with IgG within the normal range (p=0.001) (Figure 6.5).

Overall, within the group with hypogammglobulinaemia, Fnab concentrations were lower for 18 of the 19 measured antigens compared with patients with an IgG within the normal range. In relation to protection from pneumococcal disease, 79% (27/34) of patients with a normal IgG concentration had inadequate Fnab responses compared to virtually every patient with hypogammaglobulinaemia (21/22; 96%).

Similarly, Fnab results were found to be below protective levels in patients with a normal IgG for the other antigens tested; Men A: n=13 (38%), Men C: n=33 (97%); Men W n=28 (82%), Men Y n= 32 (94%), Tetanus n=13 (38%), Diphtheria n= 26 (96%) and Hib n=14 (41%). Interestingly and similar to the total serum immunoglobulin findings in section 1.3.5.1, there were few significant differences between Fnab responses in the treated and untreated cohorts; only two pneumococcal and one meningococcal serotype were found to be statistically different between the two groups (Pn4 P=0.01; Pn7F P=0.03; Men A p=0.02).

Table 6.5

The average pneumococcal responses by serotype in patients with CLL according to vaccination history and in healthy age matched donors.

	CLL Pneumovax n=37 Geomean (95% C.I.)		CLL No Vaccine n=13 Geomean (95% C.I.)		CLL Total n=56 Geomean (95% C.I.)	>65 Years Controls n=162 Geomean (95% C.I.)
Pn1	0.06	(0.03-0.16)	0.14	(0.04-0.49)	0.04 (0.02-0.07)	0.13 (0.10-0.16)*
Pn3	0.04	(0.02-0.12)	0.05	(0.02-0.11)	0.03 (0.02-0.05)	0.32 (0.25-0.40)*
Pn4	0.03	(0.02-0.05)	0.10	(0.04-0.35)	0.04 (0.03-0.06)	0.20 (0.17-0.24)*
Pn5	0.08	(0.02-0.23)	0.12	(0.04-0.35)	0.08 (0.04-0.14)	**0.92 (0.72-1.17)***
Pn6B	0.08	(0.02-0.25)	0.17	(0.07-0.39)	0.07 (0.04-0.11)	**0.66 (0.53-0.81)***
Pn7F	**0.52**	**(0.23-1.20)**	**0.76**	**(0.37-1.57)**	**0.40 (0.26-0.63)**	**0.79 (0.65-0.97)***
Pn9V	0.31	(0.16-0.62)	**0.38**	**(0.17-0.87)**	0.25 (0.17-0.37)	**0.53 (0.41-0.68)***
Pn14	**0.91**	**(0.32-2.57)**	**2.09**	**(1.01-4.35)**	**1.04 (0.64-1.68)**	**0.93 (0.72-1.20)**
Pn18C	0.26	(0.13-0.54)	**0.91**	**(0.43-1.94)**	**0.41 (0.28-0.60)**	**1.30 (1.00-1.71)***
Pn19A	0.31	(0.09-1.14)	0.32	(0.13-0.76)	0.23 (0.13-0.38)	**1.15 (0.92-1.44)***
Pn19F	0.21	(0.05-0.95)	0.20	(0.07-0.57)	0.12 (0.06-0.23)	**0.98 (0.86-1.12)***
Pn23F	0.09	(0.04-0.23)	0.26	(0.13-0.53)	0.11 (0.07-0.19)	**0.50 (0.38-0.66)***
MenA	**2.37**	**(1.29-4.37)**	**3.09**	**(1.88-5.08)**	1.98 (1.48-2.66)	1.88 (1.63-2.17)
MenC	0.11	(0.06-0.21)	0.18	(0.10-0.33)	0.09 (0.06-0.13)	0.05 (0.04-0.07)*
MenW135	0.13	(0.06-0.28)	0.24	(0.06-1.00)	0.11 (0.07-0.20)	0.08 (0.07-0.10)
MenY	0.07	(0.02-0.22)	0.21	(0.07-0.65)	0.06 (0.03-0.10)	0.78 (0.67-0.91)*
Tet	**0.10**	**(0.04-0.28)**	**0.15**	**(0.05-0.46)**	0.09 (0.06-0.16)	**0.48 (0.34-0.68)***
Dip	0.04	(0.02-0.07)	0.07	(0.03-0.13)	0.04 (0.02-0.06)	0.02 (0.01-0.02)*
HiB	0.66	(0.39-1.11)	**1.70**	**(0.54-5.32)**	0.89 (0.60-1.34)	0.21 (0.16-0.26)*

