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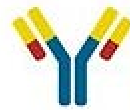

INSTITUTE OF
IMMUNOLOGY AND
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HARNESSING CD4+ T CELL EFFECTORS FOR LYMPHOMA THERAPY

By

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ABSTRACT

CD4+ T cells play a pivotal role in orchestrating immune responses. It has now been shown that some cells display direct effector functioning in the elimination of viral infections and cancers. The T cell receptor (TCR) is thought to play a part in influencing these cytotoxic mechanisms and is being investigated for optimisation of TCR gene transfer therapies. However, with the diverse TCR repertoire available, selection of TCRs conferring the greatest therapeutic potential remains a challenge.

To investigate TCRVβ usage and its effects on cellular function, in the context of the oncogenetic Epstein Barr Virus, we used MHC class II tetramers to isolate CD4+ T cell clones from healthy seropositive donor's *ex vivo*, specific for the latency III EBNA2 protein derived epitope; PRS.

We have found that the epitope specific CD4+ T cells express TCRs with various Vβ usages. These T cells had a range of functional avidities for the same MHCII-epitope combination. We have further shown there is a direct relationship between functional avidity and the efficiency of a T cell clone to recognise unmanipulated LCL targets (EBV infected B cells).

The results from these experiments highlight the importance of gaining further knowledge into the relationship between TCR usage and T cell function. This may provide steps towards future development of targeted MHC Class II restricted TCR gene transfer therapies for the treatment of EBV associated B cell malignancies.

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CHAPTER 1

1. INTRODUCTION

1.1 The Human Immune System and Cancer

The human immune system is essential for life. Alongside its pivotal role in the detection and elimination of pathogenic species, the immune system is widely acknowledged to play a significant part in cancer prevention (Blair et al 2008). The first evidence of this was produced by Elie Metchnikoff and Paul Ehrlich in 1908, who established that the immune system could naturally recognise cancer cells as altered self and initiate their destruction (Tauber et al 1992). Although these early findings were met with some scepticism, clinical research into cancer immunology subsequently developed throughout the 20th century. In the 1950s Burnet and Thomas expanded upon these initial findings, suggesting that the immune system was able to promote protection against cancer development by eliminating malignant cells before they had the opportunity to transform. This hypothesis later formed the concept known as cancer immune-surveillance (Ostrand Rosenberg et al 2008). It has now been recognized that in some cases, through immune-surveillance and anti-tumour responses, cancer can be effectively prevented and controlled. These historical research contributions have led to advances in the field of tumour immunology in the last 20 years, where the immune system has been utilised and enhanced to treat different types of cancer. Many successful immunotherapies exploiting both the humoral and acquired immune arms have now been tested in clinical trials and some are now approved for use in combination with traditional chemotherapeutic agents (Rothschild et al 2015).

However, to develop more effective novel therapeutic strategies it is fundamental to understand the mechanisms underlying interactions between immune cells and tumours.

1.1.1 The Innate Immune System

The innate immune system is the body's initial protection against invading pathogenic organisms. This primary response is rapid and involves a variety of different defence mechanisms, complement proteins and innate cell subtypes. After a pathogen penetrates through primary epithelial and mucosal barriers, they are detected by innate cells including macrophages, dendritic cells, granulocytes and natural killer cells, circulating within the infected tissue. These immune cells recognise pathogen associated molecular patterns (PAMPs) on the surface of invading microorganisms and danger associated molecular patterns (DAMPs), via membrane bound pathogen recognition receptors (PRRs) (Iwasaki et al 2004). This triggers both phagocytosis of pathogens and a range of intracellular signalling pathways the innate cells, resulting in the release of various signalling molecules including chemokines and cytokines along with a systematic pro-inflammatory response (Mogensen et al 2009). Consequentially, more innate cells migrate to the area of infection. Dendritic cells, in particular, are essential in the activation of the adaptive immune response (Section 1.1.2). This is achieved through the processing and presentation of antigenic material on their cell surface which coordinates the formation of long lasting memory T lymphocytes and protection against secondary infections (Banchereau et al 1998).

The role of the innate immune system in targeting cancer cells remains poorly understood. Natural killer cells (NK), primary innate immune lymphocytes, have been shown to be responsible for elimination of non-MHC expressing cancer cells through

the activities of their stimulatory and inhibitory NK receptors (Liu et al 2013). Furthermore, the NK receptor NKG2D, which recognises the stress ligand MHC class-I-chain-related protein A (MICA) expressed on the surface of cancer cells, stimulates the production of inflammatory cytokines, resulting in cancer cell apoptosis (Bauer et al 1999). However innate immunity is not specific and is limited in tumour control.

1.1.2 The Adaptive Immune System

The adaptive immune system is essential for elimination and long-term protection against both pathogenic organisms and cancer cells. Humoral and cellular immunity are the two lines of adaptive defence. Adaptive immune cells also express receptors, however unlike the innate cells these receptors are somatically generated via gene re-arrangements. This creates a diverse, clonally distributed receptor repertoire which is highly specific and enables adaptive immune cells to target specific antigens. Antibodies, synthesised from plasma B cells, can recognise extracellular antigens. In contrast, T lymphocytes discriminate foreign antigenic material from self, via T cell membrane bound receptors which interact with pathogen derived protein degradation products. Once activated adaptive immune cells initiate elimination of infected (or cancerous) cells and establish long lasting memory populations which are able to respond much more rapidly upon secondary encounter with a specific antigen. T cells can express either γδ and αβ T cell receptors (TCRs) (Allison et al 2002), this thesis will focus on the αβ TCR.

1.1.3 T Lymphocyte Development

To provide the most efficient cellular immune response, the T cell repertoire must be diverse enough to cover the wide range of antigens potentially encountered throughout

life. There are two types of T lymphocytes, CD8+ and CD4+ T cells, which originate from hematopoietic stem cells located within the bone marrow. Newly generated T cells migrate to the thymus, entering at the cortico-medullary junction where they initiate TCR rearrangements and undergo selection.

1.1.4 TCR Generation

To successfully produce a functional TCR and generate diversity within the TCR repertoire, each chain undergoes a process known as V(D)J recombination. This involves rearrangements of the gene segments, known as variable (V), diversity (D) and joining (J), to form a complete variable domain exon. V(D)J recombination is initiated by two proteins, RAG1 and RAG2, which together constitute the recombinase forming a complex which contain conserved recombination signal sequences (RSSs). These RSSs flank the coding sequences of individual gene segments and the formation of this complex allows for the joining of gene segments such as variable and joining. The endonuclease activity of the RAG1/2 recombinase introduces single stand DNA breaks within the 5' ends of both RSSs (McBlane et al 1995). The exposed 3' hydroxyl groups react with phosphodiester bonds located on the opposite DNA strand promoting the formation of a hairpin loop and double stand breaks within each RSS sequence (Figure 1.1). Repair proteins Ku70:Ku80 bind to the hairpin. The nuclease enzyme; Artemis is recruited, phosphorylated by a DNA phosphatase kinase (DNA-PK) and opens up the hairpin loops (Mansilla-Soto et al 2003). The cut ends are then modified through random additions and deletions of non-template nucleotides, by terminal deoxynucleotidyl transferase (TdT), producing diversity and heterogeneity in the joint between the gene segments; a process known as junctional diversification. The RSSs contain spacer sequences consisting of either 12 base pairs or 23 base pairs. These are

essential for the re-joining steps as pairing only occurs between fragments with terminal 12bp spacer sequences and terminal 23bp sequences (Van Gent et al 1996). DNA ligase IV is recruited to join the processed ends and this is followed by transcription, splicing of none coding segments and translation into a polypeptide sequence which constitutes each strand of the TCR.

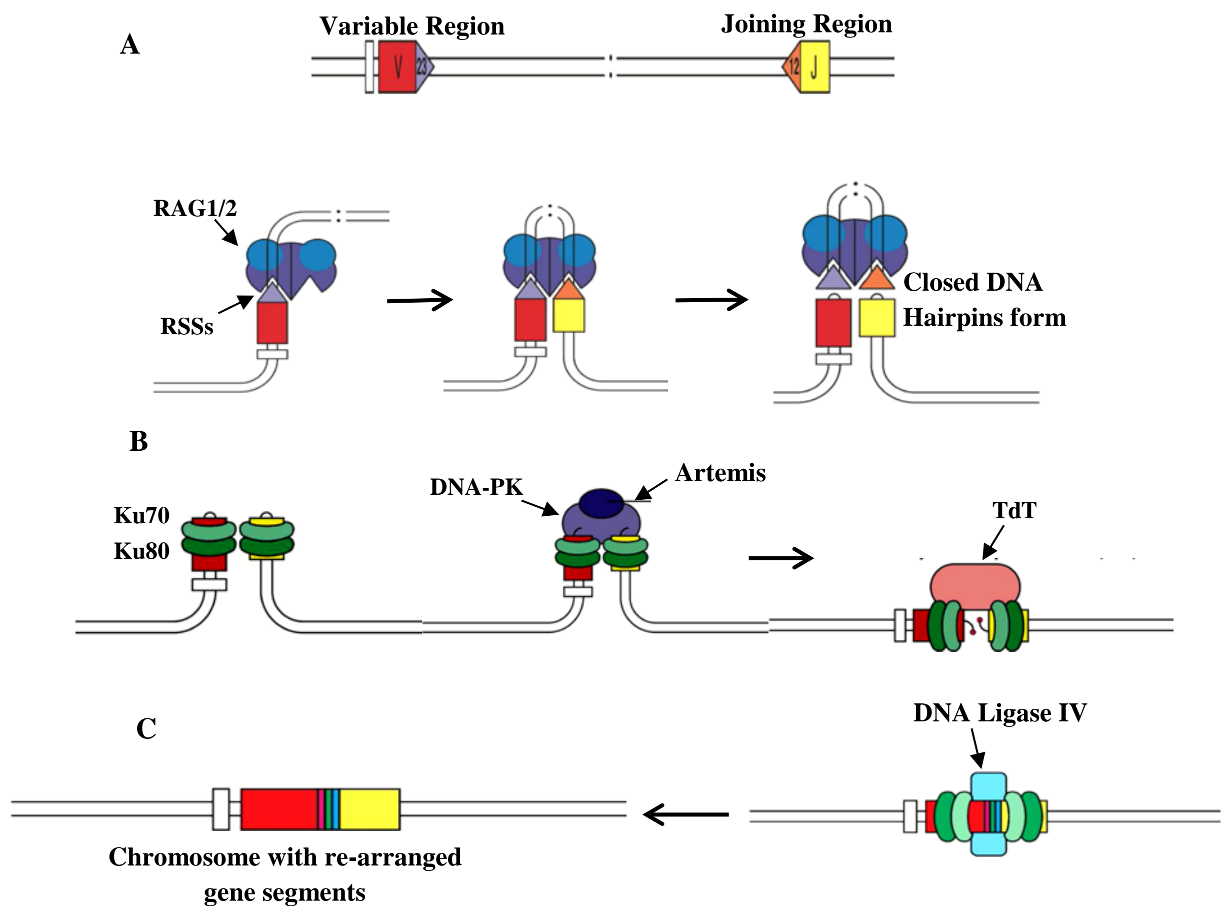


Figure 1.1 V(D)J Recombination. Both alpha and beta chain genes go through a process known as V(D)J recombination in which they rearrange gene segments to produce a large repertoire of TCRs for future immune responses. (A) RAG1/2 bind to RSS sequences introducing single stranded DNA breaks and hairpin loop formation. (B) Recruitment and activation of Artemis nuclease opens hairpin loops. Followed by additions and deletions of nucleotides within the gene sequence (C) Recruitment of DNA ligase, joining of cut modified ends and transcription of newly formed gene segments (Murphy et al 2012).

Allelic exclusion is another essential process of thymocyte development. This prevents both alleles of the same TCR chain gene, be that beta, alpha, delta or gamma chain, being expressed together and restricts a TCR to a single specificity. Hence, if a productive V(D)J gene rearrangement occurs for one beta chain gene then this promotes the inhibition of the other allele of that gene, preventing more than one chain being synthesised (Dudley et al 1994).

Once the TCR beta chain has successfully undergone somatic gene recombination and allelic exclusion it will associate with an invariant pre-TCR alpha chain forming a pre-TCR complex. This initiates alpha chain gene rearrangement and the formation of a double positive (DP) population of thymocytes expressing both CD4 and CD8 co-receptors (Van De Wiele et al 2004). DP thymocytes undergo both positive and negative selection checkpoints, thus ensuring only the most efficient TCRs (in terms of affinity) are available to induce a strong immune response.

The development and maturation of T cells is essential for determining both their own TCR specificity and affinity towards a range of Peptide/MHC complexes (pMHC), along with preventing autoimmune reactivity towards healthy cells within the body. This balance is vital in maintaining an immune system robust enough to target and eliminate infection and cancer while still preventing autoimmunity.

1.1.5 T Cell Receptors

TCRs are crucial components of adaptive immune cells and a key area of interest when investigating the therapeutic application of T cell immune responses. Each T cell expresses thousands of copies of identical TCRs which target processed antigens in order to eliminate infection. They are further involved in cancer immunosurveillance strategies when interacting with tumour associated and tumour specific antigens (TAA, TSA) presented by malignant cancer cells. Despite the diversity of the human TCR repertoire remaining unknown, the gene set encoding the TCR repertoire has the potential to create over 10^{15} TCR T cell clonotypes (A population of T cells expressing identical TCRs) (Laydon et al 2015).

X-Ray crystallography analysis of the αβ TCR has been paramount in furthering the study of its molecular structure and immune function in relation to affinity and avidity towards target antigens (Garcia et al 1996). The TCR is a transmembrane anchored heterodimer of glycoproteins consisting of distinct alpha and beta chains. These chains are composed of variable and constant regions bound to a hinge region, similar to that seen in immunoglobulins, within the humoral arm of the adaptive immune system (Figure 1.2). The variable domains of the TCR, which are folded into Beta sheet structures, contain hypervariable complementary determining regions; CDR1, CDR2, and CDR3. These are located on both alpha and beta chains of the TCR and characterised as 6 loop structures (Nielsen et al 2002). The CDR1 and CDR2 are encoded within the variable gene segments of the germline. The CDR3, on the other hand, is predetermined by the region within both the spliced VJ Alpha and VDJ Beta gene segments as a result of somatic recombination events during thymocyte development. This results in an increase in the diversity of the TCR repertoire (Wang et

al 2017). It is the most variable and primary region of the TCRβ locus for determining antigen specificity.

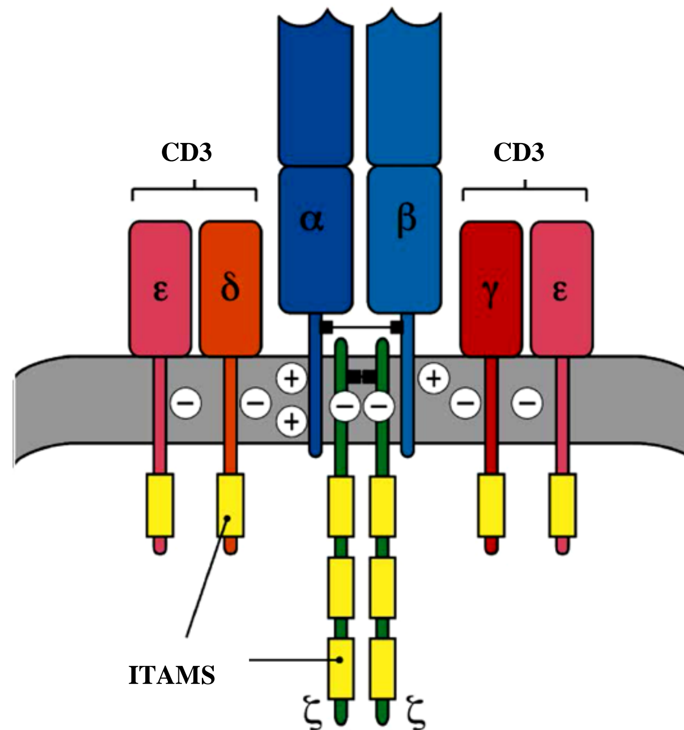


Figure 1.2 Structure of αβ T cell receptor. The αβ TCR compromised of alpha and beta chains both with variable and constant domains. A stalk segment connects the constant region with the membrane of T cell (this contains cysteine residues forming disulphide bonds which stabilise each chain). CD3 complexes are located either side of the TCR and are essential for promoting downstream intracellular signalling after initial TCR-pMHC complex interaction (Murphy et al 2012).

1.1.6 Major Histocompatibility Complexes

Major Histocompatibility Complex molecules are encoded by human leukocyte antigen genes, found on chromosome 6, within the region in closest proximity with the chromosomal centromere. These are broadly organised into 3 separate gene loci, HLA I, HLA II and HLA III, encoding 3 different MHC protein molecules (Figure 1.3). The HLA II genes (HLADR, HLADP, and HLADQ) have been found to encode the MHC II molecule presented to CD4+ T cells (Ting et al 2002). Individuals contain a number of different HLA genes and variations of these promote a polymorphic and polygenic state. The entire MHC is inherited as an HLA haplotype in a Mendelian fashion from each parent, meaning closely related individuals such as siblings have a higher probability of being genotypically HLA identical (Choo et al 2007). This thesis specifically focusses on CD4+ αβTCR interaction with define pMHC class II complexes, as such this process will be explained in greater detail.

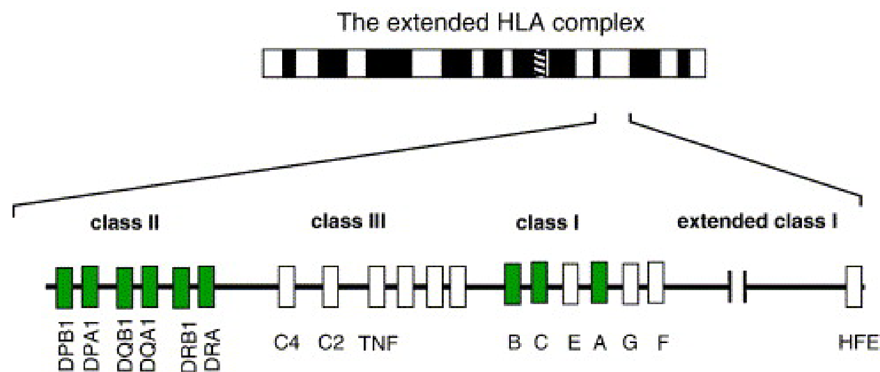


Figure 1.3 HLA genes. HLA genes encoding MHC class I, II and III molecules located within the short arm of chromosome 6. Class II genes encode MHC II, class III genes encode proteins in the serum complement system and class I encode the MHC I molecule (Thorsby et al 2005).

1.1.7 MHC Class II

The MHC II molecule, unlike the MHC I, is only expressed on the surface of antigen presenting cells. These include dendritic cells (DCs), B lymphocytes, monocytes, macrophages, and thymic epithelial cells. Notably, human T lymphocytes also express MHC class II molecules following activation. The MHC II presents exogenous antigens to CD4+ T lymphocytes and is composed of non-covalently linked glycosylated alpha and beta chains, each containing 2 domains (for example, alpha 1 and alpha 2) (Figure 1.4). The variable alpha 1 and beta 1 domains form an open-ended peptide binding groove which interacts with exogenous peptides, 10-15 amino acids in length. Hydrogen bonds form between amino acids within the binding groove and specific amino acids within the epitope of an antigen (Schmidt et al 2013). The exact amino acid and sequence the MHC molecule is composed of will determine what antigen peptides are able to bind. Thus, polymorphisms and altered amino acid sequences within its structure will modify the shape of the MHC II groove and consequentially its peptide-binding specificity.

1.1.8 Processing and Presentation of Exogenous Peptides by MHC Class II

Antigenic peptides are processed and presented to naive mature CD4+ T Cells on MHC II molecules on the target cell via a process known as the exogenous pathway (Figure 1.4). This involves the acquisition of antigenic peptides generated from proteolytic degradation in endosomal compartments from exogenous material endocytosed from the extracellular environment (Watts 2004).

MHC II molecules assemble within the Endoplasmic Reticulum (ER) and associate with an invariant chain. This chain initially prevents the binding of self-peptide fragments

prior to MHC localization within the endo-lysosome (Cresswell et al 1996). The invariable chain is released leaving behind a peptide, normally residing in its N-terminal region, known as the Class II associated invariant chain peptide (CLIP) that continues to occupy the binding groove of the MHC II. MHC-encoded heterodimeric glycoprotein HLA-DM then facilitates the dissociation of CLIP from the MHC II molecule. This promotes the formation and stabilization of exogenous pMHC class II complexes (Blum et al 2013) which are then transported to the cell membrane where they are presented to the immune system primed for TCR engagement.