Key: Bold text indicates protective levels achieved according to WHO criteria. * Indicates the 16 serotypes which differed significantly between the total CLL vaccination cohort and healthy age matched donors.

Figure 6.5

Functional antibody responses were lower amongst patients with a low total serum IgG.

Patients with a low total serum IgG level had reduced functional antibody responses to 18 out of the 19 antigens tested and demonstrated significantly reduced protection against pneumococcal serotypes, with a median of 2 serotypes protected against compared to 5 serotypes in those with a normal serum IgG level ($p < 0.001$) (Mann-Whitney).
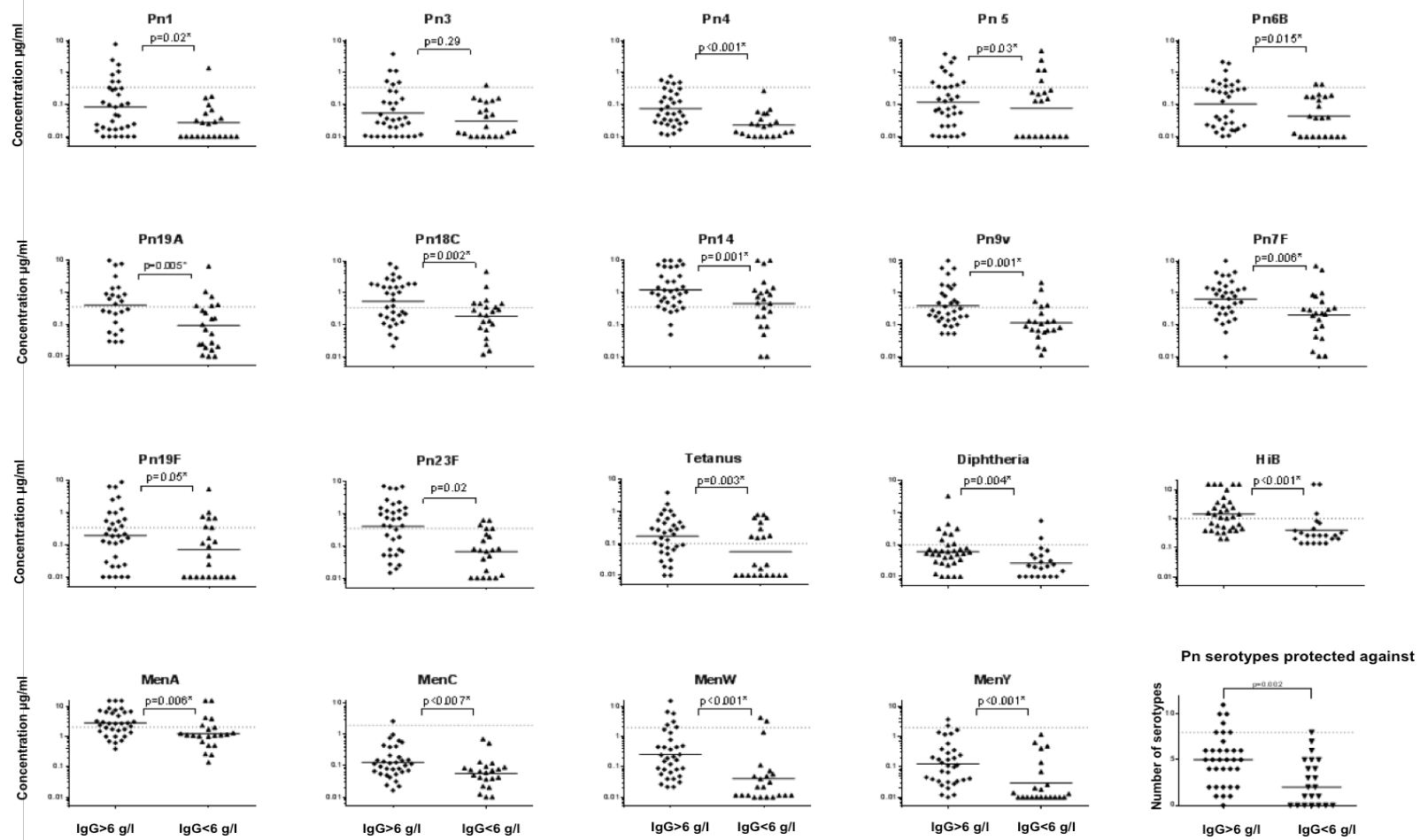
Key: dotted line represent the WHO level deemed adequate for protection