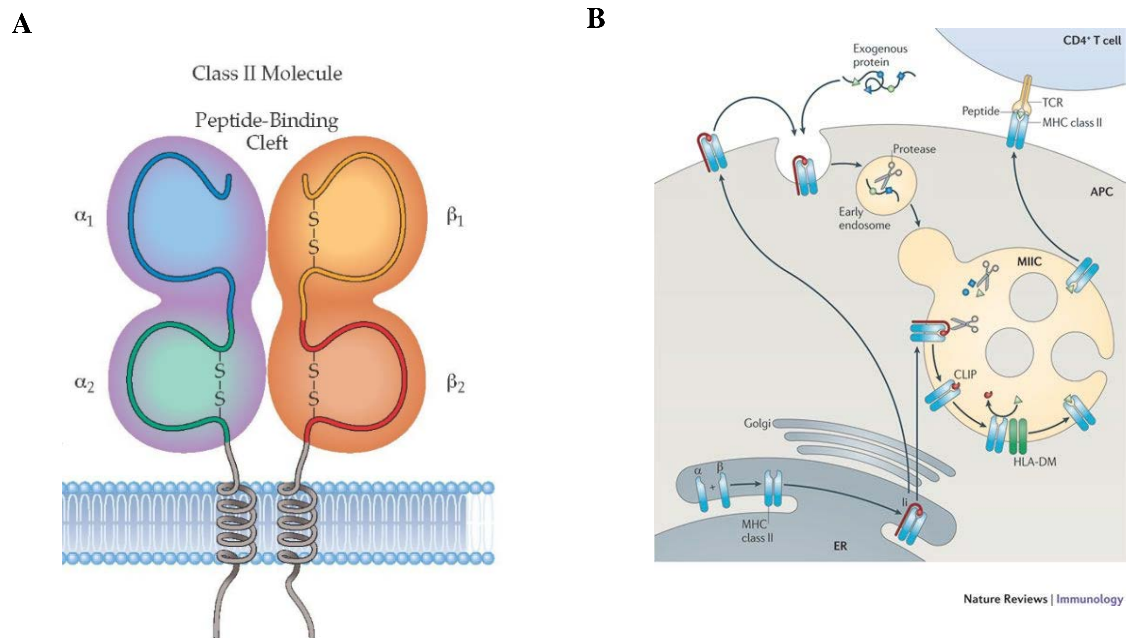


Figure 1.4 MHC II structure and the exogenous pathway. (A) Structure of the MHC Class II molecule (Murphy et al 2012). (B) Diagrammatic representation of the exogenous pathway deployed by MHC II in processing and presentation of antigens (Neefies et al 2011).

For a CD4+ T cell to display effector or helper functions, it must interact with these pMHC complexes. Along with direct TCR binding and appropriate co-stimulation, phosphorylation of signalling domains induces various intracellular activation pathways involving STAT proteins which mediate transcription of genes regulating their differentiation status (Vahedi et al 2012). A range of CD4+ T cells subsets can be produced (Figure 1.5). The type of subset a naive CD4+ T cell will differentiate into is determined by the cytokines and co-stimulatory molecules present at the time of activation. There are a number of CD4+ T cells that have been defined; Th1, Th2, Th17, Th22, T regulatory cells (Tregs), follicular helper T cells (Tfh) and CD4+ T cells with cytotoxic activity (CTLs). In particular, the cells of the Th1 subset expressing T-bet have shown to display direct anti-viral and anti-cancer cytotoxic properties through their capacity to synthesise effector cytokines such as IFNγ and TNFα (Swain et al 2006). Despite the majority of research focussing on IFNγ producing Th1 cells, there is now evidence to show polyfunctional CD4+ T cell populations which produce a range of different cytokines are essential in anti-viral immune responses (Makedonas et al 2006).

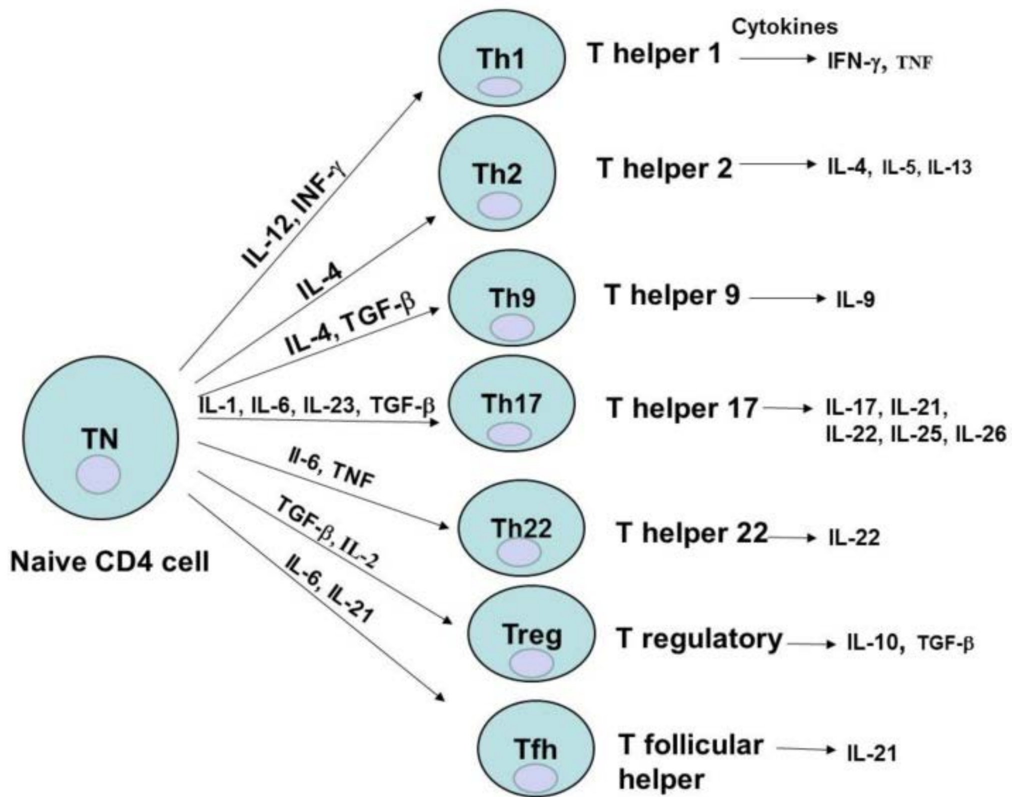


Figure 1.5 CD4+ T cell subsets and functions. Summary of the terminally differentiated subsets of effector CD4+ T helper cells (Golubovskaya et al 2016).

1.2 Epstein Barr Virus (EBV)

The Epstein Barr Virus (EBV) is a 172Kb, double stranded linear DNA, gamma herpesvirus that is globally prevalent. It has been shown to establish persistent infection in more than 90% of the adult human population (Rowe et al 2010). Infection normally occurs in childhood as an asymptomatic infection, however it can be delayed until adolescence when it can result in acute Infectious Mononucleosis (IM). This disease is characterised by pharyngitis, cervical lymph node enlargement, and fever along with abnormally high numbers of CD8+ T cells (Dummire et al 2015). In immunocompetent individuals, the infection is brought under control by EBV specific T cell immune responses.

1.2.1 EBV Life Cycle and Infection

Transmission of EBV is via oral salivary exchange. As illustrated in figure 1.6, the virus enters the lymphoid tissue and crosses the epithelial barrier where it is directed towards both target squamous epithelial cells and naive B lymphocytes circulating within the oropharynx (Williams et al 2006). Initial interaction occurs via two viral glycoproteins, gp350 and gp220, which bind to the complement B cell receptor; CD21. For successful cell entry the activity of another viral glycoprotein complex, gp25/28 follows, promoting the interaction between EBV and the B cells MHC class II molecule (Taylor et al 2015). This mediates the activation of intracellular signalling pathways resulting in viral replication and release of virions as part of the virus's lytic cycle. The EBV genome does not integrate into the host cell's DNA but instead forms circular episomes that occupy the nucleus and induce a persistent infection.

At the same time the virus instigates a cascade of intra-nuclear events leading to the expression of various latency genes from the EBV viral genome, which encode 10 proteins including six Epstein barr nuclear antigens (EBNA1, EBNA2, and EBNA3A, 3B, 3C, and -LP), three latent membrane proteins (LMP1, LMP2A, and LMP2B) and BHRF1 (Price et al 2015). The expression of this latent growth transforming programme is known as latency III.

The outgrowth of the EBV infected B cell population, expressing both lytic and latent immunogenic antigens, is brought under control by immune responses mediated by CD8+ and CD4+ lymphocytes and NK cells. However, the expression of viral genes by some cells, within this latently infected B cell population, is downregulated inducing a state known as latency 0. These cells become part of a differentiated genome positive, but antigen negative, pool of resting memory B cells, able to circulate between the nasopharyngeal lymphoid system and peripheral blood indefinitely. This allows a degree of immune evasion and escape by the virus. At certain sites within the oropharynx, some latently infected B cells become permissive for the lytic infection cycle, releasing EBV virions into the oropharynx, which go on to infect naive B lymphocytes and epithelial cells or enter the saliva to be orally transmitted to other susceptible individuals. In this way the virus is able to remain dormant and protected from EBV specific immune responses, establishing persistent latent infection in memory B cells with occasional replication and lytic cycle entry. (Lawson et al 2001, Ralf Kupper 2003).

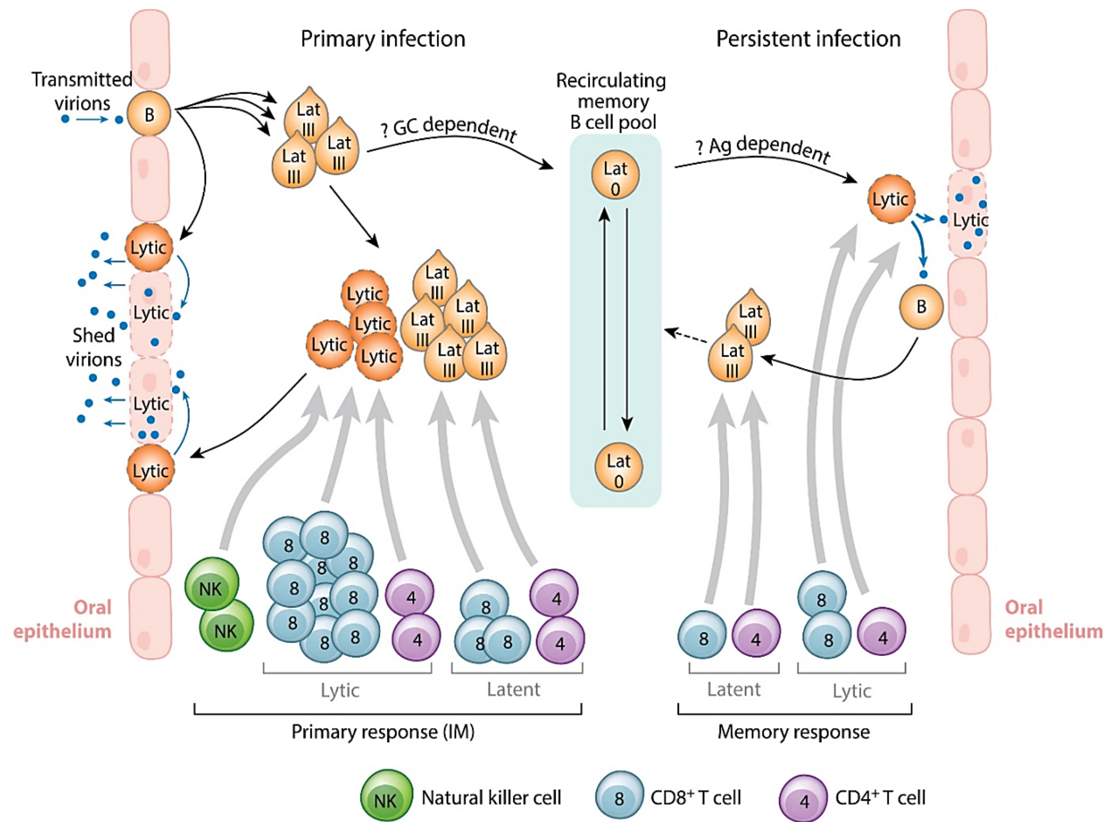


Figure 1.6 EBV life cycle. EBV initially infects epithelial cells and B lymphocytes within the oropharynx. Following primary infection, the outgrowth of lytic and latently infected b cells is controlled by an EBV specific immune response. EBV infected B cells switch to latency 0 in response to NK, CD4+ and CD8+ T-cell pressure. At certain sites lytic infection can be established from these latently infected B cells. This results in the release of infectious EBV virions which can either infect other B or epithelial cells or enter the saliva to be orally transmitted to other individuals. In this way a persistent infection is established (Taylor et al 2015).

1.2.2 Immune Control of EBV

The clinical identification of acute Infectious Mononucleosis (IM), has enabled researchers to investigate the EBV specific immune responses towards primary infection. EBV specific T cells have additionally been detected in long-term healthy seropositive carriers, making EBV a model system when studying the anti-viral immune responses occurring within humans (Rowe et al 2010).

Adaptive immune responses towards EBV promote the latency 0 state, as infected B cells expressing immunogenic lytic and latent antigens are readily cleared (Taylor et al 2015). In response to primary infection, plasma B cells are stimulated to secrete antibodies such as IgG. These immunoglobulins target virus capsid antigens (VCA), along with the EBV nuclear antigen proteins, EBNA1 and 2. Many of the antibodies secreted during primary infection, including IgG, are maintained throughout persistent infection. This has facilitated in identification of EBV seropositive carriers to be used as healthy donors when investigating EBV specific immune responses (Henle et al 1987).

Along with these initial B cell responses, both CD4+ and CD8+ T lymphocytes proliferate, increasing in number during primary infection and IM. These cellular immune responses involve the interaction between the T cell receptor and pMHC complexes expressed by the infected B cell (Rickinson et al 2014). Most of the T cell expansions seen in IM patients, are CD8+ T cell driven, with large expansions occurring within the peripheral blood (Steven et al 1996). The majority of the immunodominant CD8+ T cell expansions recognise antigens associated with the lytic life cycle of the virus. Responses of up to 40% being specific for a single EBV epitope, derived from early lytic cycle proteins have been reported (Abbots et al 2013). Through analysis of T

cell clones, epitopes of immediate early (IE) and some early (E) lytic cycle proteins, including BMLF1 and BZLF1, have also been revealed to be key targets of these CD8 T cell responses (Steven et al 1997, Abbots et al 2013). These findings have been facilitated through the use of MHC class I tetramer reagents, allowing the phenotypic characterisation and quantification of epitope specific CD8+ T cells. (Rickinson et al 1997, Callan et al 1998). A lower frequency of up to 5% of the total peripheral CD8+ cell population have been shown to successfully recognise some of the latency III proteins. These include the Epstein Barr nuclear proteins; EBNA 3A, 3B, 3C and LMP1 which induce a cytotoxic immune response and elimination of EBV infected B cells (Hislop et al 2007).

As primary EBV infection is resolved, the number of cytotoxic CD8+ T cells within the peripheral blood decline to a level seen in long term healthy carriers, leaving behind a small proportion which form the EBV specific memory CD8+ T cell pool that go on to control persistent chronic infection. Once this virus specific memory pool has been established, the phenotypic, functional and TCR repertoire composition is stabilised and maintained for several years (Klarenbeek et al 2012).

Recent technological improvements in MHC class II tetramer usage has enabled efficient analysis of live EBV specific CD4+ T cells, on a single cell level. Furthermore, this has allowed for the determination of their epitope specificity and HLA restriction (Long et al 2011, Long et al 2013). Despite individual epitope frequencies being much lower than that of CD8+ T cell responses to the same antigenic proteins during primary infection and IM, CD4+ T cells recognise a broader range of lytic and latent EBV derived peptides, meaning the antigen epitope repertoire covered is much larger (Long et al 2011).

Along with their helper functions for other immune cells, research has begun to explore the immune responses of these less pronounced HLA class II restricted CD4+ T cells, to act independently of CD8+ T cells, in a direct cytotoxic manner against EBV infection. Effector functions are of key interest and importance to anti-viral immune responses, especially for viruses such as EBV that occur in cells expressing MHC II. Investigations into the primary differential process, along with proliferative capacity of CD4+ T cells, have demonstrated that these cells differentiate from naive cells into a Th1 subset of EBV specific CD4+ T cells, producing many common cytotoxic cytokines including IFNγ, TNFα and IL-2, and can induce the elimination of EBV-transformed lymphoblastic cell lines (LCLs) i*n vitro* (Appay et al 2002). However, further research is required to determine whether this cytotoxic function similarly occurs *in vivo*.

1.2.3 EBV Associated Lymphoproliferative Diseases

EBV contains cellular growth transforming abilities through its expression of key latency III genes encoding proteins which drive cellular proliferation, inducing B cell transformation and tumorigenesis (Table 1.1) (Kang et al 2015, Kempkes et al 2015).

EBV latency III genes	Role in B cell transformation
EBNA1	Replication and maintenance of the episomal EBV genome.
EBNA2	Transcriptional activation of B cell antigens and LMP1/2. Transactivation of viral C promotor.
EBNA3A, B, C	Transcriptional regulators which bind the host RBP-Jκ transcription factor supressing binding to cognate Jκ sequence.
EBNA-LP	Required for efficient lymphoblastic cell outgrowth.
LMP1	LMP-1 is a six-span transmembrane protein which induces the expression of cell surface adhesion molecules and activation antigens along with driving latently infected B cells to leave the germline centre and become memory cells.
LMP2A/B	Delivers a ligand independent BCR signal driving both survival of resting B cells along with differentiation, proliferation and development of plasma cells secreting immunoglobulins.
BHRFI	This is a homologue of the anti-apoptotic protein Bcl-2 and an inhibitor of cellular apoptosis.
BART	Encode microRNAs which facilitate long term persistence of the virus in an infected host.
EBERS (EBV-Encoded RNA)	These noncoding RNAs not only induce growth but also provide resistance to protein kinase dependant apoptosis.

Table 1.1 EBV latency III genes (Kang et al 2015, Kempkes et al 2015).

In healthy immunocompetent individuals, the development of malignancy is prevented by the previously described EBV specific T cell immune surveillance mechanisms. However, in immunosuppressed individuals, this can result in a loss of circulating virus specific T cells, which has been shown to increase the EBV viral load and increase the number of viral episomes generated within infected B cells (Thorley-Lawson et al 2016). Consequentially, this can trigger the onset of various types of EBV associated B cell lymphomas including, Hodgkin's lymphoma (HL), Burkitt's lymphoma (BL), Post-transplant lymphoproliferative disease (PTLD) and Diffuse Large B cell lymphoma (DLBCL) (Babcock et al 1999).

The different forms of EBV associated lymphomas have different patterns of latency, involving the expression of differing viral genes. Burkitt's lymphoma is associated with latency type I, with EBNA-1 being highly expressed by these cancer cells. Latency type II has been linked to the development of HL and the epithelial derived cancer: nasopharyngeal carcinoma (NPC). They are characterized by the expression of latent genes EBNA-1, LMP-1 and LMP-2. EBVs Latency III gene profile involves the expression of all EBV antigens and is a key characteristic of PTLD. The action of these latency genes is essential for growth transformation and over proliferation of infected B lymphocytes (Grywalska et al 2015, Lowe et al 2017).

1.2.4 EBV Positive Post-Transplant Lymphoproliferative Disease

Post-transplant lymphoproliferative disease (PTLD) is of key interest, with EBV infection being associated with 90% of all PTLD cases (Gottschalk et al 2005). Being a heterogonous disease, PTLD presents with a diverse set of clinical symptoms and morphologies. This can include IM type symptoms and the development of polyclonal lymphoproliferative lesions. It can be divided into three categories, early lesions, polymorphic PTLD and monomorphic lymphomatous PTLD, with the majority of the latter being diffuse large B cell lymphomas (Gottschalk et al 2005). The development of PTLD has been linked to a deficiency in EBV specific T cell immune responses. This is the result of immunosuppressive drug administration to prevent graft rejection in solid organ transplantation (SOT), and both high dosage chemotherapy and the use of immunosuppressive medication, to prevent graft-vs-host disease, in allogeneic haematopoietic stem cell transplant (HSCT) recipients (Mansour et al 2013). Due to immunosuppression and functional impairment in immune surveillance, the EBV infection is allowed to be reactivated in seropositive individuals and results in the outgrowth of genetically unstable lymphoblastic B cells and clinical symptoms to appear. The majority of PTLD cases in SOT and HSCT patients occur within the first-year post-transplant. Early onset PTLD has been highly associated with EBV and in many cases, shows high expression levels of the latency III proteins. Whereas late onset monomorphic cases have additionally been linked to cellular mutations in tumour suppressors and oncogenes such as c-Myc (Mansour et al 2013).

The expression of latency III proteins in early onset PTLD makes this lymphoproliferative disease an ideal candidate for adoptive immunotherapies, as they

can be directly recognised and eliminated by infused EBV specific cytotoxic T cells (Gottschalk et al 2005).

1.3 Immunotherapy for EBV Associated Cancers

EBV related B cell malignancies, such as PTLD, can be treated with a range of immunotherapies due to the expression of multiple EBV latency III antigenic proteins and their epitopes which can be targeted specifically.

1.3.1 Standard Therapy

Initially, monoclonal antibody (mAb) therapies in combination with chemotherapy were administered to SOT and HSCT patients suffering from PTLD. Rituximab, a mAb against the B cell antigen CD20 has been used extensively in patients with a high viral load (Oertel et al 2005). However, a significant proportion of patients fail to respond to this treatment, showing relapse and an increased risk of mortality. Due to this and limitations in relation to rituximab's bio-distribution, modest tumoricidal activity and significant B lymphocytic depletion this has led to research into more effective T cell based immunotherapies.

1.3.2 Adoptive T Cell Immunotherapy

To combat the issues of standard therapies, there has been an increased interest in the modulation of T cells which are able to directly recognise, target and destroy cancer cells. The main goal of cellular adoptive immunotherapy is to utilise the immunomodulatory capabilities of T cells in the elimination of viral infections and cancer, whilst, preventing any adverse effects such as graft vs host disease (GvHD).

1.3.3 Cytotoxic T Lymphocyte Infusions

Harnessing the activity of EBV specific cytotoxic T lymphocytes (CTLs) has been shown to be clinically effective in treating both SOT and HSCT PTLD, even in patient's refractory to monoclonal antibody-based therapies (Papaddopoulous et al 1994).

CTLs are generated by *in vitro* stimulation of patient or donor PBMCs with autologous lymphoblastic cell lines (LCLs). LCLs are in vitro EBV transformed B cells, which express a full range of latent III proteins, and resemble the tumour cells that outgrow in PTLD. The antigen specific CTLs are then expanded and infused into patients. By isolating HLA matched epitope specific CTLs, this reduces the risk of GvHD. Clinical success has been observed through trials utilising EBV-specific CD8+ CTLs to target various B cell lymphomas. EBNA3A, B, C and LMP2 specific CTL infusions have been used in the treatment of Hodgkin's lymphoma, with results showing adoptively transferred CTLs induced complete tumour responses in some patients (Bollard et al 2004). Furthermore, clinical trials on patients undergoing HSCT, with a high risk of developing B cell lymphoma, successfully showed prophylactic treatment with polyclonal EBV specific CTL lines and reconstituted immunity against EBV. This was displayed through a decrease in EBV-DNA and an expansion in viral specific immune activity leading to long term remission of patients (Heslop et al 2010).

1.3.4 Therapeutic Importance of CD4+ CTLs

CD4+ T cells are well known for their helper roles in enhancing the activity of cytotoxic CD8+ T cells along with their ability to be functional memory cells. There is also research investigating the direct effector function of cytotoxic CD4+ T cells (CD4+ CTLs), in responses to viral infection and cancer, and the therapeutic potential for these

immune cells to be used to control EBV induced B cell malignancies such as PTLD, where the tumour cells present antigens via MHC class II.

CD4 CTLs have been shown to be most closely related to the Th1 subset in terms of transcription factor differentiation regulation and cytokine production (Takeuchi et al 2017). The cytotoxic effects of these cells have been associated with various previously established CD8+ mechanisms including Fas-FasL interactions, the perforin/granzyme pathway, and granulysin release (Appay et al 2002). During differentiation and expansion, CD4+ CTLs are able to lose the expression of co-stimulatory molecules such as CD28 and CD27. In doing so, they gain the expression of various cytotoxic granular proteins including granzymes and perforin, along with memory markers such as CD45RO and the activation marker CD69, associated with cytotoxic functioning. These are seen similarly in antigen experienced CD8+ T cell development. Thus, CD4+ CTLs gain the cytotoxic phenotypic features used to target and eliminate virally infected cancer cells (Appay et al 2002, Vanhoutte et al 2009).

In the context of EBV, in vitro studies have demonstrated the direct cytotoxic capacity of isolated and cultured EBV specific CD4+ T cells. These cells can directly lyse epitope loaded LCL targets and inhibit their outgrowth, in vitro (Münz et al 2000, Long et al 2005, Taylor et al 2006).

The importance of EBV specific CD4+ T cells in adoptive immunotherapy has been exemplified in clinical trials, using 3rd party cytotoxic CTLs generated from HLA-matched allogenic EBV-seropositive donors, to treat patients with SOT PTLD. All CTL infusions, contained populations of both CD8+ and CD4+ T cells. From 33 patients treated, the overall response rate was 64% at 5 weeks, showing a better response in

patients receiving CTL infusions, that comprised of a greater proportion of CD4+ T cells. This trend was successfully maintained at 6 months after the first infusion with a total response rate of 52% and no long term off target adverse effects observed (Haque et al 2007). This demonstrates the importance of CD4+ T cells in providing help to CD8+ cytotoxic T cells. Whether CD4+ cells within CTL infusions are helper or direct effector cells is still unknown, however it does provide a starting point for future alternative approaches for the treatment of various viral infections and cancers.

There are now a growing number of third party donor derived EBV specific CTL cell banks that have been manufactured both within the UK and USA, showing to offer an effective curative strategy for the treatment of EBV associated lymphomas (Leen et al 2013) (Vickers et al 2014). Although this adoptive immunotherapy has shown some success in EBV+ lymphoma patients, previously failing to respond to standard therapies, there are various constraints surrounding this therapeutic approach. Forth most, *in vitro* generation of donor derived EBV specific CTLs and target EBV transformed B cell lines takes 40-60 days to complete, and a further 28-35 days of expansion and elimination of alloreactive T cells (O'Reilly et al 2016). With CD4+ T cells being much lower in frequency within donor blood this can additionally result in a poor yield of virus specific CTLs being obtained. Furthermore, the final CTL population produced has to be patient specific with regards to screening for HLA allele matches. For this to be most therapeutically efficient, it requires the identification of an individual matched allele that also confers antiviral activity against the infecting virus in both donor and recipient. This has led to investigations into other novel methods, such as TCR gene transfer, in order to attempt to eliminate some of these issues.

1.3.5 TCR Based Therapies-TCR Gene Transfer

The limitations surrounding adoptive transfer of EBV specific CTLs, has led to exploration of the T cell receptor molecule and its therapeutic relevance in adoptive T cell- based transfer applications.

Research has begun investigating TCR gene transfer as a therapeutic approach to target many forms of cancers, this holds great therapeutic potential. Theoretically the T cells being subject to this gene transfer approach could be engineered to respond to virtually any tumour specific antigen. Currently the identification of candidate TCRs involves the isolation of tumour specific T cell clones. Genes encoding both the alpha and beta chains of the TCR can be identified and sequenced on a single cell basis. These are inserted into retroviral or lentiviral vectors and transferred into T cells, allowing for the rapid generation of antigen specific T cell populations. Currently, genetically transferred TCRs are being used to treat several forms of advance stage cancer directed against both tumour and viral antigens. Many of these have shown some degree of clinical success in terms of direct killing capacity by the genetically modified T cells (Rapoport et al 2015, Yao et al 2016).

Identifying antigen targets which are shared amongst many patients, presented through the same HLA allele, expressed explicitly on tumour cells and are highly immunogenic is key to success. This forms the basis for the safety and efficiency of TCR based gene therapy, however it still remains a significant challenge. The majority of TCRs clinically tested previously have been directed against tumour associated antigens (TAAs) such as the melanoma associated antigens; MART1 and gp100, and the squamous cell carcinoma antigen NY-ESO1. Many of these antigens are normal,

nonmutated self-proteins selectively expressed or overexpressed on cancers cells (Johnson et al 2009). Unfortunately targeting TAAs has resulted in on-target toxicity with normal tissue expressing low levels of a target antigen (Bendle et al 2010).

Exploration into virus specific antigens associated with cancer development has enabled some of these previous challenges to be overcome in a subset of cancers derived specifically from viral infections. Cancers associated with transforming viruses, express viral protein products which are attractive antigen targets for TCR gene therapies, as they are not expressed by normal healthy cells.

With regard to TCR gene transfer therapy for EBV associated malignancies, this is a useful system as the antigens expressed on infected B lymphoblast's are truly foreign meaning off-target adverse effects are less likely to occur. Work conducted by Jurgans and colleagues demonstrated the transferal of HLA-A2, A23, and A24 restricted TCRs, derived from human CD8+ CTL clones led to a strong immune response and cellular lysis of target LCLs presenting the latency III protein LMP2, *in vitro* (Jurgans et al 2006). More recent studies have shown clinical success in the treatment of nasopharyngeal carcinoma, using LMP2 specific TCRs restricted though HLA A11, with robust antigen specific function being observed in transduced CD8+ CTLs (Zheng et al 2015). However, in the context CD4+ TCR gene transfer, little is known regarding therapeutic potential and to date there has been no development of an MHC class II restricted TCR gene transfer therapy targeting any EBV epitopes.

1.4 TCR Repertoire Analysis

The adaptive immune system generates a significant diversity of unique antigen specific TCRs due to V-D-J gene segment recombination events which occur during T cell development (Section 1.1.4). Following thymic selection, this process results in the formation of a vast αβTCR repertoire, however the number of different clonotypes expressed by the estimated 10^{12} T cells within the human body is still unknown (Arstila et al 1999). TCR selection for gene transfer therapies remains a challenge due to the broad TCR repertoire available. Recent in-depth analysis of the TCR repertoire of antigen specific T cell immune responses, however, has meant the most therapeutically relevant TCRs within an epitope specific population could be selected for in the future.

Advances in high throughput DNA sequencing has led to the determination of hundreds of thousands of CDR3 sequences, defining phenotypes and subsets of T cell clonal populations, and their TCR repertoire usage responding most significantly to single peptide epitopes (Wang et al 2010, Warren et al 2011). This has allowed for the comparison of individual T cell clones and their frequency in a given population of antigen specific T cells.

Recent studies have attempted to determine the immune TCR repertoire status under different disease statuses, including viral infection and cancers, selected for by specific peptide antigens. Work conducted by InYoung Song et al, investigating immune responses towards the influenza A M1 epitope in HLA-A2+ healthy individuals, demonstrated that epitope specific CD8 T cell populations express a broad repertoire of differing TCRs specific for the same pMHC complex. Most donors were found to have several hundred different TCRα and TCRβ sequences used by CD8+ T cells in resting

memory pools. However, a single public TCR beta variable (TRBV) gene segment, TRBV19, associated with a few specific CDR3 motifs, did appear to dominate 65% of the overall CD8+ T cell response to the M1 epitope (InYoung Song et al 2017). Point mutations within the TRBV19 gene, during early recombination events, will have promoted the production of these differing CDR3 motifs. This highlights the importance of structural similarities in peptide specific T cell populations responding to the same epitope. Further analysis of anti-viral T cell immune responses has utilised mass spectrometry, multiplex tetramer and antibody staining to quantify and phenotypically characterise multiple antigen specific CD8+ and CD4+ T cells at a single cell level within infected murine models (Fehlings et al 2018). The broad combination of phenotypic markers detected emphasises the diversity available during antigen specific immune responses.

The combination of both conserved and diverse structural and phenotypic components of an antigen specific TCR repertoire could be influential in preventing individual clonotypic loss. This has been shown to contribute to the maintenance of repertoire diversity and overall memory effector T cell pool generation.

With regards to CD4+ T cells although antigen specific CD4+ TCR repertoire usage is less well defined, it has been identified that antigen specific T cell populations can display differences in recognition of single peptides based on differences in their TCR contact residues located within the epitope peptide (Pu et al 2002).

To date, the only work attempting to investigate the relationship between TCR sequence and function of T cells, at a single cell level, was conducted by Han and colleagues. This involved the sequencing of tumour infiltrating lymphocytes TCRα and TCRβ

genes along with *in vitro* phenotypic analysis of transcription factors conferring function, in relation to subset differentiation, within single antigen specific T cells (Han et al 2014). This provided some suggestion that different TCRs activate antigen specific T cells differently increasing the diversity of differentiated effector memory T cell pools.

In the context of EBV infection and the TCRVβ usage during immune responses, early studies demonstrated that identical Vβ gene sequences were positively expressed by different CD8+ T cell clones in response to EBNA3 antigen epitopes, FLRGRAYGL (FLR) and QAKWRLQTL (QAK) (Burrows et al 1995). Thus, despite an enormous repertoire, it was shown that responses, could be conserved to a single TCRVβ sequence within the same healthy individual or separate HLA matched people.

More recently, the availability of large panels of monoclonal antibodies to TCRs, mainly against Vβ epitopes, has enabled the analysis of the TCR repertoire of an epitope specific population via flow cytometry. Using this technology, it has been shown within our laboratory that in healthy EBV seropositive donors epitope-specific CD4+ T cell populations can comprise T cells expressing multiple TCRs, however, there is often an over-representation of single Vβ chain usage (Figure 1.7).

These findings provide some indication that there is a form of precise TCRVβ repertoire usage in antigen specific immune responses, towards single EBV peptide epitopes, that may provide a structural/functional advantage for TCR recognition. However, in spite of this previous TCRVβ repertoire analysis, much less is known regarding the relationship between TCR sequence usage and the function of parent T cells.

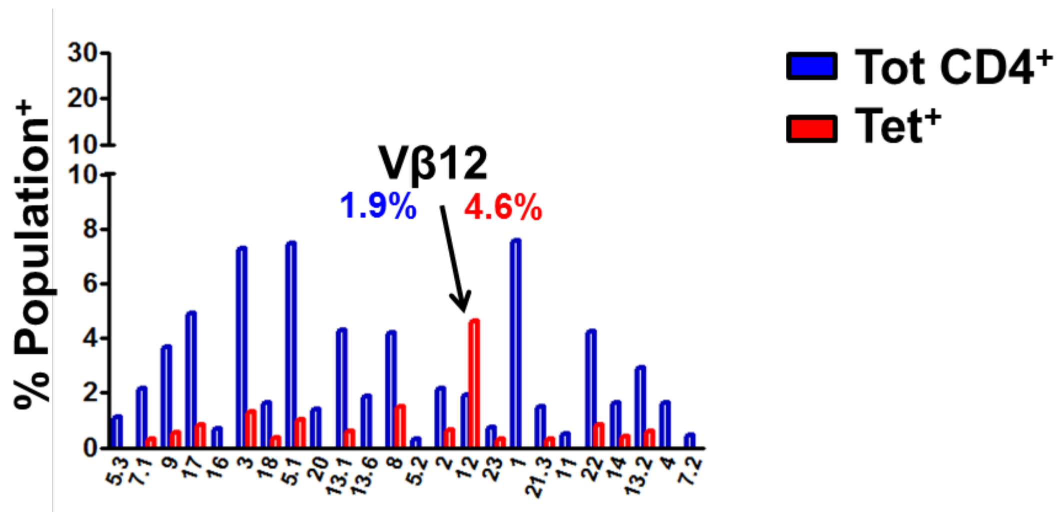


Figure 1.7 Clonogram of TCRvβ repertoire usage in EBV antigen specific CD4+ T cell populations. In tetramer positive T cell populations (red) there is an increased representation of 4.6% for Vβ12. (Unpublished data Benjamin Meckiff University of Birmingham).

1.5 The Relationship between TCR Usage and T cell function

The use of immunodominant TCRs which promote optimal anti-viral and anti-tumour activity have been proposed to enhance the effectiveness of gene-modified T lymphocytes. Research investigating the different TCR properties that confer the most efficient immune responses is thus essential for further improvements in TCR gene transfer therapy.

The parameters used to describe the ability of T cells to recognize and respond to target antigens include TCR affinity, and functional avidity. TCR affinity refers to the physical strength of monomeric interactions between TCRs and pMHC complexes (Vigano et al 2012). In immune responses these interactions are generally of low affinity, which makes it difficult to determine relationships between initial binding parameters and T cell function. However, assessment of the strength of these interactions has shown a slower MHC dissociation rate during TCR binding leads to a better T cell immune response (Holler et al 2003). T cell activation remains critically dependent on this functional parameter. Through optimising structural components within an introduced TCR, it is thought this could lead to an increase in TCR surface expression and improve the efficiency of TCR gene transfer therapies. Enhancing the TCR affinity through *in vitro* maturation, has been associated with more efficient immune responses in transferred T cells (Thomas et al 2011). However, defining the optimal affinity threshold between anti-tumour and autoimmune activity is essential to prevent adverse cross-reactivity effects seen previously in many *in vitro* enhanced affinity TCRs.

Initial TCR binding interactions have been associated with influencing the functional avidity of a T cell. Functional avidity is defined as the biological measure of the *ex vivo* response produced by a T cell to a given concentration of target peptide and comprises the efficacy and sensitivity of recognition and the cellular response.

Biological responses include proliferative capacity, cytokine production and cytotoxic activity of a responding cell. Antigen specific T cells producing the best responses to target cells presenting a lower density of peptide, are considered to be high avidity. In the context of the HIV virus, it has been shown *in vitro* that epitope specific CTLs exhibiting high functional avidity can mediate a robust immune response and superior control of viral replication (Almeida et al 2007).

The relationship between the functional parameters of a T cell and its TCR usage has not been explored in depth, *ex vivo*. This is important to study, as the identification of those antigen specific T cell populations exhibiting the most efficient anti-viral and anti-cancer immune responses is essential for the future optimisation and development of effective TCR gene transfer immunotherapies. For CD4+ T cells, this is especially true for malignancies expressing antigens through MHC class II, such as EBV associated PTLD.

1.6 EBNA2 Model

This project addressed these issues focusing on an epitope derived from the EBV latency III protein, EBNA2. This antigenic protein is one of the first to be expressed by infected cells and is important in B lymphocytic transformation, as it drives the expression of other crucial viral and cellular genes. It has been shown to directly transactivate the CP promotor involved in early B cell infection, and upregulate

oncogenes such C-Myc previously associated with unregulated cellular over-proliferation (Young et al 2003, Kim et al 2016).

Despite the evidence of its oncogenetic functioning, EBNA2 is still yet to be explored in depth as a therapeutic biomarker for immunological based treatments. Due to its high levels of immunogenicity in most people infected and presentation of epitopes on MHC class II molecules by infected lymphoblast's, this makes it an ideal antigen target for cytotoxic CD4+ T cell immune responses and EBV specific TCR gene transfer immunotherapies (Khanna et al 1997). The previous determination of many epitopes within EBNA2 combined with development and optimisation of EBNA2 MHC II tetramer reagents, makes it a useful model when investigating epitope specific T cell populations, *ex vivo.*

Therefore, we explored the EBNA2 epitope; PRS which has been shown to elicit strong cytotoxic CD4+ immune responses in the context of several different HLA class II alleles (Long et al 2005). PRS specific CD4+ T cell clones have now been successfully generated which have the capacity to directly recognise LCL target, along with the ability to eliminate their outgrowth, *in vitro*. (Long et al 2005). The EBNA2 epitope PRS, is thus an ideal epitope target for analysing the relationship between TCRVβ repertoire usage and T cell function in a therapeutically relevant setting.

1.7 Aims of the Project

We hypothesised that TCR usage may affect T cell function, making some TCRs of greater therapeutic benefit than others.

The project aims were therefore:

(i) To isolate a panel of PRS specific CD4+ T-cell clones from 2 healthy HLA matched EBV seropositive donor by, *ex vivo* MHCII tetramer selection.

(ii) To analyse the TCRVβ repertoire usage of the epitope specific CD4 T cell clones, using flow cytometry.

(iii) To investigate the function of the PRS-specific T cell clones with known Vβ usage, through assessing their functional avidity, and the ability of the clones to recognise physiological levels of epitope expressed by infected B cell targets (LCLs).

CHAPTER 2

2. MATERIALS & METHODS

2.1 Ethics Statement and Donors

All blood samples used were obtained from healthy laboratory volunteers who provided written informed consent to participate in the project. Apheresis were provided by Birmingham NHS Blood transport. All experiments were in concordance with the University of Birmingham and South Birmingham LREC (14/WM/1245).

2.2 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood of HLA-DR7+ seropositive healthy donors, and separated by Ficoll-Paque centrifugation. 30ml of Peripheral blood was combined with plain RPMI 1640 medium at a 1:1 ratio and layered on Ficoll-Paque; a density gradient media used to separate mononuclear cells from whole blood. Following centrifugation at 1800rpm for 30 minutes (no brake) at room temperature, PBMCs were extracted from the mononuclear/lymphocyte cell layer at the plasma-Ficoll interface using a clean transfer pipette. PBMC were washed 3 times in RPMI 1640 medium and resuspended at $1x10^6$ cells/ml in standard media (Table 2.1).