6.3.6.2 Functional antibody responses are inadequate in CLL patients who have received prior vaccination

Prior pneumococcal vaccination history was available on 53 of the 56 patients (95%). Thirteen (24%) patients were unvaccinated against Pneumococcus, despite UK guidelines. 37 had received the Pneumovax polysaccharide vaccine and 3 patients had been given the Prevenar13 conjugate vaccination. Surprisingly, patients who had received Pneumovax had protective levels against 2 of 12 pneumococcal serotypes, compared with 4 of 12 pneumococcal serotypes for the unvaccinated patients, although the median age of the vaccinated cohort was 73 years compared to 66 for the unvaccinated group (Table 6.4). Due to the cross sectional nature of this study the time from 'vaccination to analysis' was variable. However, no difference was seen in Fnab levels between patients that had received Pneumovax within the last 5 years (n=9) compared to those who had received the vaccine more than 5 years ago (range 6-15 years) (n=27). In this cohort, no difference in Fnab levels was observed between patients that were vaccinated within 2 years, 2-5 years and more than 5 years since vaccination.

6.3.6.3 The impact of CMV on functional antibody responses

Using Mann-Whitney testing, the impact of CMV status on pneumococcal functional antibody responses was next investigated. Firstly, the number of serotypes protected against was compared between CMV positive and negative participants. No difference was found between these however, with CMV positive participants demonstrating protection against an average of 5 serotypes, whilst CMV negative participants were protected against 3 serotypes on average (p=0.463). Next, the mean pneumococcal functional response of all 13 serotypes tested was compared between CMV negative and positive participants. Again, no difference

was found (CMV negative participants, 0.301 µg/ml vs CMV positive participants 0.498µg/ml; p=0.595) (data not shown).

6.4 Discussion

In patients with immunosuppression, CMV has long been recognized to be an important disease causing pathogen but more recently, in otherwise immunocompetent individuals, it has also been associated with a variety of medical conditions and increased mortality (Spyridopoulos et al., 2015, Kilgour et al., 2013, Savva et al., 2013). Patients with CLL are known to have global immunosuppression, which is multifactorial and yet, paradoxically an increase in CMV specific T cell responses are seen amongst patients who are CMV positive compared to healthy age matched controls (Pourgheysari et al., 2010, Mackus et al., 2003). This work represents the first investigation into the relationship between CLL and CMV, with outcome measures including overall survival, time to first treatment and infection risk explored.

In each of the three cohorts investigated, the prevalence of CMV was found to be between 57 and 75%, which is comparable with studies of viral seroprevalence in healthy people at a similar median age of 64 years (Kilgour et al., 2013). From this finding, CMV infection does not appear to be associated with the development of CLL but it is noteworthy that chronic viral infection has been suggested as a potential antigenic stimulus to account for the finding of shared immunoglobulin gene sequences in tumours from different patients. In particular, the recurrent IGHV1-69 sequence, which is common in CLL was reported to react with the pUL32 phosphoprotein of CMV (Steininger et al., 2012).

On univariable analysis of the discovery cohort, a significant two-fold increase in the risk of death was found in CMV positive patients compared to CMV negative patients. However, after adjusting for other variables, this significance was lost. In particular, age was found to be

an important variable, with CMV positive patients being 4 years older than CMV negative

patients. This is not surprising given CMV prevalence is known to increase with age but as

samples were collected soon after diagnosis, an alternative possibility is that CMV infection

could potentially serve to delay the diagnosis of CLL. To address this, a healthy age matched

cohort is needed in the same geographical areas as the participants with CLL. Unfortunately

this was not available at the time of the study. An alternative option might be a larger patient

cohort with longer follow up time to identify if these factors act independently. However, the

fact no relationship was found between CMV and age in the confirmatory cohort, suggests

any potential benefit of CMV in delaying diagnosis is unlikely. The discrepancy between the

two cohorts in relation to age is likely to be the result of socio-economic and geographical

differences impacting on the CMV prevalence in the two cohorts (Cannon et al., 2010).

In contrast to studies in healthy elderly people, no association between survival and CMV

infection was found. This study was limited by the lack of long term follow up and small

number of patient deaths. In both cohorts there was clearly a trend to shorter survival in CMV

seropositive patients so it is not possible to definitely exclude an effect of CMV on mortality

in CLL based my work. This limitation is illustrated by the fact that not all established

prognostic factors in CLL were found to be significant in the multivariant analysis. It may

also suggest that any negative effect of CMV infection in the elderly is outweighed by a

diagnosis of CLL. Another factor may be the younger age of the discovery and confirmatory

cohorts compared to healthy elderly studies, where participants were aged between 70 and

100 years (Roberts et al., 2010, Savva et al., 2013, Hadrup et al., 2006). Inflammatory

markers may contribute to the mortality impact of CMV in healthy elderly individuals.

Indeed, elevated levels of inflammatory markers, including IL-6 have been attributed (Wikby

et al., 2006, Kilgour et al., 2013, Trzonkowski et al., 2004). This is of note, as patients with

CLL often already have increased levels of IL-6 and may therefore confound any impact of CMV on outcomes measured (Adami et al., 1994, Callea et al., 1996). It may also suggest that any negative effect of CMV infection in the elderly is outweighed by a diagnosis of CLL. In addition, patients within these cohorts were only checked for the presence of CMV infection around the time of diagnosis and any later acquisition of CMV infection during disease progression was not be accounted for within the analysis. In contrast, an alternative possibility is that CMV infection may actually play a potential beneficial role on immune function in patients with CLL. The virus stimulates a strong Th1 immune response and leads to accumulation of large numbers of cytotoxic cells. An example of such benefit is seen in the setting of stem cell transplantation, where CMV infection has been shown to reduce the relapse rate (Ito et al., 2013, Elmaagacli et al., 2011).

High CMV-specific antibody titres have been correlated with poor clinical outcome in elderly donors (Roberts et al., 2010) but this work failed to demonstrate this in patients with CLL. Interestingly, progressive hypogammaglobulinaemia is a feature of progressive CLL but paradoxically unlike other herpes viruses such as VZV and EBV, CMV IgG titres have been shown to increase with disease progression (Vanura et al., 2013). Indeed, almost 50% demonstrated an increase in their CMV-specific IgG titre 4.6 years later and all CMV positive patients were still found to have detectable CMV IgG. As such it is unlikely that any patients with hypogammaglobulinemia were incorrectly identified as seronegative for latent CMV. The increase in CMV IgG observed over time may suggest episodes of reactivation are occurring. Furthermore, no significant difference in the total IgG was noted between CMV positive and negative individuals and neither was there a difference in the CMV titre in patients who were deficient in IgG compared to those that had a normal serum IgG level. This work has also found no difference between patients who are CMV positive and CMV

negative in terms of the average number of infections documented in hospital notes or the time to first infection. Neither was there any difference in the mean pneumococcal Fnabs response or total number of serotypes protected against in patients who were CMV positive compared to those who were CMV negative by serology. Overall, this indicates that the humoral immune system is able to respond to CMV challenge despite the overall state of immune suppression (Vanura et al., 2013) and that CMV does not appear to impact on the global immune response towards other common pathogens.