2.3 Generation of EBV Specific T cell clones

2.3.1 Isolation of PRS Specific CD4+ T Cells by MHC Class II Tetramer Staining and Fluorescent Activated Cell Sorting

CD4+ T cells were negatively isolated from PBMCs (Section 2.2) utilising a Dynabeads isolation kit. The isolated cells were re-suspended in 1ml of Human serum, centrifuged (1600 rpm for 5 minutes) and incubated for 1 hour with 1µl of HLADR7/PRS tetramer complex conjugated to PE, to reactivate tetramer specific CD4+ T-cells. The cells were then washed with MACs buffer to remove any unbound HLA class II tetramer/peptide complex and stained for CD3+ and CD4+ surface markers for 20 minutes in darkness. Appropriate compensation tubes were prepared including anti-CD3 APC, anti-CD4 ECD and anti-CD4 PE for tetramer positive cell populations. The cells were fluorescent activated cell sorted (FACS) in order to obtain frequencies of tetramer positive CD4+ T Cell populations from each healthy donor.

2.3.2 Limiting Dilution Cloning

Clones were established by seeding tetramer positive CD4+ T cells (Section 2.3.1) at 0.3, 3 and 10 cells/well into round bottom wells of ninety-six well culture plates. This method increased the likelihood of obtaining a monoclonal daughter cell population derived from a single T cell, from this initial polyclonal mass of CD4+ cells. Irradiated autologous HLA-matched LCL cells (10^5/well) and feeder cells (10^6/well) were added to the tetramer positive CD4+ T cells along with Human Serum and RPMI supplemented with a Pen/Strep antibiotic mix (CD4+ Cloning mixture). The HLA–matched LCLs were pulsed with cognate PRS peptide (Table 2.2) for 1 hour prior to co-culturing with CD4+ T cells. The feeder cells were mononuclear preparations derived

from 3 pooled fresh buffy coats, which had been previously exposed to phytohemagglutinin (PHA) at 10µg/ml for one hour, and then washed twice in standard media (Table 2.1). Both the PHA treated and peptide pulsed autologous HLA-matched LCLs were γ-irradiated at 4000 rads and washed twice before use. The seeded cells were left for 2 weeks and screened for peptide specificity as described below (2.6.2). Any epitope specific clonal populations were expanded further via transfer into 24 well plates, with 10^5 LCL cells/well and 10^6 feeder cells/well, kept in 5% CO_2 and at 37^0C.

2.3.3 Maintenance of T cell clones

The T cell clones were maintained in T Cell media (Table 2.1), fed twice weekly and split into new wells when proliferation confluence was apparent. If growth rate was poor, re-stimulation was performed which involved the addition of irradiated (4000 rads) LCLs and PHA treated feeder cells.

2.4 Generation and Culture of Lymphoblastic Cell Lines (LCL)

LCLs were generated *in vitro* from donor PBMC derived B lymphocytes with the use of the marmoset B59.8 producer. LCLs were obtained by centrifugation of 4mls of B59.8 supernatant which was filtered through a 0.45µm syringe and incubated overnight at 37^0C 5% CO_2. The next day the PBMCs were centrifuged and resuspended in 2ml of standard culture media supplemented with 1µg/ml cyclosporine A for a period of two weeks in a 24 well plate. Cells were split once sufficient proliferation had occurred. All LCL lines were maintained in standard media. The LCLs used in this project were either generated specifically for these experiments or previously had been made and stored in liquid nitrogen via cryopreservation (Section 2.5).

2.5 Cryopreservation and Restoration of Cells

Cryopreservation involved centrifugation of cells (LCL and T cells) at 1600rpm for 5 minutes. The cells were resuspended in freezing media (Table 2.1) and transferred to a sterile 1ml cryovials. These were then transferred to a Mr. frosty containers holding isopropanol which was placed into a -80°C freezer.

Restoration of cells began with removal from of cryovials -80^{0}C freezers and transferal into a water bath heated to 37°C. Post thawing, the cells were resuspended in relevant culture media dropwise and washed twice via centrifugation at 1600rpm for 5 minutes in order to remove freezing media. Cells were then either used in experimental analysis or transferred to a 24 well plate and incubated at 37°C and 5% CO2.

2.6 Functional Analysis of T cell clones

2.6.1 IFNγ ELISA

IFNγ ELISA was performed to measure many factors of functionality of the T cell clones. Ninety-six well ELISA plates were pre-coated with anti-IFNγ monoclonal antibody (MAb) and left overnight at 4^{0}C. The next day the plates were blocked for 1 hour with blocking buffer (Table 2.1) to prevent any non-specific binding. Following washing the plates with PBS buffer (Table 2.1), 50µl of supernatant from the T cell clone/LCL mixtures being tested (Sections 2.6.2, 2.6.3 and 2.6.4) was added along with IFNγ standards into separate wells, and left for 3-4 hours at room temperature. Standards were made by double dilutions of IFNγ from 20,000 pg/ml to 0pg/ml in standard media. This incubation was followed by another PBS wash and the addition of 50µl of biotinylated anti-IFN-γ MAb to each well which was incubated at room temperature for 1-2 hours. Any unbound secondary antibody was then removed through

washing, and replaced with 50µl of streptavidin horseradish peroxidase to each well, left to incubate for an additional 30 minutes. After a final wash, 100µl of TMB (3,3',5,5'-tetramethylbenzidine) soluble substrate was added to each well and yielded a blue reaction product. The reaction was stopped after a period of 20 minutes with the addition of 100µl 1M Hydrochloric acid. The amount of IFNγ produced by each clone was analysed using a MPM6 exe plate reader and excel software package measuring A450. IFNγ release was calculated by comparing A450 from T cell clones against a standard curve.

2.6.2 Screening Polyclonal T Cell Populations for Peptide Specificity

50µl of each isolated CD4 T cell clonal culture was incubated overnight in V bottom micro test plate wells with HLA II matched LCL target cells (50,000 cell/well), media and PRS peptide (Table 2.2). The HLADR7 matched LCLs were either un-manipulated, or pre-exposed for 1h to 5µl of PRS epitope peptide. The clones were additionally tested with media alone and media combined with 10µg/ml peptide. After overnight incubation the test supernatant medium from the T cell and LCL co-cultures was assayed for IFNγ production by the previously described ELISA method (Section 2.6.1). A positive result was higher IFNγ production from the LCL/peptide compared with LCL alone and media/peptide compared to media alone.

2.6.3 Peptide Titration Assays

The functional avidities of T cell clones were similarly tested in ELISA assays of IFNγ release, against titrated concentrations of individual defined PRS peptide epitope presented by autologous HLA-matched LCL targets. Equivalent volumes of LCL media and T cell clones were added to separate wells as negative and positive controls. T cell

clones (100µl/well) were added to triplicate wells at known cell numbers (2000cells/well) in the presence of 80µl HLA matched LCL targets (50,000cells/well) and 20µl PRS peptide epitope at dilution range of $1x10^{-5}$ to $1x10^{-11}$ M (including no peptide). The plates were incubated overnight at 37°C in 5% CO2. The supernatant medium from the T cell and LCL co-cultures was harvested after 12-16 hours and assayed for IFNγ by ELISA (Section 2.6.1).

2.6.4 LCL Recognition Assays

Individual T cell clones were incubated in v bottom micro test plate wells with 50,000 autologous HLADR7 matched or HLA mismatched LCL (100µl/well). This was performed in triplicates. The LCLs were either un-manipulated or pre-pulsed with 5µl of exogenous PRS peptide epitope for 1 hour and then washed with standard media. These were co-cultured with 100, 500, 1000 or 5000 cells of a given T cell clone at 37^0C 5% CO_2. LCL and T cell controls were included in separate wells in which they were incubated with standard media alone. The supernatant was harvested after 12-16h and an ELISA assay measuring IFNγ was performed (Section 2.6.1).

2.7 TCRVβ Analysis of PRS Responsive T cell clones

2.7.1 T cell TCRVβ Usage Analysis

From each donor, PRS specific CD4+ T cell clones, were analysed in order to phenotypically characterise their TCRVβ expression and specificities. A matrix of pools of T cell clones for each donor was constructed. Each pool within this matrix, contained 6 clones (Donor 1) or 5 clones (Donor 2) and each clone was present in 2 different pools. This analysis was performed using an IOTest Beta Mark TCR Repertoire staining kit covering around 70% of the normal human TCRVβ repertoire

(Beckman Coulter). The kit contained 8 vials of mixtures of conjugated TCRVβ antibody complexes; A to H, each composed of 3 separate TCRVβ antibodies labelled with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or doubly labelled with FITC and PE (Table 2.3). These antibody complexes corresponded to 24 different Vβ subfamilies.

T cell clones for a given pool were counted and a volume to obtain 700,000 cells (70,000cell/tube x 10 tubes) was extracted and dispensed into a 15ml tube. Once this was completed for all clones within a pool the volume within the 15ml tube was made up to 10ml with MACs buffer and distributed evenly between 10 FACS tubes; 1ml per tube. These were made up of an unstained sample, anti-CD3/CD4 gating antibodies only stained sample, along with the 8 TCRVβ antibody stained samples (A-H). An additional 1ml of MACs buffer was added to all tubes and each sample was centrifuged at 1600rpm for 5 minutes. Following centrifugation, 1µl of Viability Dye was added to relevant tubes and incubated at 4^{0}C for 30 minutes. The samples were washed and stained with 1µl of both APC conjugated anti-CD3 and ECD or PE-Cy7 conjugated anti-CD4 gating antibodies followed by 5µl of the relevant TCRVβ antibody complexes from the IOTest Beta Mark TCR Repertoire staining kit (Table 2.3). The cells were incubated at room temperature for 30 minutes followed by a final wash and resuspension in 250µl of MACs buffer prior to LSRII flow cytometer analysis.

Along with the test samples, compensation samples were additionally set up to compensate for fluorochrome overlap. This included; 1µl of APC-Cy7 conjugated anti-CD14, 1µl of APC conjugated anti-CD3, 1µl of ECD or PE-Cy7 conjugated anti-CD4, 2µl of PE conjugated anti-CD4 and 2µl of FITC conjugated anti-CD4. Compensation beads were distributed evenly between FACS tubes and the antibody conjugates were

added in relevant volumes. The samples were then analysed via flow cytometry using the LSRII; 20,000 events were recorded for each sample. The samples being tested and unstained control were gated on forward (FSC) and side scatter (SSC) after which any doublets and dead cells were excluded and CD3+ CD4+ T lymphocytes were gated on specifically. The data was then further analysed on Kaluza software. Confirmation of TCRVβ expression on individual clones was achieved through; the use of 7µl single conjugated TCRVβ antibodies (Table 2.4).

2.8 TCR Profiling

Certain T cell clones with known TCRVβ usage and displaying interesting results from functional analysis were further investigated via sequencing of both their alpha and beta chains.

2.8.1 RNA Extraction and 5'-RACE-Ready cDNA Formation from T Cell Clones

RNA was extracted from PRS specific T-cell clonal cell pellets of $5x10^5$ cells following the RNeasy plus microkit protocol, as per the manufacturer's instructions (Qiagen). RNA concentration was then measured using a Nanodrop microvolume spectrophotometer in ng/µl. Following RNA extraction, 5' and 3' RACE Ready cDNA was synthesised via reverse transcription of the eluted RNA utilising a SMARTer II A oligonucleotide primer and SMARTScribe reverse transcriptase, as per the manufacturer's instructions (Qiagen). This provided a 5' anchor for subsequent PCR procedures. Samples were stored at -20^0C.

2.8.2 Rapid Amplification of cDNA ends (RACE)

PCR 5'-3' RACE reactions were performed on the synthesised 5' RACE Ready cDNA samples (Section 2.8.1) to generate 5' and 3' cDNA fragments. This used gene specific primers for both the alpha and beta chains of the clones TCR. PCR cycling parameters included; 5 cycles at 94 degrees for 30 seconds, 72 degrees for 3 minutes. 5 cycles at 94 degrees for 30 seconds, 70 degrees for 30 seconds, 72 degrees for 3 minutes. 20 cycles of 94 degrees for 30 seconds, 68 degrees for 30 seconds, and 72 degrees for 3 minutes. These cycles of denaturation, annealing and extension were performed utilising a PCR system.

2.8.3 Characterisation of RACE Products

Agarose gel electrophoresis was performed on the RACE PCR DNA products (Section 2.8.2). This included negative controls for each TCR alpha and beta chain. Gel images were obtained using a UV-image analyser and bands of the correct size (200bp) were cut out. DNA of interest was recovered and purified using a gel extraction spin column method kit as per manufacturer instructions (Qiagen). DNA concentration was measured using a Nanodrop microvolume spectrophotometer in ng/µl.

2.9.4 DNA Sequencing

11µl of reaction mixture containing 4ng of the recovered and purified DNA (Section 2.9.3) and relevant alpha or beta primers were sent to the University of Birmingham Biosciences Genomics Laboratory for sequencing of the extracted DNA.

2.9 Tables of Reagents

Reagents and Medias	Ingredients
Standard Media	RPMI 1640 media (Sigma) supplemented with 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 10% Fetal bovine serum (FBS).
T Cell Media	RPMI 1640 media (Sigma) supplemented with 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 10% Fetal Calf Serum (BioSera), 1% Human serum (TCS Biosciences), 30% Monkey Leukocyte Antigen 144 (MLA 144) cell line and 50ug/ml interleukin 2.
CD4 T Cell Cloning Media	Standard culture media supplemented with 1% HuS, 50U/ml IL2 and 30% filtered supernatant harvested from the Monkey Leukocyte Antigen 144 (MLA 144) cell line.
Cyclosporine A Media	Standard culture media supplemented with cyclosporine A (1ug/ml).
Freezing Media	RPMI 1640 containing 2mM L-glutamine, 20% FBS and 10% DMSO.
Coating Buffer	10x stock solution was made of 1.36g Sodium Carbonate (Sigma), 7.35g Potassium Bicarbonate (Sigma) and 100ml sterile H2O. The buffer was adjusted to pH9.2 with 1M HCL (Sigma) or 1M NaOH (Sigma).
Blocking Buffer	1 1xPBS tablet (Thermo Scientific) was added to 500ml of sterile H20. To this 5g of Bovine serum albumin (BSA) (Sigma) and 250ul of tween (Sigma) was added.
Phosphate Buffer Saline (PBS) Wash Buffer	10 1x PBS tablets (Thermo Scientific) was added to 5Litres of sterile H20 and 2.5ml of tween (Sigma) was then added.
MACs Buffer	0.5% Bovine Serum Albumin (BSA) and 2.5mM Ethylenediaminetetraacetic acid (EDTA) was added to PBS.

Table 2.1. Summary of reagents used

Latency III Protein	Amino acid Coordinates	Epitope	HLA restriction
EBNA2	276-295	(PRS)TVFYNIPPMPLPPSQL	DRB1*07 (DR7)

Table 2.2. Summary of HLA-allele EBV epitope combination

2.10 Tables of Antibodies

Beckman Coulter TCRVβ antibody complexes:	Vβ:	Conjugated Fluorochrome:
A	Vβ 5.3 Vβ 7.1 Vβ 3	PE PE+ FITC FITC
B	Vβ 9 Vβ17 Vβ16	PE PE+ FITC FITC
C	Vβ18 Vβ 5.1 Vβ 20	PE PE+ FITC FITC
D	Vβ 13.1 Vβ 13.6 Vβ8	PE PE+ FITC FITC
E	Vβ 5.2 Vβ 2 Vβ 12	PE PE+ FITC FITC
F	Vβ 23 Vβ 1 Vβ 21.3	PE PE+ FITC FITC
G	Vβ 11 Vβ 22 Vβ 14	PE PE+ FITC FITC
H	Vβ 13.2 Vβ 4 Vβ 7.2	PE PE+ FITC FITC

Table 2.3. TCRVβ antibody complexes for TCR Vβ repertoire analysis

TCRVβ antibody	Source	Clone	Volume used per sample
PE conjugated anti-vβ1	Beckman Coulter	BL37.2	7ul
FITC conjugated anti-vβ2	Beckman Coulter	MPB2D5	7ul
FITC conjugated anti-vβ5.1	Beckman Coulter	IMMU 157	7ul
PE conjugated anti- vβ17	Beckman Coulter	E17.5F3.15.13	7ul
FITC conjugated anti-vβ21.3	Beckman Coulter	IG125	7ul

Table 2.4. Individual TCRVβ antibodies for staining of T cell clones

Antibody	Source	Clone	Amount used
APC conjugated anti-human CD3	Invitrogen	7D6	1µl
PE Cy7 conjugated anti-human CD4	BD Biosciences	RPA-T4	1µl
ECD conjugated anti-CD4	Beckman Coulter	SFCI12T4D11	1µl
PE conjugated anti-human CD4	BD Biosciences	RPA-T4	2µl
FITC conjugated anti-human CD4	BD Biosciences	RPA-T4	1µl
APC Cy7 conjugated anti-human CD14	BD Biosciences	HCD14	2µl

Table 2.5. Summary of surface antibodies used

CHAPTER 3

3. RESULTS

3.1 Isolation of Peptide Specific CD4+ T-cells from Healthy EBV Seropositive Donors

In order to isolate PRS specific CD4+ T-cell clones, we used a peptide:MHC class II tetramer (pMHC) loaded with the EBV PRS peptide, derived from the latency III protein EBNA2 and presented through HLADR7 (DR7/PRS). Fluorescent activated cell sorting (FACS) and limiting dilution cloning was performed on PBMCs, *ex vivo*. To generate CD4 T cell clones representative of cells within the peripheral circulation without in vitro expansion, PBMCs were isolated from the peripheral blood of 2 healthy EBV seropositive, HLADR7+ donors. CD4+T cells were negatively isolated from these. The CD4+ T cells were then exposed to the PE-conjugated pMHC tetramer complex (DR7/PRS) and surface stained with APC conjugated anti-CD3 and ECD conjugated anti-CD4 antibodies. The gating strategy for FACS is shown in Figure 3.1A. We gated on singlets, followed by lymphocytes and CD3+ CD4+ T cells. The DR7/PRS tetramer positive CD4+ T cells within this gate were collected.

The results, as indicated in Figure 3.1B and 3.1C, show the frequencies of DR7/PRS tetramer positive CD4+ T cell populations present within the peripheral blood of each healthy donor. The percentage frequencies were 0.1% of donor 1 and 0.025% of donor 2's total CD4+ T cell populations.

In order to isolate CD4 T cell clones originating from single epitope specific populations, FACS sorted cells were plated out by limiting dilution cloning at 0.3, 3 and 10 cells per/well into 96 well plates containing 100,000 peptide-loaded HLA matched

autologous LCLs and 1 million PHA-treated allogeneic buffy coat feeder cells per well (both previously irradiated with 4000 rads). The aim was to generate monoclonal populations of T cells originating from single circulating cells.

FIGURE 3.1

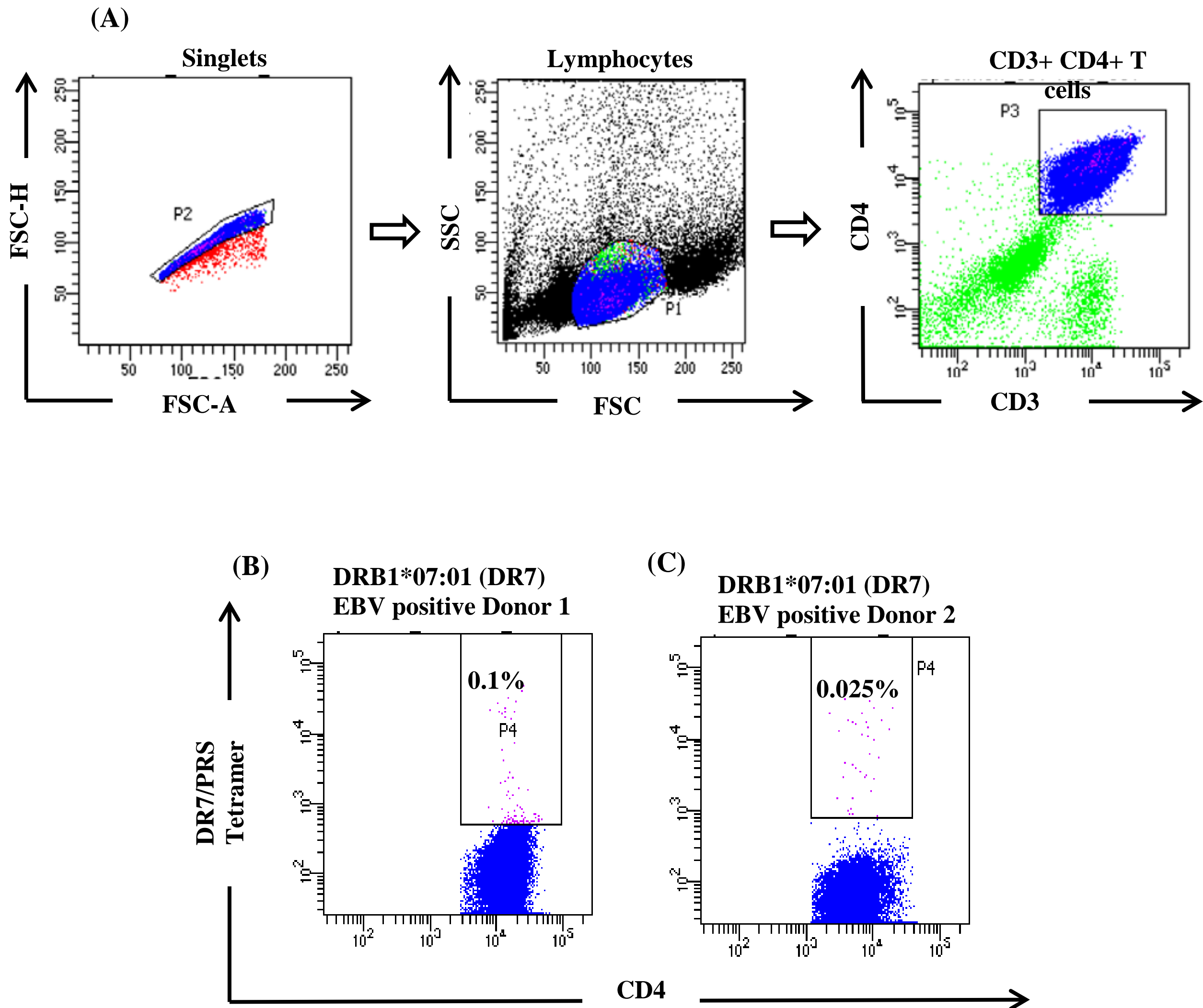


Figure 3.1 Fluorescent activated cell sorting of DR7/PRS positive CD4+ T cell populations

Analysis of CD4+ T cells populations from 2 EBV seropositive healthy donors stained with DR7/PRS tetramer conjugated to PE, followed by anti-CD3 and anti-CD4 antibodies. (A) CD4+ T cell gating strategy. From left to right: singlets, lymphocytes, and CD3+ CD4+ T cells. (B) Donor 1 sorted CD4+ T cells. The sorting gate shows 0.1% of the total CD4+ T cell population. (C) Donor 2 sorted CD4+ T cells. The sorting gate shows 0.025% of the total CD4+ T cell population.

3.2 Characterisation of T Cell Clones.

3.2.1 IFNγ Production in Response to Target PRS Peptide

From each healthy donor, the growing CD4+ T cell clones were screened for their specificity against the PRS peptide epitope. After a period of 2 weeks of T cell expansion, 155 growing clones from donor 1 were screened. To increase the probability that the proliferating cells originated from single cells, we concentrated on the growing clones from the 0.3c/w and 3c/w micro-culture plates. T cells were co-cultured with 50,000 HLADR7+ autologous LCL targets (per well) either un-manipulated or pre-loaded with 5μg/ml of PRS peptide epitope. T cell clones were also co-cultured with media alone or containing 5μg/ml of PRS peptide epitope. Positive CD4 T cell clones were identified by an increased response to LCL target pre-loaded with PRS peptide epitope over un-manipulated LCL and standard media combined with peptide epitope over media alone.

As illustrated in Figure 3.2, for the first 50 clones of the 155 T cell populations screened for donor 1, only a small proportion showed a greater response towards the LCL pre-loaded with PRS epitope than without peptide and to media with peptide above media alone. This represented 25% of the total 155 screened clones (Table 3.1). This was a relatively low yield obtained, most likely as a result of the gating strategy used in the FACs cell sorting for this donor (Figure 3.1), which incorporated a higher proportion of none specific CD4+ T cells.

After 2 weeks of T cell expansion 192 growing clones derived from donor 2 were similarly selected from the 0.3c/w and 3c/w micro-culture plates and screened for their specificity towards the PRS peptide. The results generated for this second donor are

displayed in Figure 3.3, which shows the first 50 clones screened. As illustrated out of the 192 clones screened 186 showed a response towards PRS which represented a yield of 97% (Table 3.1). This increased yield reflects the tighter gating applied during the FACS sorting (Figure 3.1).

PRS specific clonal populations from each donor were re-stimulated with HLADR7 matched LCL and Feeder cells and expanded further in vitro. The unresponsive non-specific clones were discarded. A summary of the FACS sorting, limiting dilution cloning and specificity screening is shown in Table 3.1. As indicated in the table, 31 PRS specific clones from donor 1 and 28 PRS specific clones from donor 2 were then analysed further for their TCRVβ usage.

FIGURE 3.2

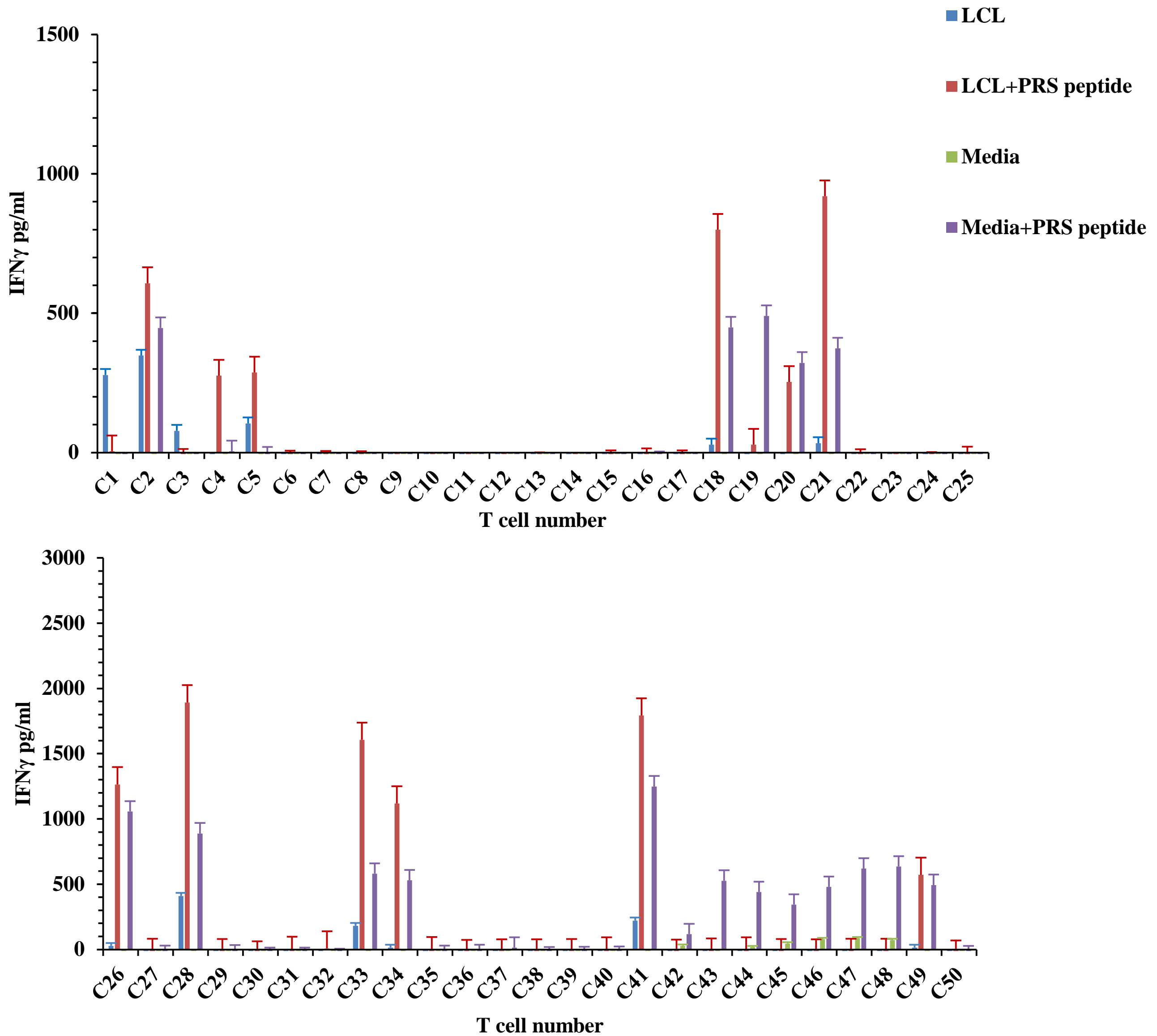


Figure 3.2 IFNγ ELISA screening of T cell clones from Donor 1

T cells from proliferating micro-cultures were co-cultured with autologous LCLs and LCLs pre-exposed to PRS epitope peptide**.** T cells were also co-cultured with standard media alone and standard media containing epitope peptide. Analysis of supernatant was conducted via IFNγ ELISA method. The first 50 clones of the 155 screened are represented. Error bars representative of the single experiment conducted.

FIGURE 3.3

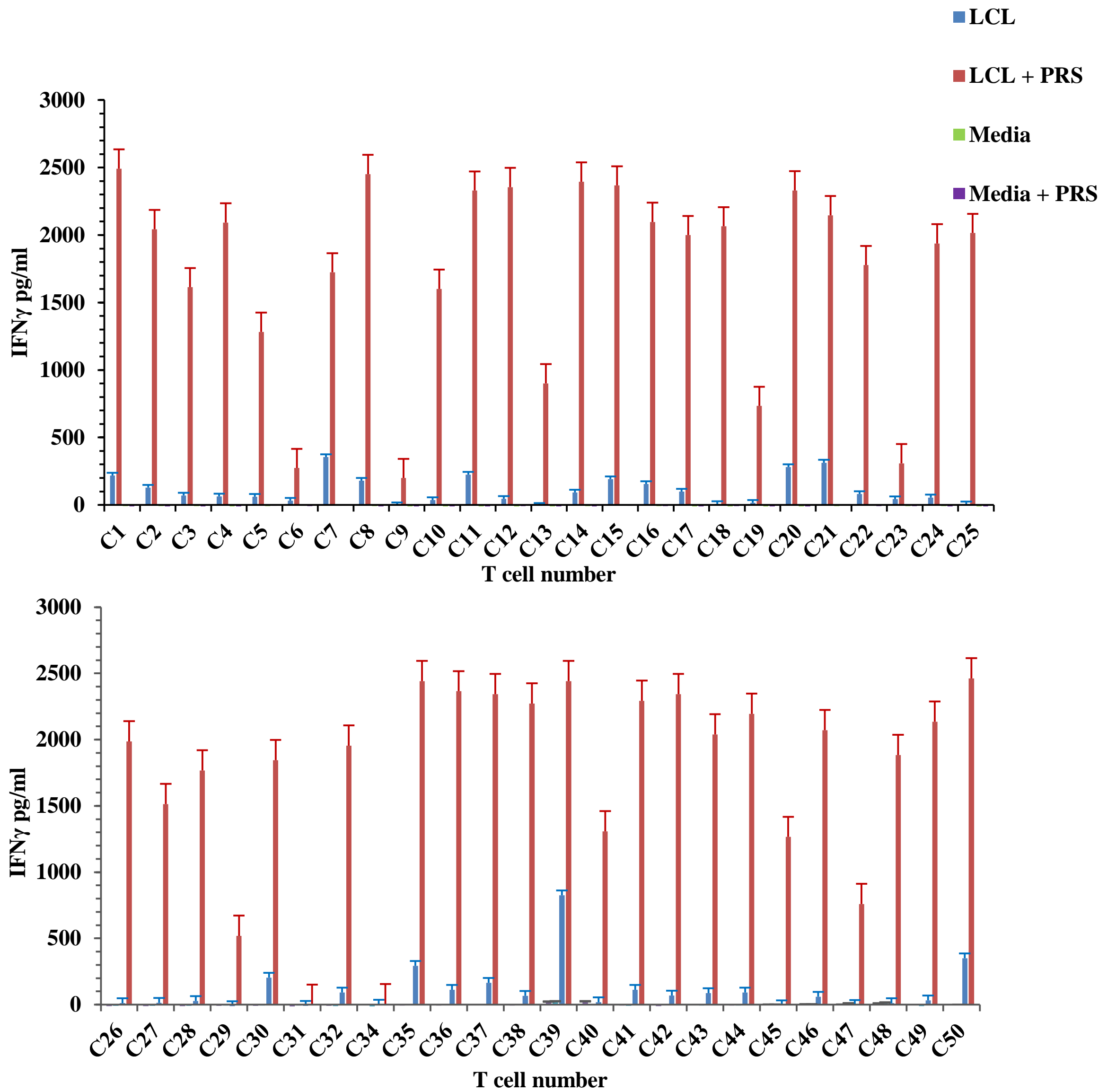


Figure 3.3 IFNγ ELISA screening of T cell clones from Donor 2

T cells from proliferating micro-cultures were co-cultured with autologous LCLs and LCLs pre-exposed to PRS epitope peptide. T cells were also co-cultured with standard media alone and standard media containing epitope peptide. Analysis of supernatant was conducted via IFNγ ELISA method. The first 50 clones of the 192 screened are represented. Error bars are representative of the single experiment conducted.

TABLE 3.1

	Donor 1	Donor 2
PBMCs extracted	20 million	50 million
Isolated CD4+ T cells	6 million	40 million
FACS sorted DR7/PRS positive CD4+ T cells	8000 (0.1%)	10,000 (0.025%)
Growing Clones screened for PRS specificity	155	192
Number & percentage of PRS specific clones	39 (25%)	186 (97%)
Number of PRS specific clones taken forward for vβ usage analysis	31	28

Table 3.1 Summary of peptide specific CD4 T cell clones isolated from whole blood, *ex vivo*

This summarises the number of original PBMCs extracted from each HLADR7+ matched donor, the number of isolated CD4 T cells, FACs sorted populations, growing clones assessed for PRS specificity, number and percentage of PRS specific clones and those taken forward for TCRvβ repertoire analysis.

3.3 Determination of T Cell Clonal Vβ usage

3.3.1 TCRVβ Repertoire Staining

After confirming the clones retained their PRS specificity following expansion, we then went on to examine their TCRVβ usage. These experiments used the multi-parametric IOTest Beta Mark TCR Repertoire Kit (Beckman Coulter) in flow cytometry, which contains a panel of 24 monoclonal antibodies specific for different TCRVβ families. The kit provides approximately 70% coverage of the human TCRVβ repertoire, and the Abs (antibodies) are provided in mixes. This allows for the detection of 3 different TCRVβs within the same tube. To limit the analysis required, a matrix was constructed enabling analyses of multiple clones together. The matrix for donor 1 is displayed in Figure 3.4, and was constructed such that each clone was present in a unique combination of 2 pools. Pools of clones were stained using the repertoire kit. This meant that each T cell clone was stained twice with the antibody mixes, in two different pools of clones.

As illustrated by Figure 3.5, pools of clones were gated on live CD3+ T lymphocytes. This gating strategy was used throughout all repertoire staining. As a control, an aliquot of each pool of clones was viability and surface stained with a CD3 surface antibody, but not stained with the IOTest Beta Mark Kit antibody mixes. There was no positive staining in the absence of these TCRVβ antibody mixes (Figure 3.5B).

Figure 3.6 shows density plots for two pools of clones from donor 1; iv and x stained with the panel of TCR Vβ antibodies. As indicated by the density plots (A-H), each pool of clones was divided out among 8 separate tubes and stained with relevant TCRVβ antibody mixes. Within pool iv positive expression for Vβ12 was seen in tube E

(14.46%) and of Vβ1 was seen within tube F (29.21%) (Figure 3.6A). Within pool x positive expression for Vβ5.1 (1.89%) in tube C and Vβ1 within tube F (46.89%) are also shown (Figure 3.6B). Interestingly, these two pools both revealed Vβ1 usage indicating that one of the clones in each of these pools expressed a TCR with Vβ1 usage. Similar analysis was performed for all pools of clones; i-xi.

Figures 3.7 summarises the findings gained from TCRVβ repertoire staining of pools of clones from donor 1. To decipher which of the clones within the pools were expressing the TCRVβ detected, we cross matched any percentage expression of Vβ usage, within the matrix. For example, after cross matching the Vβ1 expression detected in pools iv and x, this was indicative of clone 99. Similar cross-matching of all the results in the pools i-x, allowed for the determination of specific clones to be taken forward for individual TCRVβ staining.

FIGURE 3.4

Pool of Clones	i	ii	iii	iv	v	vi
vii	2	4	18	19	21	26
viii	28	33	41	49	53	57
ix	64	74	78	79	83	91
x	94	96	97	99	101	110
Xi	122	127	131	149	153	154

Figure 3.4 Matrix strategy for analysis of TCRVβ usage

A matrix was constructed to enable analysis of PRS specific CD4+ T cell clones in pools. Each individual T cell clone is present in 2 unique pools. Example of donor 1 clonal matrix is shown.

FIGURE 3.5

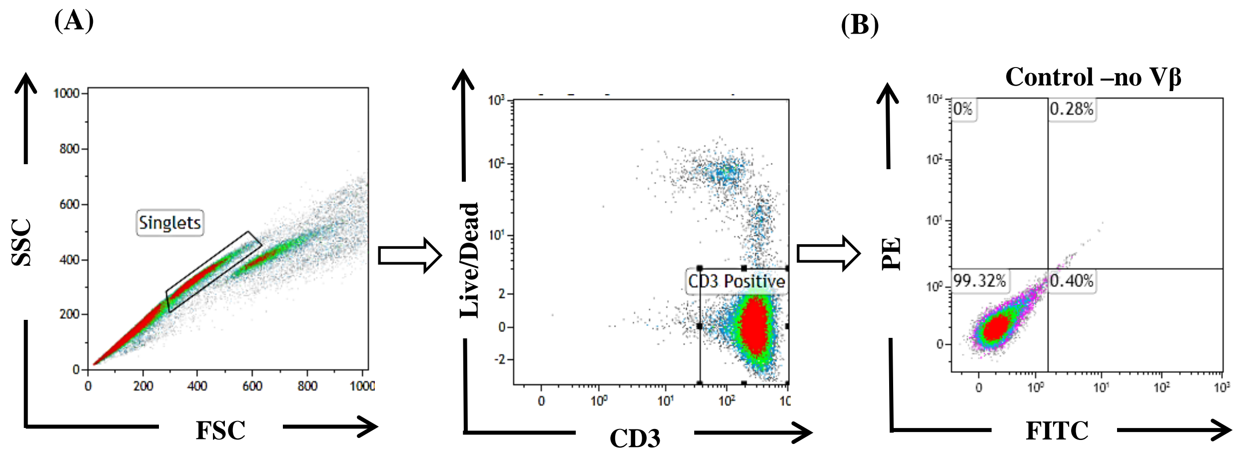
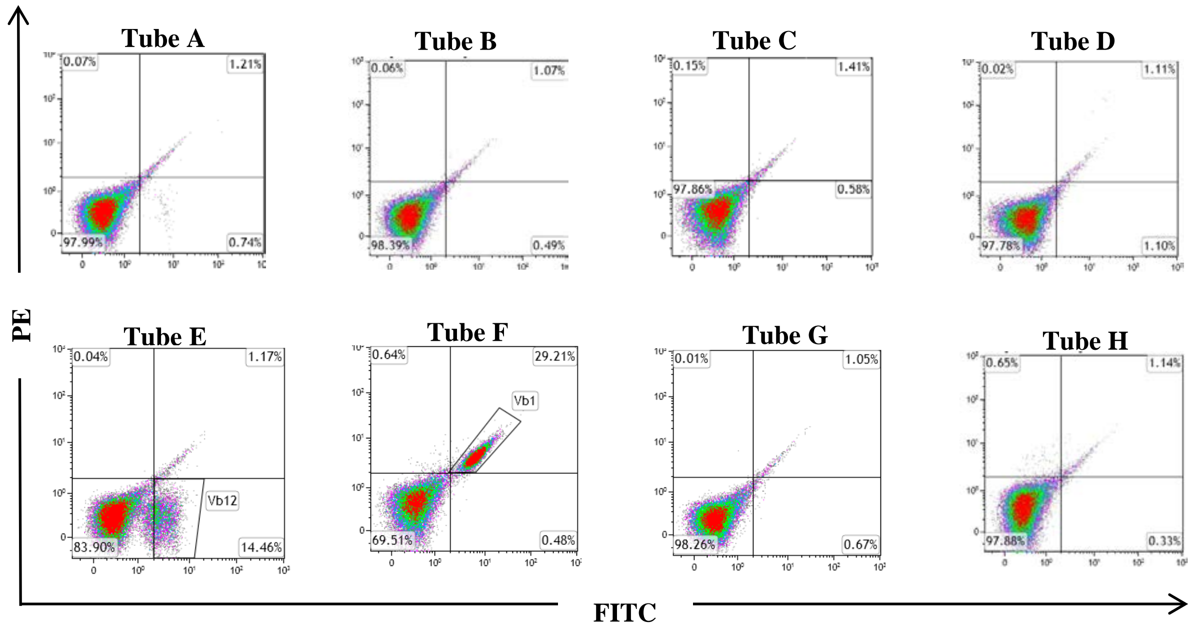


Figure 3.5 TCRVβ repertoire analysis gating strategy

(A) TCRVβ repertoire analysis gating strategy. From left to right: singlets and alive CD3+ lymphocytes. (B) Control; no Vβ. This gating strategy was used throughout all repertoire staining.