Up to 80% of all deaths in CLL are attributed to infection (Itala et al., 1992, Molica, 1994). Hypogammaglobulinaemia contributes to this infection risk and consistent with previous reports, this work found almost 40% of patients with CLL had low serum IgG and this was associated with an increased risk of infection and respiratory symptoms, even amongst stage A untreated patients (Parry et al., 2015). Total serum IgG also predicted time to first infection in patients at diagnosis, with those deficient in IgG demonstrating a shorter time to documentation of a hospital recorded infection by a median of 5.3 years. The type of infections reported in hospital notes was also indicative of a humoral deficit and predominantly consisted of lower respiratory tract infections (Parry et al., 2015).

As expected, low IgG levels were frequently associated with deficient Fnabs in the vaccination cohort. However, even amongst those with normal IgG, almost 80% demonstrated inadequate protection by Fnab testing for pneumoccocal serotypes. As patients with a normal IgG can have poor functional response, it is likely that functional antibody status gives independent, additional information relating to infection risk in addition to measuring serum IgG levels. Further work with a larger patient cohort is now needed to verify this. Currently, the BCSH recommend testing for serum IgG at diagnosis, and only suggest testing Fnabs following vaccination (Oscier et al., 2012). In following these guidelines, a substantial

proportion of patients with CLL would not be identified as being at risk for pneumococcal disease at the point of diagnosis. Testing with Fnabs would also be useful at diagnosis as a baseline for assessing response following vaccination.

Fnabs are known to diminish with age, but my results show that in comparison to an unvaccinated healthy elderly population, patients with CLL have significantly poorer Fnab responses. As the healthy elderly cohort were vaccine naïve, they represent an age matched cohort with only natural immunity from previous exposure and infection. Despite this, the mean number of serotypes protected against in patients with CLL was significantly lower than that in the healthy elderly cohort. This comparison suggests it is the CLL disease and not age which results in the profoundly low Fnab concentrations giving further evidence for this cohort's susceptibility to infection and degree of immune impairment (Parry et al., 2015). This work also demonstrates how prominent the immunodeficiency in CLL is, even at a very early stage of the disease (Moreira et al., 2013).

Despite the average age being above 65, and an underlying diagnosis of CLL, only 74% of patients had been vaccinated against Pneumococcus, regardless of the vaccine type. This emphasizes not only a need for more studies on vaccination but a more robust system of vaccination is required with clear guidelines on whether this should occur in primary or secondary care. Given the differences seen in my work, future studies need to differentiate between the optimal program of vaccination for both the healthy elderly population and for patients with CLL. The timing of vaccination was not addressed in this work, but others have demonstrated increased vaccine efficacy is achieved when it is delivered at diagnosis. (Sinisalo et al., 2007). At the time samples were being collected for this work, the guidance was changing to receive Prevenar 13, the conjugate vaccine (PCV), rather than the polysaccharide, 23vP vaccine in patients with haematological disorders and a secondary

immunodeficiency, so this may account for the low uptake of Prevenar13 seen in this work (Oscier et al., 2012, England, 2014).

The need to establish the clinical utility of pneumococcal vaccination using the newer conjugate vaccine is now required in patients with CLL. This study found that prior vaccination (mostly with 23vP vaccination) against pneumococcus did not improve Fnab pneumococcal concentrations, conversely concentrations were lower in this group. The age of this cohort was slightly higher but no other explanation for this finding was obvious from this study and warrants further investigation. Studies that have examined pneumococcal vaccination in CLL have only looked at short term response to vaccination so this study provides a useful insight into the poor long term immunogenicity of 23vP vaccination in CLL (Hartkamp et al., 2001, Sinisalo et al., 2003). With the exception of the Influenza, Pneumococcus and Haemophilus Influenza type B vaccination, no other vaccines are currently recommended in CLL (Oscier et al., 2012). This work highlights the inadequate functional antibody levels found against diphtheria and tetanus though and suggests vaccinating against these bacteria should also be recommended and included in future research vaccination studies. Studies have found that the use of PCV yields a protection rate of up to 47% in CLL patients (Sinisalo et al., 2007). The variable vaccine response rate highlights the need for Fnab testing one month following vaccination, as is currently recommended by BCSH guidelines (Sinisalo et al., 2007, Kroon et al., 2000, Oscier et al., 2012). This will identify those patients who require closer monitoring for infection and potentially prophylactic interventions. Future work is required now to assess whether those deficient in functional responses following initial vaccination would benefit from a booster vaccination, as is given routinely to infants.