FIGURE 3.6

(A) Pool iv



(B) Pool x

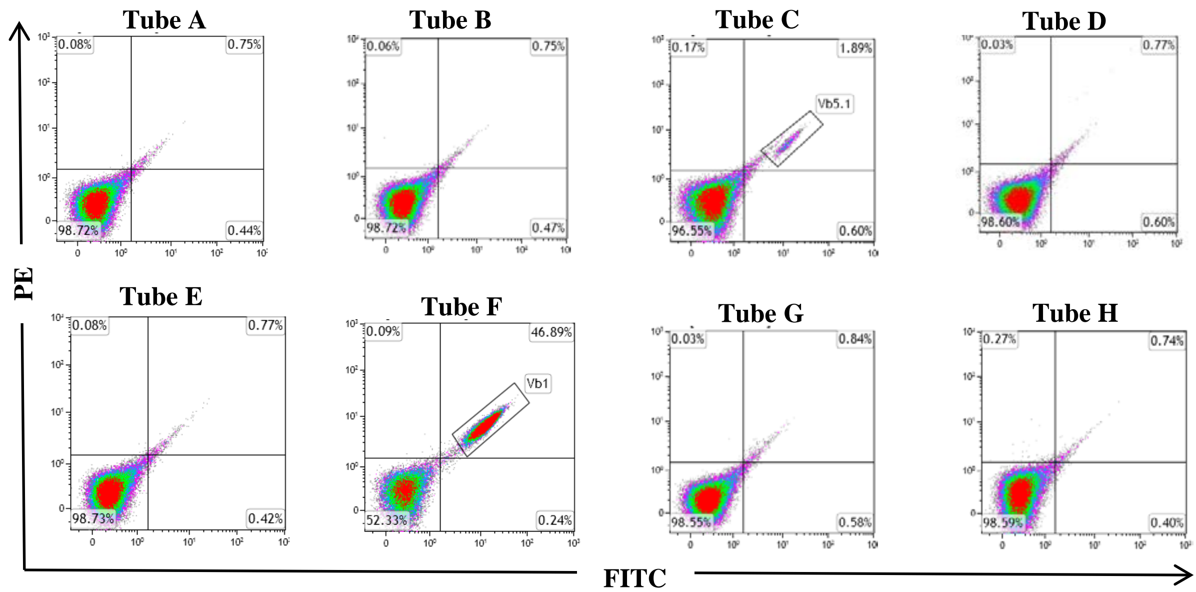


Figure 3.6 Donor 1 TCRVβ repertoire analysis

TCRVβ repertoire staining of clonal pools iv and x from donor 1. Pools of clones were made, divided among 8 tubes (A-H) and stained with relevant Vβ repertoire antibody mixes. Results are gated on live CD3 lymphocytes and any positive Vβ staining is presented as an increase in percentage of single/double staining. (A) Pool iv contained positive staining in tube E, indicating Vβ12 usage (14.46%) and tube F indicating Vβ1 usage (29.21%). (B) Pool x contained positive staining in tube C indicating Vβ5.1 usage (1.89%) and in tube F indicating Vβ1 usage (46.89%).

FIGURE 3.7

Clonal Pools	i	ii	iii	iv	v	vi	Positive Vβ usage %
vii	2	4	18	19	21	26	Vβ5.1 (5%) Vβ21.3 (12%) Vβ23 (11%)
viii	28	33	41	49	53	57	Vβ1 (20%) Vβ22 (7%)
ix	64	74	78	79	83	91	Vβ1 (25%) Vβ2 (11%)
x	94	96	97	99	101	110	Vβ1 (47%) Vβ5.1 (3%)
Xi	122	127	131	149	153	154	Vβ1 (18%) Vβ12 (16%)
Positive Vβ usage %	Vβ21.3 (10%)	Vβ2 (4%) Vβ5.1 (8%) Vβ21.3(3%) Vβ22 (11%)	Vβ1 (15%) Vβ2 (12%)	Vβ1 (29%) Vβ12 (15%)	Vβ1 (46%) Vβ21.3 (8%)	Vβ1 (16%) Vβ2 (13%)	

Figure 3.7 Donor 1 TCRVβ repertoire analysis summary

Summary of TCRVβ repertoire staining on donor 1 clones. Any positive Vβ usage found in each clonal pool is presented as a percentage.

3.3.2 TCRVβ Individual Staining

To confirm the Vβ usage of individual T cell clones, staining was performed using antibodies against individual TCRVβ. Live CD3+ CD4+ T lymphocytes were gated on (Figure 3.8A). This gating strategy was used throughout all individual Vβ staining. As a control, cells were viability and surface stained with anti CD3 and anti CD4 surface antibodies but not stained with Vβ antibodies. There was no positive staining in the absence of these individual Vβ antibodies (Figure 3.8b). Figure 3.8 displays the individual TCRVβ staining performed on clone 99, as previously indicated by the matrix strategy to express Vβ1 (Figure 3.7). The results show a shift in the staining in the presence of Vβ1 Abs, with 94.47% positive expression for Vβ1 usage, indicating that clone 99 used Vβ1.

Similar experiments were repeated on all clones where positive staining was seen in the matrix (Figure 3.7). Figure 3.9 provides a summary of the positive results obtained for donor 1. Using this strategy, we successfully found the Vβ usages of 7 clones. Among these, 4 clones (clone 53, clone 83, clone 99 and clone 110) were positive for Vβ1 usage, 2 clones (clone 2 and clone 21) were positive for Vβ21.3 usage and clone 91 was positive for Vβ2 usage. In each case over 90% of the T cell population was positive for the relevant Vβ.

Similar analysis was performed on the T cell clones isolated from donor 2. Figure 3.10 summarises the findings from TCRVβ repertoire analysis utilising a similar matrix strategy as used for on donor 1. Again, Abs staining was used to confirm the results obtained. Figure 3.11 displays Vβ usage of 5 individual clones where the TCRVβ usage

could be confirmed. Among these, 2 clones (clone 2 and clone 49) were positive for Vβ2 usage, 2 clones (clone 14 and clone 15) were positive for Vβ5.1 usage and clone 46 was positive for Vβ17. Again, all of which showed a shift in staining above 90%. Table 3.2 summarising the findings gained from this individual TCRVβ staining for both healthy donors.

Interestingly, from these results T cells specific for the same peptide epitope-MHCII combination but with different Vβ usages were isolated from each donor. Both donors had T cells utilising Vβ2 (Donor 1 clone 91) (Donor 2 clones 2 and 49) and within donor 1 there was also preferential usage of Vβ1 from 4 out of the 7 clones assessed.

FIGURE 3.8

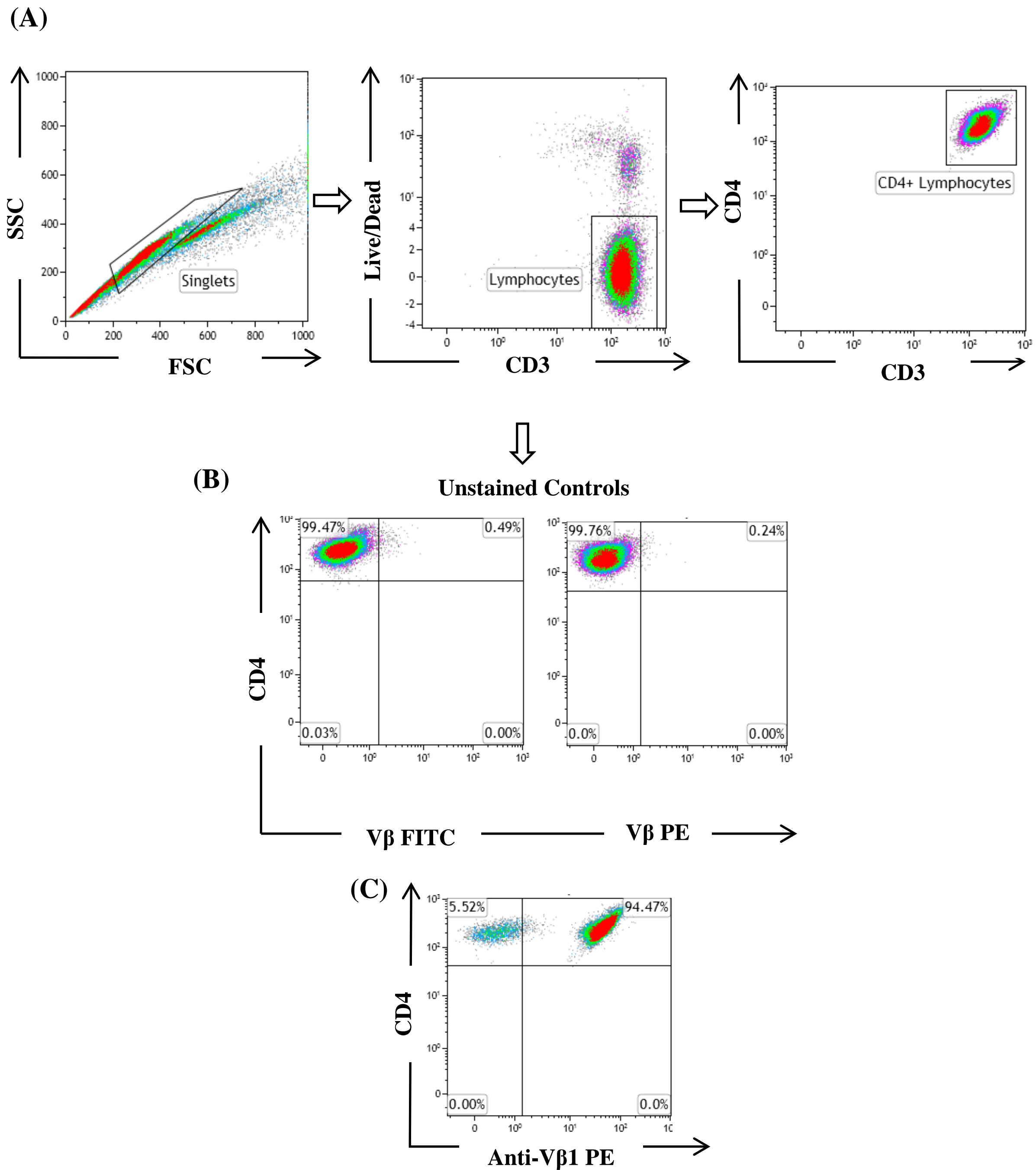


Figure 3.8 Individual TCRVβ usage analysis

(A) Individual TCRVβ analysis gating strategy. From left to right: singlets, alive lymphocytes and CD3+ CD4+ T cells (B) Controls; no Vβ. This gating strategy was used throughout all individual staining. (C) Clone 99; positively stained with anti-Vβ1 conjugated to PE as indicated by a shift in staining of 94.47%.

FIGURE 3.9

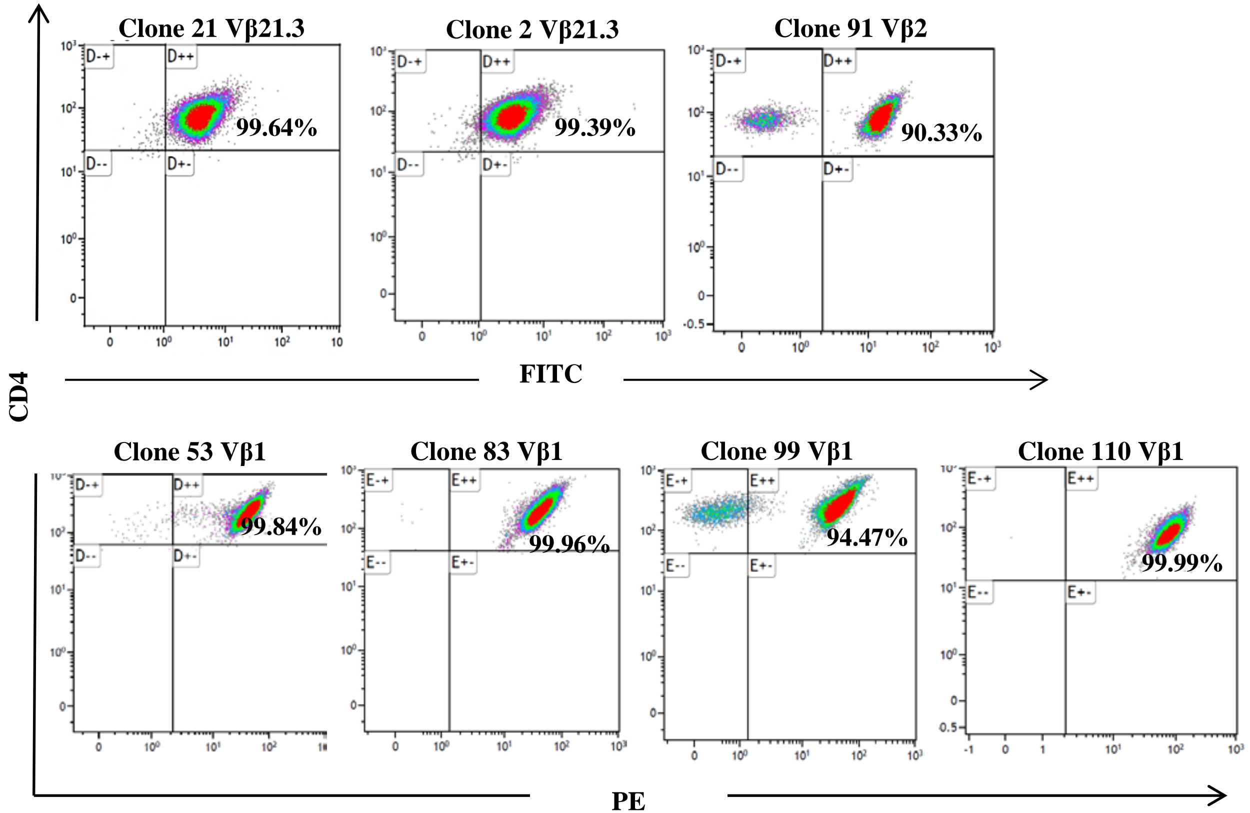


Figure 3.9 Donor 1 summary of individual TCRVβ staining

Histograms represent individual T cell clones (200,000 cells) stained with viability dye, anti-CD3 APC, anti-CD4 ECD and 7µl of individual TCRVβ monoclonal antibodies, conjugated to either PE or FITC. Percentages within each histogram represent frequencies of PRS specific CD4+ T cells in each clonal population stained positively for a specific Vβ.

FIGURE 3.10

Clonal Pools	i	ii	iii	iv	v	Positive Vβ usage %
vi	1	2	3	5	7	Vβ5.1 (9%) Vβ2 (27%)
vii	10	11	12	14	15	Vβ5.1 (43%)
viii	17	27	28	30	35	
ix	38	39	40	41	42	Vβ8 (7%) Vβ2 (28%)
x	43	44	45	46	47	Vβ17 (26%) Vβ5.1 (3%) Vβ2 (11%)
Xi	48	49				Vβ2 (44%)
Positive Vβ usage %	Vβ5.1 (12%) Vβ2 (6%)	Vβ2 (44%)	Vβ2 (13%)	Vβ17 (10%) Vβ5.1 (21%)	Vβ5.1 (32%)	

Figure 3.10 Donor 2 TCRVβ repertoire analysis

Summary of donor 2 TCRVβ repertoire staining. Any positive expression for a particular Vβ usage within a given pool is displayed within the matrix and presented as a percentage.

FIGURE 3.11

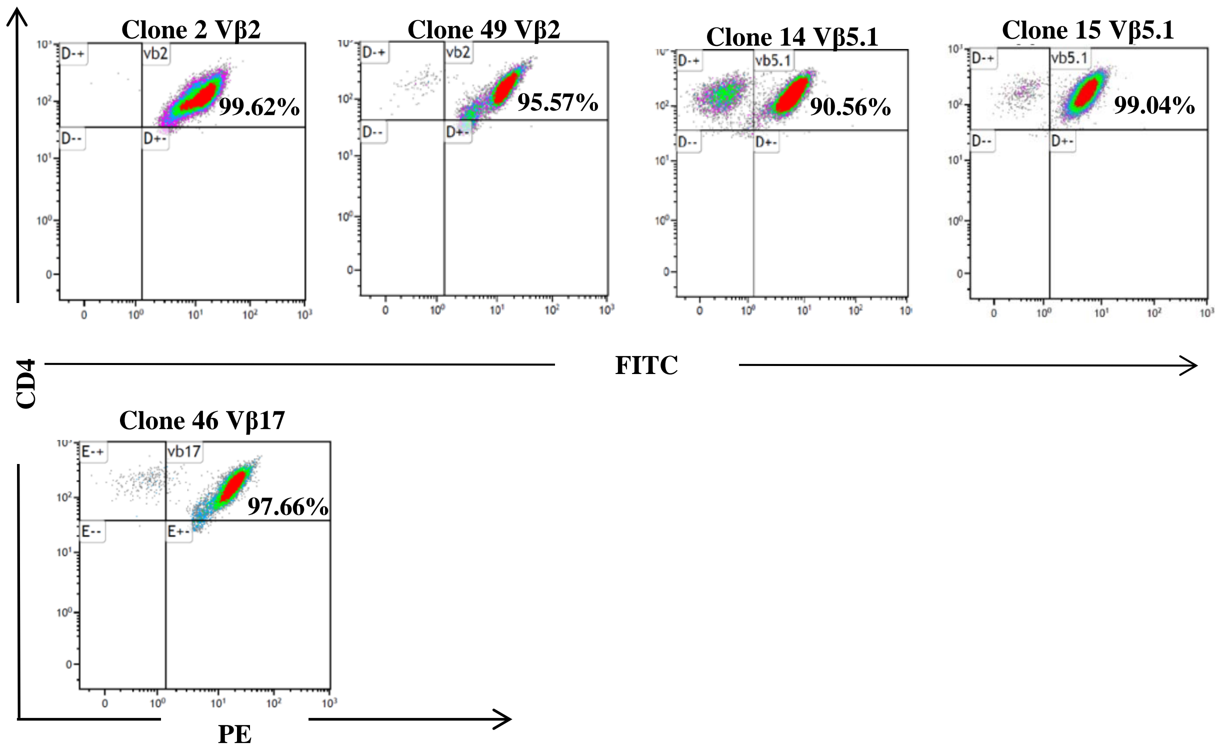


Figure 3.11 Donor 2 individual TCRVβ usage analysis

Histograms represent individual T cell clones (200,000 cells) stained with viability dye, anti-CD3 APC, anti-CD4 ECD and 7µl of individual TCRVβ monoclonal antibodies, conjugated to either PE or FITC. Percentages within each histogram represent frequencies of PRS specific CD4+ T cells in each clonal population stained positively for a specific Vβ.

TABLE 3.2

Donor 1 Vβ Usage	
Vβ	**Positive Clones**
Vβ1	**C53, C83, C99 & C110**
Vβ2	**C91**
Vβ21.3	**C2 &C21**
Donor 2 Vβ Usage	
Vβ	**Positive Clones**
Vβ2	**C2 & C49**
Vβ5.1	**C14 & C15**
Vβ17	**C46**

Table 3.2 Summary of TCRVβ usage analysis.