6.5 Conclusion

In conclusion, I have shown no evidence that CMV infection can predispose toward the development of CLL or that established infection can impact on time to first treatment or overall survival in newly diagnosed patients with CLL. Neither does CMV appear to have any impact on infection rates or time to first infection. This work has however, highlighted antibody dysregulation and poor vaccination uptake as key areas for concern in patients with CLL and further work is now required to optimise immunity against common vaccine preventable diseases, which are well established to be a leading cause of death in patients with CLL.

7 GENERAL DISCUSSION

7.1 General discussion and conclusions

Using class I and II tetramers, a phenotypic description of both CD8+ and CD4+ CMV-specific T cells and their contribution to the total T cell repertoire has been shown in patients with CLL and compared to healthy donors. In particular, novel class II tetramers have provided opportunity for in-depth characterization of CMV-specific CD4+ T cells both in CLL patients and healthy donors. A limitation to the use of tetramers though, is the small number of epitope peptides for which tetramers can be generated and this is particularly the case for the stable production of class II tetramers (Vollers and Stern, 2008). In this regard, phenotypic features described in this work have been interpreted with the knowledge that CD8+ responses were targeting the CMV proteins IE-1, pp65 and pp50, whilst CD4+ responses were identifying gB and pp65 and these may not always be reflective of the total T cell responses directed at CMV.

The importance of understanding the biology of CMV in the context of CLL has recently been highlighted through the use of the novel agent idelalisib (a PI3 Kinase inhibitor). Idelalisib has shown great clinical efficacy in the relapsed setting but its use in first line and combination therapy has been halted as a result of fatalities and serious toxicities including CMV reactivation. Understanding the impact of idelalisib on CMV specific CD4+ and CD8+ T cell phenotype and function is therefore now warranted.

In my work, I have compared the phenotype of CMV-specific CD8+ and CD4+ T cells and demonstrated that both have great cytotoxic capacity with high levels of perforin and granzyme. No difference was observed between HD and patients with CLL for these molecules. Expression of perforin and granzyme is common amongst effector CD8+ T cells, but is unusual amongst CD4+ T cells. However, HIV, EBV and CMV-specific CD4+ cytotoxic cells rich in perforin and granzyme have previously been described (Zaunders et al.,

2004, van Leeuwen et al., 2004, Appay et al., 2002). It has been postulated that cytotoxic CD4+ T cells arise as a surveillance mechanism to combat attempts by virus to evade MHC class I presentation (Appay, 2004).

In comparing CMV-specific CD4+ and CD8+ T cells, a difference in memory phenotype was noticed, with a greater proportion of terminally differentiated T cells that re-expressed CD45RA observed amongst the CD8+ cells. In contrast, CD4+ CMV-specific T cells were predominantly effector memory in phenotype and negative for CD45RA. This supports previous work, which showed perforin positive CD4+ T cells in patients with CLL were T_{EM} in phenotype (Walton et al., 2010). Interestingly, CD45RA expression has been shown in CD8+ T cells to correlate with the time elapsed since antigen exposure (Carrasco et al., 2006) and in this work I have shown that patients with CLL demonstrated a reduced percentage of CD45RA positive cells CD8+ CMV-specific T cells compared to healthy donors. This therefore suggests they have been exposed to CMV antigens more recently, most likely in the context of subclinical viral reactivation. The expression of CD45RA and time since antigen exposure has not been reported for CD4+ T cells but if parallels are drawn, this might suggests CD4+ T cells are being more frequently exposed to viral epitopes in their T helper cell capacity via cross-presentation as result of exogenous processing by profession antigen presenting cells.