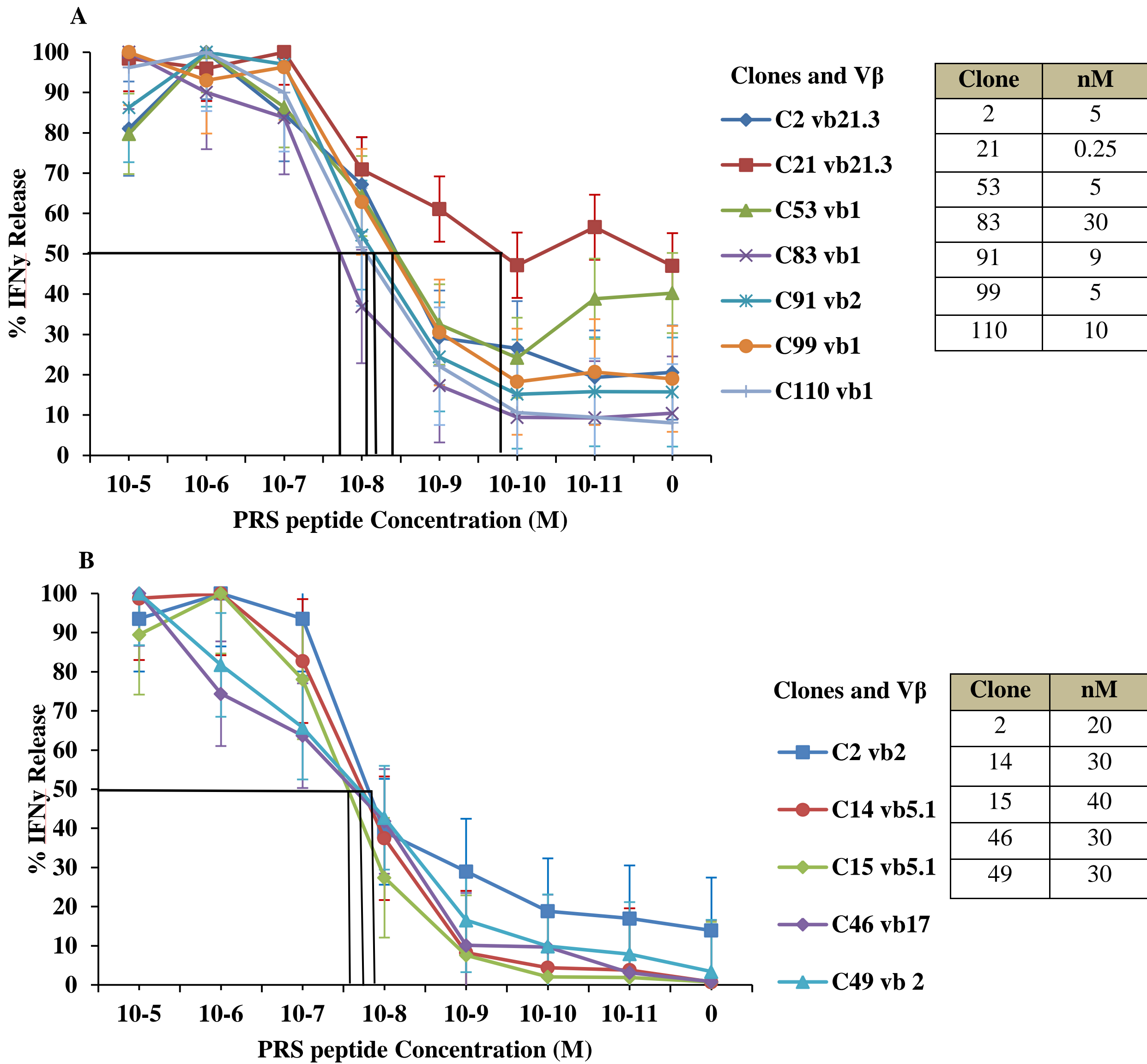
Summary tables of displaying known Vβ usage of clones in each healthy donor.

3.4 Determination of T-cell Functional Avidities

Having isolated individual PRS specific T cell clones with different Vβ usages, we were now interested in whether TCR usage affects T cell function. We therefore measured the functional avidity of each clone with known Vβ usage in peptide titration assays. In these assays, the T cells were exposed to a series of peptide dilutions ranging from $1x10^{-5}$ to $1x10^{-10}$ M, in the presence of autologous LCL as antigen presenting cells. As indicated by Figure 3.12, within these experiments, functional avidity was defined as the peptide concentration required to produce 50% of the maximum IFNγ release. The final functional avidities were converted from M to nM concentration. Figure 3.12 demonstrates that the PRS specific clones from both healthy donors displayed a range of differing functional avidities. In donor 1 these ranged from 0.25nM to 30nM, whereas in donor 2 these were consistently much lower and ranged from 20nM to 40nM.

Importantly, these experiments also showed that certain clones produced a IFNγ response towards autologous LCL in the absence of exogenously supplied PRS peptide as a response was seen at 0M. This suggested that these T cell clones could recognise naturally processed peptide epitope on the EBV infected LCL targets. This response appeared to differ between the two individuals, with all clones from donor 1 able to recognise the un-manipulated autologous LCL cells, whereas only 2 clones from donor 2 (clones 2 and 49) were similarly able to produce a response. These results gave the first indication that certain clones within each healthy donor might function more effectively than others, as demonstrated by the higher levels of IFNγ release, seen in response to the un-manipulated LCL target.

FIGURE 3.12



Clone	nM
2	5
21	0.25
53	5
83	30
91	9
99	5
110	10

Clone	nM
2	20
14	30
15	40
46	30
49	30

Figure 3.12 Functional avidities of PRS specific clones

CD4+ T cell Clones of known Vβ usage (2000 cells per well) were stimulated overnight with HLADR7+ autologous LCL (5×10^{4} cells per well) pre-loaded with PRS peptide at $1x10^{-5}$ to $1x10^{-10}$ M concentrations. Controls used included T cells co-cultured with culture media alone and HLADR7+ autologous LCL co-cultured with media alone. All conditions were performed in triplicates. Responses were assayed by IFNγ release in picograms per millilitre. Functional avidities were determined as the peptide concentration required to produce 50% maximal response (A) Donor 1 clonal functional avidities ranging from 0.25 to 30nM (B) Donor 2 clonal functional avidities ranging from 20 to 40nM. Results are representative of 2 replicate experiments.

3.5 T Cell Clonal Recognition of Naturally Processed and Presented PRS Peptide Epitope

The ability of a T cell to respond to peptide exposed LCL targets *in vitro* is important, as it indicates the specificity of a T cell and its functional avidity. However, for antigen specific T cells to have therapeutic relevance *in vivo*, as direct effectors, the T cell must also be able to recognise antigens presented by naturally infected B lymphocytes. Recognition of un-manipulated autologous LCL replicates how the CD4 T cells within the peripheral blood, may respond to virus infected cells, *in vivo*. Success of this would imply that such T cells may have direct effector functioning in the immune control of EBV infection and B cell lymphomas. Therefore, we further explored the capability of the PRS specific T cell clones to recognise autologous un-manipulated LCL target.

The LCL recognition efficiency of each T cell clone was examined through co-culturing T cells with HLADR7 matched or mismatched LCL targets, with no prior exogenous PRS peptide treatment, compared to the same LCL targets pre-exposed to PRS peptide epitope. The efficiency with which each clone recognised the un-manipulated autologous LCL target, as displayed in Figure 3.13, was expressed as a percentage of the maximal response seen against the same HLADR7 matched LCL target pre-loaded with optimal PRS peptide.

Figures 3.13 shows representative LCL recognition efficiencies for the clones derived from each healthy donor. To obtain accurate results and prevent anomalies, three replicate LCL recognition assays were performed and a mean was formulated from these. As expected, donor 1 derived clones showed strong recognition of matched LCL target pre-loaded with peptide epitope, and no recognition of mismatched LCL even

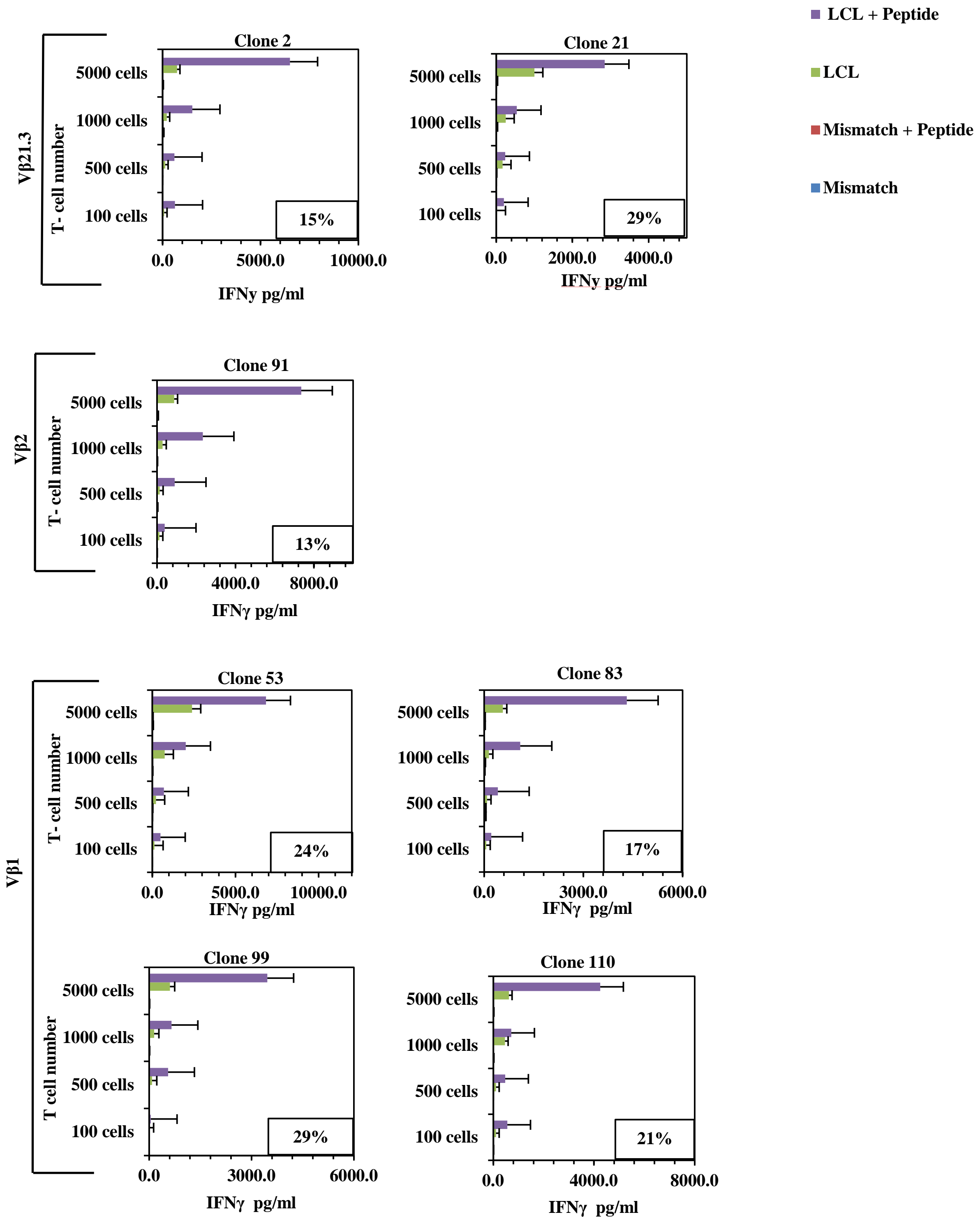
when pre-exposed to peptide. All clones from donor 1 also recognised the un-manipulated autologous LCL at differing percentages ranging from 13% to 29%. In particular, clone 21, clone 53 and clone 99 all produced concentrations of IFNγ at high percentage efficiencies compared to peptide loaded LCL (clone 21; 29%, clone 53; 24%, and clone 99; 29%), and in previous assays displayed similarities in functional avidity (Figure 3.13). Interestingly, clones 53 and 99, both showing high percentage LCL recognition efficiency, both expressed TCRs using Vβ1. However, two other clones with this same Vβ usage (clone 83 and clone 110) had lower efficiency of LCL recognition (Summarised in Table 3.3).

Similar experiments were performed on the clones from donor 2. Figure 3.13 illustrates that these clones showed an overall weaker recognition of the matched LCL target pre-loaded with PRS peptide epitope, compared to donor 1 clones, indicated by the lower IFNγ release. Again, there was no recognition of the control un-manipulated mismatched LCL. In donor 2, 4 out of the 5 clones analysed recognised the un-manipulated autologous LCL target cells at percentage efficiencies ranging from 5%-12%, however clone 46 was unable to do this. Interestingly, the percentage recognition efficiencies of donor 2 were all lower than the clones isolated from donor 1, matching with their lower functional avidities, as shown in Figure 3.13, in the peptide titration assays and thus their inability to see LCL targets.

Table 3.3 and table 3.4 summarise the findings from both Vβ usage and functional analysis of the clones from each healthy donor.

FIGURE 3.13

(A)



(B)

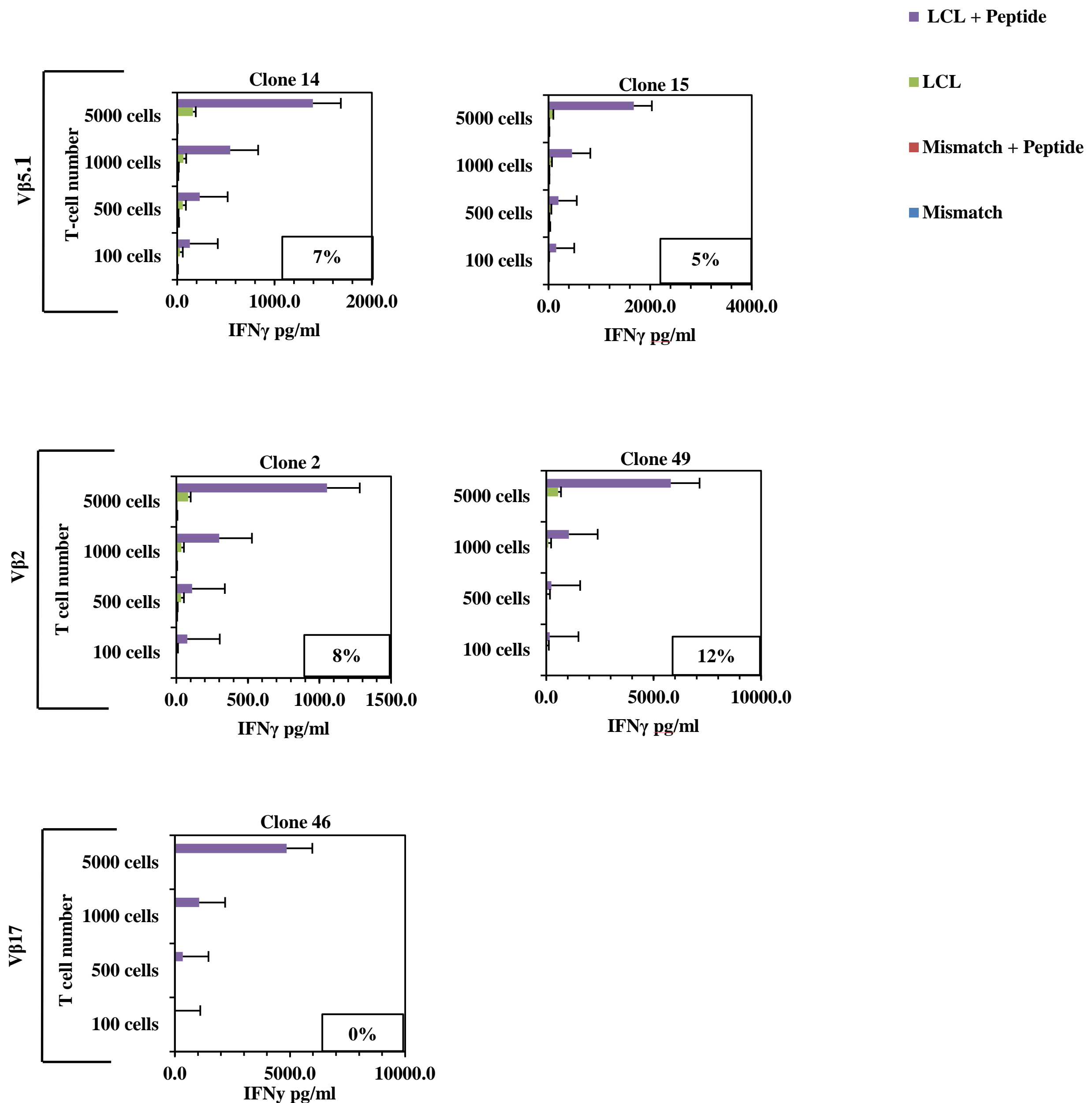


Figure 3.13 T-cell efficiencies.

T cell clones (100 to 5000 T cells per well) were co-cultured with HLADR7 matched LCL or mismatched LCL ($5x10^4$ cells per well), both previously exposed to PRS peptide epitope and compared to the same LCLs targets with no prior exogenous peptide treatment. Responses are shown as IFNγ release in picograms per millilitre. Boxes show percentage efficiency of responding cells. Percentage efficiency is the mean of replicate assays. (A) LCL recognition of donor 1 clones. (B) LCL recognition of donor 2 clones.

TABLE 3.3

Donor 1 HLA/DR7 restricted EBNA2/PRS clones			
Clones	**Vβ Usage**	**Functional avidity (concn (nM))**	**% Recognition of LCL**
Clone 2	**Vβ21.3**	**5**	**15**
Clone 21	**Vβ21.3**	**0.25**	**29**
Clone 53	**Vβ1**	**5**	**27**
Clone 83	**Vβ1**	**30**	**18**
Clone 99	**Vβ1**	**5**	**29**
Clone 110	**Vβ1**	**10**	**21**
Clone 91	**Vβ2**	**9**	**13**

TABLE 3.4

Donor 2 HLA/DR7 restricted EBNA2/PRS clones			
Clones	**Vβ Usage**	**Functional avidity (concn (nM))**	**% Recognition of LCL**
Clone 2	**Vβ2**	**20**	**8**
Clone 49	**Vβ2**	**30**	**12**
Clone 14	**Vβ5.1**	**30**	**5**
Clone 15	**Vβ5.1**	**40**	**7**
Clone 46	**Vβ17**	**30**	**0**

Tables 3.3 & 3.4 Summary of EBNA2 PRS specific CD4+ T cell clones' Vβ usage and functionality.

Functional avidity is defined as the concentration of epitope peptide mediating 50% of the maximal IFNγ produced in peptide titration assays. Values shown are the means of the results of single assays for each clone using triplet replicates of each peptide concentration. Recognition of the un-manipulated autologous LCL, is expressed as a percentage of the IFNγ production seen in the same assay against the same matched LCL target but optimally loaded with PRS peptide epitope. The percentage values for each clone are a mean obtained from several assay replicates.

3.6 TCR Profiling

To further investigate the relationship between TCRVβ usage and T cell function, alpha and beta chains of TCRs from clones were sequenced to determine if there were similarities in nucleotide sequences within the beta variable domain CDR3 region. Donor 1 clones using Vβ1 and Vβ21.3 were selected for this TCRseq. These showed interesting results in relation to TCRVβ usage and function in terms of functional avidities and percentage LCL recognition efficiency.

In order to isolate the alpha and beta genes from these clones, an RNAeasy plus microkit and a SMART RACE cDNA amplification kit were used. Cellular RNA was extracted using the RNAeasy plus microkit and converted via reverse transcription into cDNA. Both the alpha and beta TCR DNA was then amplified using rapid amplification of cDNA ends (RACE) PCR with primers specific for each alpha and beta chains, along with negative controls.

As shown in Figure 3.14 gel electrophoresis was performed and from each clone we successfully amplified PCR products with the correct size from both alpha and beta chains of the T cell receptors (200bps in length). Alpha and beta chain DNA from each clone was then extracted and purified using a gel extraction spin column method and prepared for sequencing. Unfortunately, from the sequencing data obtained we were unsuccessful in generating any conclusive data, therefore further troubleshooting is required for future TCRseq replicates to be performed.