A similarity noted in both CD4+ and CD8+ CMV-specific cells was a dramatic reduction in the telomere lengths. This finding indicated that CMV-specific T cells have undergone numerous rounds of cell replication. Memory cells re-expressing CD45RA are known to have the shortest telomere lengths amongst memory T cells, therefore it is unsurprising that CD8+ CMV-specific T cells demonstrated this feature (Romero et al., 2007, Hamann et al., 1997). In contrast, the finding that populations of effector memory CD4+ T cells also had greatly

reduced telomere lengths compared to the total CD4+ T cell population was less expected. Indeed, amongst the CMV-specific CD4+ T cell populations that had statistically shorter telomere lengths, one of the three donors consisted of CMV-specific T cells that were entirely CCR7-CD45RA- in phenotype.

PD-1 expression differed amongst CD4+ and CD8+ CMV-specific T cells in patients with CLL, with more PD-1 observed on CD4+ CMV-specific T cells. This increased expression was greater than observed on healthy control CMV-specific CD4+ T cells and was not associated with classical Treg markers or markers of recent activation. This contrasts with published work that found PD-1 on CD4+CD27-CD28- CMV-specific T cells was associated with FoxP3 expression (Tovar-Salazar et al., 2010). Why increased expression of PD-1 was observed on CD4+ but not CD8+ CMV-specific T cells is unexplained. It may reflect the differentiation status of the CMV-specific CD4+ T cells, as PD-1 is reported to be on T cells which express a T_{EM} phenotype (Riches et al., 2013). It may also occur on CD4+ T cells to minimize co-stimulatory support to cytotoxic CD8+ T cells in order to prevent an overwhelming CD8+ T cell response that could potential damage host. Although cytokine responses were suboptimal in PD-1 positive cells and could be interpreted as a feature of exhaustion, reports have found a high expression of CD57 on CMV-specific CD4+ T cells, suggesting this is not the case (Pourgheysari et al., 2007, Casazza et al., 2006). Furthermore, the transcription factor analysis of Tbet and Eomes did not find any Eomes high, Tbet dim cells, which have been described in CD8+ T cells to have an exhausted phenotype in the context of HIV infection (Buggert et al., 2014). Thus, from the work presented in this thesis, it is difficult to ascertain the function of PD-1 in the context of CMV-specific CD4+ T cells. With the advent of class II tetramers, more attention is now required to phenotype these PD-1

positive CD4+ T cells further, including the presence of other inhibitor receptors and to understand their role in both health and patients with CLL.

An increase in frequency of CMV-specific T cell responses with stage of disease has previously been reported for CD4+ but not CD8+ T cells. This work found patients with stage C disease had higher CMV-specific CD8+ T cell responses compared to stage A. Together, these findings suggest that an increased CMV directed T cell response occurs under conditions where immunosuppression is more prevalent. In addition, the finding that stage C patients with CLL also demonstrated higher levels of CMV viral load by ddPCR of monocyte DNA suggests subclinical reactivation may account for this T cell expansion. An alternative reason for these findings could relate to the poor functional responses observed. CMV specific T cells may maintain the capacity to deliver an effective anti-CMV response but without an intact synapse formation, a poor response will prevail (Ramsay et al., 2012, te Raa et al., 2014). This would potentially lead to more episodes of viral reactivation and boost CMV-specific T cells frequencies. Finally, with advanced stage disease, the increased number of CLL tumour cells could also potentially provide stimulus for T cell expansion. However, there is no direct evidence for this (Mackus et al., 2003). To address this further, serial measurements and correlation of CMV viral load and CMV-specific T cell responses is now required over time. The addition of CMV IgM testing may also help to validate periods of CMV reactivation.

Despite its prevalence amongst patients with CLL, this work finds no evidence that CMV infection impacts on clinical outcome measures including time to first treatment and overall survival in 2 large cohorts of patients with CLL. I have also shown no relationship exists between the titre of CMV IgG antibody and the same outcome measures in patients who are CMV positive. Neither is there any evidence to support more infections in patients who are

latently infected with CMV. As such there is no suggestion that anti-viral therapy to target CMV would impact on outcome measures in patients with CLL.

Finally, I have shown that functional antibody testing can provide the clinician with additional information for identifying patients at risk of infection, even at an early stage of disease.

7.2 Future work

The interesting observation of increased expression of PD-1 on CMV-specific CD4+ T cells now needs in-depth characterization, particularly with regards to co-expression of other alternative inhibitory markers, expression of CD57 and natural killer receptor markers. These findings would help to confirm if any exhaustive phenotype exists amongst CMV-specific CD4+ T cells (Wherry and Kurachi, 2015). Using RNA sequencing, sorted populations of PD-1 positive and negative CMV-specific CD4+ T cells could also be examined to identify any difference in the whole genome transcription profile. To investigate the functionality of the PD-1+ CMV-specific CD4+ T cells further, PD-1 blockade is also now required following either peptide stimulation or peptide pulsed LCLs at a controlled B:T cell ratio. LCL peptide presentation would also aid understanding of the contribution of immunological synapse formation and whether PD-1 is still a contributing factor under these circumstances to any impaired functionality seen.

To understand the phenotypic T cell changes observed in CLL patients, the transcription factor profile needs to now be studied in more detail. From the limited number (n=20) of HD and patient CMV-specific CD4+ T cells examined for Eomes and Tbet expression, it appeared that CMV-specific T cells account for much of the total CD4+ Eomes expression seen in CMV positive donors and raises the question whether CMV negative donors have reduced frequencies of Eomes positive CD4+ T cells in their peripheral blood. This has not previously been reported and now needs to be investigated in a larger cohort of donors.

Finally, I have shown functional antibody testing is useful and effective at identifying patients at risk for pneumococcal disease and this is a leading cause of death in patients with CLL. Inadequate protection against pneumococcal disease was demonstrated in most patients and vaccination uptake for the recommended Prevenar13 was poor. To address this, I have now initiated a project which will identify the functional antibody response before and after Prevenar13 vaccination in order to identify those at risk and to investigate the impact of a booster vaccination to improve responses.

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CLL GENERAL HEALTH SURVEY

Please give your age: _____

Please indicate your gender: Male ☐ Female ☐

Please tell us your ethnicity: _____

Approximately when did we diagnose you with CLL (month and year)?: _____

Do you have any other medical conditions (If so, please specify):_____
_____
_____
_____

1. MY USUAL HEALTH

Please indicate which statements best describe your health **today** by placing a tick (thus ✔) in one box in each group below,:

a) Cough
I normally cough on

Most days.................................. ☐

One or two days a week......... ☐

A few days a month................. ☐

Only with chest infections...... ☐

Never... ☐

b) Sputum
In a normal week I cough up sputum on

Most days.................................. ☐

One or two days a week......... ☐

A few days a month................. ☐

Only with chest infections...... ☐

Never... ☐

c) In the last 6 months I have suffered from the following types of infection:

Chest.. ☐   How many times?   ☐

Urinary tract infection (water infection).............. ☐   How many times?   ☐

Skin Infection.. ☐   How many times?   ☐

Other (please specify below)................................ ☐   How many times?   ☐

Specify 'other' infection: _____

I have not suffered an infection in the last 6 months... ☐

2.VACCINATION

a) Do you have the flu vaccine every year? ☐ Yes ☐ No ☐ Sometimes

b) Have you had the pneumonia vaccine every year? ☐ Yes ☐ No

If yes, when did you have the pneumonia vaccine (if you remember)?

3.ANTIBIOTICS

a) Do you take antibiotics every day? (please tick)? ☐ Yes ☐ No

b) How many courses of antibiotics have you had in the last 6 months to treat an infection?

(Please include those prescribed by the hospital or the GP. If you have had 2 courses for the same infection this would count as 2 courses, not 1).

Number of courses: _____