FIGURE 3.14

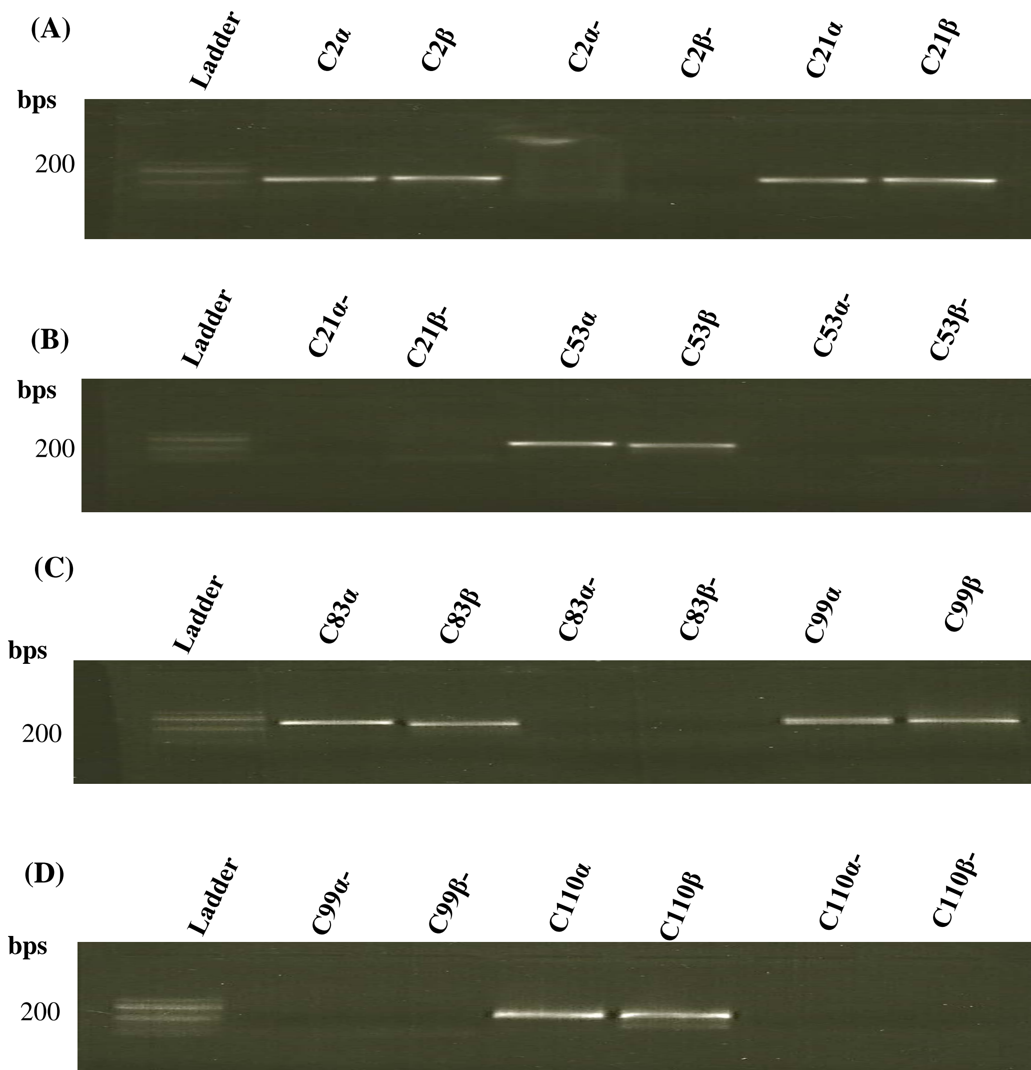


Figure 3.14 Gel electrophoresis of clonal TCR alpha and beta chain cDNA

Alpha and beta TCRs from the CD4+ T cell clones were amplified using alpha and beta gene specific primers and a universal primer mix, via RACE PCR. The size of RACE DNA products was confirmed on a 1% agarose gel, via gel electrophoresis. For all CD4+ T cell clones each α and β primer yielded a PCR product of expected size (200bp). Controls containing no DNA (-) did not produce any PCR product. (A) From left to right; positive DNA products of clone 2 alpha and beta chains (C2α+) (C2β+), clone 2 alpha and beta chain negative controls (C2α-) (C2β-) and positive DNA products of clone 21 alpha and beta chains (C21α+) (C21β+). (B) From left to right; clone 21 alpha and beta chains negative controls (C21α-) (C21β-), positive DNA products of clone 53 alpha and beta chains (C53α+) (C53β+), and clone 53 alpha and beta chain negative controls (C53α-) (C53β-). (C) From left to right; positive DNA products of clone 83 alpha and beta chains (C83α+) (C83β+), clone 83 alpha and beta chain negative controls (C83α-) (C83β-), and positive DNA products of clone 99 alpha and beta chains (C99α+) (C99β+). (D) From left to right; clone 99 alpha and beta chains negative controls (C99α-) (C99β-), positive DNA products of clone 110 alpha and beta chains (C110α+) (C110β+), and clone 110 alpha and beta chain negative controls (C110α-) (C110β-).

CHAPTER 4

4 DISCUSSION

The αβTCR is essential for determining the antigen specificity of T lymphocytes of the adaptive immune system. Previous research into T cell-based therapies for viral diseases and cancers includes the development of novel methods, such as TCR gene transfer therapy, in which antigen specific TCRs, are genetically introduced into cytotoxic or helper T lymphocytic populations. However, currently TCR selection from the broad αβTCR repertoire remains a significant challenge. Epitope specific populations within this repertoire are known to be extremely diverse and contain a substantial proportion of cells expressing low affinity and avidity TCRs.

Analysis of cytotoxic T cell responses towards single peptide epitopes, in HLA matched humans has revealed identical TCR sequence usage, in the context of various viral models (Crompton et al 2008, InYoung Song et al 2017). However, the biological mechanisms by which clonal restriction occurs and how this distinct TCR usage influences effector mechanisms remain unclear. Further, assessment of which TCR Vβs and TRBV gene segments, induce the most effective antigen-specific immune responses, based on T cell function, is vital for optimisation of future gene transfer immunotherapies for various cancers.

We sought to investigate these concepts, through analysis of the less well studied CD4+ T cell immune response, in the context of the globally prevalent gamma-herpes virus, Epstein Barr Virus. In spite of the robust innate and adaptive immune responses controlling the virus in the majority of people infected, EBV has been associated with various B cell malignancies. Importantly, infected malignant B cells retain expression of

the MHC class II molecule. This is essential for subsequent processing and presentation of EBV exogenous proteins, enabling successful CD4+ TCR recognition and subsequent anti-viral immune control. Despite previous belief that direct effector functioning was entirely related to CD8+ T cells, it has now been established that certain subtypes of CD4+ T cells can also directly target antigen-positive cells through MHCII. These helper type cells have been reported to express various phenotypic markers of known cytotoxicity including perforin and granzyme A linked to CD8+ functioning (Appay et al 2002) and thus are of significance importance during viral and cancer cell elimination. Unfortunately, within patients suffering from B cell malignancies such as post-transplant lymphoproliferative disease (PTLD), peripheral EBV specific CD4+ T cell populations are of low frequency due to immunosuppression. However, they have now been observed in healthy seropositive individuals to respond to a large array of EBV derived antigens, with cytotoxic effects (Long et al 2005, Long et al 2011).

This project focussed on the EBV latency III protein, EBNA2 restricted through HLADR7. Many of the proteins' epitopes have now been identified and have been further shown to be presented well by MHCII on infected B lymphocytes (Long et al 2005). EBNA2 is expressed in latency III positive tumours, such as PTLD, therefore it is thought to have the potential to be an important immunotherapeutic target for the long-term treatment of this lymphoproliferative disease.

Based on these considerations we aimed to investigate the relationship between TCR repertoire usage and effector functioning of epitope specific CD4+ T cells in healthy EBV carriers, utilising the EBNA2-derived PRS epitope as a model. Previous work in our laboratory has shown within healthy seropositive carriers that CD4+ T cell

populations specific for PRS, contain T cells with multiple different Vβ usages. However, further analysis of TCR sequence homology is yet to be performed. We hypothesised that the TCR usage may affect T cell function, with some TCRs therefore being of greater therapeutic benefit than others.

4.1 Generation and Screening of PRS specific CD4+ T cell clones

To address this hypothesis, we successfully isolated PRS specific CD4+ T cells from two healthy HLA DR7+ seropositive donors using MHC class II tetramer staining and FACS sorting, directly *ex vivo*. Previous generation of clonal populations, has focussed on isolation from peripheral blood mononuclear cells (PBMCs) with a period of antigen stimulation and culturing leading to higher enrichment but lower purity levels.

The *ex vivo* generation implemented within this study was important to maintain the TCR repertoire diversity of the sorted CD4+ T cells to reflect that present *in vivo*. Prior *in vitro* stimulation may lead to skewing of the TCR repertoire as some subclonal populations may expand more than others affecting the phenotype and functioning, thus having negative implications on subsequent TCR usage analysis.

The percentage frequencies of DR7/PRS tetramer positive CD4+ T cells detected in each healthy donor was low, 0.1% in donor 1 and only 0.025% in donor 2. This would imply that within the total EBV specific CD4+ T cell immune responses of HLADR7 positive healthy carriers, PRS specific responses constitute a very low proportion. However, similar low frequencies of MHC class II tetramer positive CD4+ T cell populations (0.005 – 0.085% of the total CD4+ T cells) have been observed in previous

in vitro work on healthy long term EBV carriers (Long et al 2013). Generally, within EBV specific immune responses, the magnitude of CD4+ T cells within the peripheral blood is much lower compared to the immunodominant CD8+ populations (Long et al 2011).

To obtain clonal populations, the T cells were FACS sorted and subjected to limiting dilution cloning. The growing clones were then screened for their specificity against the PRS epitope. The functional readout for peptide epitope specificity screening and upcoming assays was determined by IFNγ production. CD4+ T cells producing this particular cytokine have been observed to display both anti-viral and anti-tumour therapeutic effects, being Th1 in subtype (Swain et al 2012). Within each healthy donor, not all the growing clones made IFNγ in response to the PRS peptide epitope. Analysis of T cell responses during both chronic viral infection and cancer, has revealed that repeated cellular stimulations and expansions can render some specific T cells exhausted meaning proliferative capacity and cytokine production can consequentially be reduced (Wherry et al 2011). These fatigued memory effector cells have limited persistence, reducing their response rates and therapeutic effectiveness. This may have occurred within our limiting dilution cultures, which were cultured for 2-3 weeks prior to peptide specificity screening.

However, more likely, the non-responsive wells contained non-specific T cells. We found differences in percentage yield of PRS-specific clones generated from donor 1, 25%, compared to donor 2, 97%. These differences can be explained by the gating strategy used during FACS sorting of the DR7/PRS tetramer stained populations. The gate in donor 1 clearly incorporated a higher proportion of non-specific cells meaning a

lower percentage would be specific for the PRS epitope. This gating was corrected for donor 2.

A further factor to consider is the potential outgrowth of polyclonal T cell populations due to more than one cell being plated into each well of the limiting dilution cloning. Although the clones were preferentially selected from the 0.3c/w plates reducing the probability of selecting polyclonal populations, some clones within these populations may have contained two or more T cells, with non-specific TCRs, outgrowing the PRS specific cells.

Another potential reason for a lack of IFNγ production in some of the clones screened could have been the result of other subsets of PRS specific CD4+ T cells being present within the cultures screened, producing other types of cytokines not assayed for, such as IL-4 linked to the helper subtype; Th2. sRNA sequencing and cyTOF mass spectrometry has revealed that T cells within the same monoclonal population containing the same TCR, have the capacity to downregulate certain transcription factors for one differential status, and upregulate a different one to produce a new subset of cells (Hans et al 2014). Within this study, MHC class II tetramer staining performed prior to dilution cloning and screening selected T cells based on specificity of their TCR, thus this had no influences on the activated cells subset lineage and effector function.

There was the additional possibility of polyfunctional T cells growing within the cultures screened, producing multiple different cytokines not assayed for. Previous work conducted by Betts et al demonstrated this phenomenon in the context of HIV infection. It was found in a substantial proportion of viral specific CD8+ T cell immune

responses there was little or no production of IFNγ but in fact many other cytokines were secreted instead, highlighting the functional heterogeneity of anti-viral T cell immune responses (Betts et al 2006). Within our own laboratory it has been further shown in the context of EBV specific CD4+ T cells in healthy carriers, these cells can produce high levels of the cytokine TNFα along with IFNγ, and sometimes in its absence. (Benjamin Meckiff, unpublished data). In the context of CMV specific CD8+ T cell immune responses, work conducted by Sanberg and colleagues also reported within polyfunctional T cell populations, the type of cytokine being secreted determined functional differentiation of a T cell, with TNFα production representing an earlier stage of cellular development compared to IFNγ and IL-2 (Sanberg et al 2001). Therefore, the use of ELISplots would be a beneficial form of future analysis to determine whether any of the unresponsive T cell populations within our growing cultures were in fact other subsets producing different cytokines, polyfunctional populations or at a differing stage of functional development.

Interestingly, we noticed that some clones did additionally appear to produce IFNγ in response to the unmanipulated LCL target in these screening assays, which suggested they might have the ability to recognise naturally processed and presented exogenous cognate antigen epitope, from the resident EBV genome. This is extremely important from a clinical standpoint, as the ability to recognise naturally infected B cell targets would imply that such T cells may have direct effector functions in the long-term control of EBV driven lymphoproliferations, *in vivo.*

4.2 TCRVβ usage analysis of PRS specific CD4+ T cell clones

We next explored the TCRVβ repertoire usage of the PRS-specific T cell clones, in order to assess the range of TCRs specific for the PRS epitope. This additionally enabled us to confirm the clonality of the T cells as all cells within a clonotypic population should express the same V/D/J segment and identical TCRVβ protein.

Interestingly, we were only able to determine the Vβ usage of a small subset of the clones screened. This conservation of TCR usage may have been due to the limitation of the IOTest Beta Mark Kit utilised, in which the monoclonal antibody panel, identifying 24 separate TCRVβ families, only covers up to 70% of the normal human αβTCR repertoire. Therefore, less common TCRVβs being used by clones not covered for by the kit, may have been consequentially missed. Previous assessment of TCRVβ repertoire diversity using the IOTest Beta Mark Repertoire kit, has similarly identified significant proportions of T cells within a population failing to react to any TCRVβ antibody tested (Tembhare et al 2011).

Nevertheless, we successfully identified the Vβ usage of a number of clones from each healthy donor. From donor 1 out of the 31 clones assessed, 7 showed Vβ usage for Vβ21.3, Vβ1 or Vβ2; 22.5%. In comparison, within donor 2 only 5 clones from the 28 clones analysed showed Vβ usage for Vβ2, Vβ5.3, or Vβ17; 17.9%. Most interestingly, similarities in Vβ2 usage between the donors was apparent. The distinct Vβ usage observed in both donors is indication that a particular mechanism is favouring their occurrence.

During epitope specific CD8+ T cell immune responses against chronic EBV infection, TCR repertoire analysis has identified several recurrent Vβ subsets being used

including, Vβ2, Vβ4, and Vβ16. (Lim et al 2000). In the context of other chronic viral infections, previous sequence analyses have similarly observed immunodominance of individual TCR gene segments associated with a defined sets of CDR3 motif sequences in both epitope-specific CD8+ T cell and CD4+ T cell populations, despite broad TCR repertoires available (Chrompton et al 2008, InYoung Song et al 2017). These findings highlight the importance of structural immunodominance within differing TCRs responding to the same MHC-peptide combination. In the context of CD4+ T cells, TCR repertoire analysis has demonstrated that restriction to particular subsets of Vβ subfamilies such as Vβ2 and Vβ17 does occur within populations of antigen experienced CTLs (Appay et al 2002). Similarly, within our own laboratory investigations into the diversity of the TCR repertoire usage of CD4+ T cells in healthy EBV carriers has shown overrepresentation of single Vβ chains (Benjamin Meckiff, unpublished data).

The biological basis for this TCRVβ repertoire focussing and clonal selection observed, remains subject to further in-depth characterisation and investigation. EBV is reactivated throughout the lifetime of an infected individual, thus it is likely that PRS specific CD4+ T cells have been chronically exposed to this particular latent antigen epitope, resulting in the continuous activation of specific clones with conserved TCR structural components. Subsequently this would result in antigen driven selection of a distinct subset of immunodominant TCRs arising within seropositive healthy individuals.

It has been further speculated that the processing and presentation pathways antigen presenting cells along with HLA allele restriction of epitope specific CD4+ T cell clonal populations may enhance their potential for clonal restriction and conservation of TCR

usage (Chrompton et al 2008). However further investigations are required to assess these possibilities.

From our own results and others, this therefore suggests that, despite the initial diverse TCRVβ repertoire availability within CD4+ T cell immune responses, clonal selection along with immunodominance of individual TCR structural components does occur within epitope specific populations. Homogenous TCR responses to immunodominant epitopes such as PRS may be important for activation of T cells conferring the most robust immune responses. However, further analysis is required in order to determine the exact factors having greatest influence on this TCR immunodominance.

4.3 Functional analysis of PRS specific CD4+ T cell clones with known Vβ usage

Having established PRS specific CD4+ T cell clones and confirmed their Vβ usages, we were next interested in gaining a better understanding of their functional characteristics. The initial parameter investigated was functional avidity; the biological measure of the *ex vivo* response produced by a T cell to a given concentration of target peptide. The potency of cellular immune responses strongly depends on this functional characteristic. Within the context of various viruses, epitope specific T cells displaying high functional avidity have been associated with better viral clearance. Analysis was achieved through measuring the IFNγ production in response to stimulation with an autologous LCL target pre-loaded with titrating amounts of PRS peptide.

The clones displayed various avidities ranging from 0.25 to 40nM. Interestingly, the functional avidities of the clones isolated from donor 1 were generally higher than those from donor 2. Previous work conducted by Long et al reported that EBV specific CD4+

T cell clones against the same epitope-HLA allele combination appeared functionally alike to one another, with regards to peptide titration curves. However, these previous experiments investigated this functional characteristic on only a small number of clones for each epitope, and similarly to other studies, these cells were subject to repeated prior *in vitro* stimulations and expansion before cloning. This would have increased the likelihood of preferential expansion of certain clones inducing bias to the avidity measurements produced. Despite this, in the context of PRS-specific CD4+ T cell clones, restricted through different HLA alleles functional avidities were reported to range from 3-30nM (Long et al 2005). Similarly, peptide titration analysis has reported avidities of CMV epitope specific CD4+ T cell clones ranging from 10nM-100nM, in line with CD8+ cytotoxic cellular responses (Chrompton et al 2008). In the context of other viruses such as HIV, work conducted by Billeskov et al found that low antigen dosage in adjuvant-based vaccination selectively induced CD4+ T helper cells with enhanced functional avidity, increasing cytokine production, along with improving the efficacy of CD8 T cell anti-viral activity (Billeskov et al 2017). This coincides with the results generated from these peptide titration assays, that certain clones with high functional avidity could respond with higher levels of IFNγ cytokine release to low levels of peptide. These results, therefore, infer that certain clones may be associated with more potent responses against infections and cancers and thus are of more therapeutic relevance than others of lower functional avidity.

The differences observed in the functional avidities between clones of the same epitope specificity and HLA allele restriction could be related to variations in the levels of TCR expression. T cells with low surface TCR expression may not have been able to respond fully due to less binding interactions, resulting in lower activation thresholds.

Alternatively, it has been previously reported that T cells expressing enhanced affinity TCRs have a direct influence on the strength of an immune response and thus functional avidity of a T cell (Thomas et al 2011). This means T cells with high affinity TCRs would recognise much lower levels of the PRS peptide epitope presented through MHC class II on target cells and still induce a robust immune response.

Other factors to be considered include the efficiency of exogenous peptide epitope processing and presentation within the target cell, along with interactions between TCR and pMHC as a result of specific binding anchor residues. This would subsequently lead to more efficient binding kinetics and functional outputs of specific clonal populations. X-ray crystallography analysis of epitope specific CD4 T cell immune responses has reported the effects of exogenous peptide processing and presentation by MHC II molecules resulting in enhanced flexibility. These present peptides able to adopt a variety of conformational states, displaying amino acid side chains which promote better recognition and TCR binding interactions associated with more efficient immune responses (Pu et al 2002).

Structural analysis of TCR binding to pMHC has further revealed the T cell coreceptor has implications on the sensitivity of immune responses with regards to functional avidity. In the context of CD8 T cell responses, studies have highlighted the influences of the CD8 co-receptor on association and dissociation rates, enhancing antigen recognition and binding efficiency particularly in T cells expressing low affinity TCRs, along with increasing the rate of subsequent intracellular activation processes induced by the TCR/CD3 complex (Artyomov et al 2010). However, the exact mechanisms by which this is achieved have yet to be elucidated.

Importantly, within these peptide titration assays and the earlier screening assays, it was apparent that many of the CD4+ T cell clones could recognise the unmanipulated LCL target cell without the addition of cognate PRS epitope peptide. This is an important *in vitro* indication that such clones may be able to directly recognise EBV derived lymphoproliferations *in vivo,* and be of therapeutic benefit. Within the literature it is already apparent PRS specific CD4+ T cell clones are able to recognise and eliminate autologous LCL targets (Khanna et al 1997, Omiya et al 2002, Long et al 2005). We therefore investigated this further by assessing the efficiency of LCL recognition among these clones of known functional avidity and Vβ usage. Previous work conducted by Omiya et al generated PRS specific clones, which similarly were able to recognise autologous unmanipulated LCL target, determined through IFNγ cytokine production. Though, these response levels were not compared to that of a maximal peptide-induced response (Omiya et al 2002). Further to this, work conducted by Long et al, investigating LCL recognition efficiency as a percentage of that seen in parallel against the same LCL target pre-loaded with optimal PRS epitope peptide, reported that PRS specific CD4+ T cell clones of varying MHCII restriction had percentage LCL recognition efficiencies ranging from 1-35%. In particular, HLA DR7 restricted clones had an efficiency of 15% (Long et al 2005). However, these earlier studies assessed a limited number of clones, with unknown TCRVβ usage. Therefore, we sought to expand on these previous findings by analysing the function of our T cell clones with known Vβ usage, in order to determine if there was an association between the two factors.

Here, we found that the 7 PRS specific CD4+ T cell clones analysed from donor 1 all had an efficiency of LCL recognition above 10%, and 3 clones, in particular, had relatively high efficiencies ranging from 27 to 29%, in line with previous findings.

Surprisingly, the clones generated from donor 2 all had much lower percentage recognition efficiencies with only one showing an efficiency above 10%.

Importantly, the differences in LCL recognition between clones within each donor were consistent with the pattern of functional avidities previously determined in the peptide titration assays. Predominantly, within donor 1, the PRS-specific clones with high functional avidity had increased ability to recognise physiological levels of PRS epitope, endogenously processed and presented by autologous LCLs, as observed through the higher LCL recognition efficiencies. Many investigators have similarly demonstrated this correlation between T cell avidity and target recognition of T cell populations that are able to recognise virally infected antigen presenting cells, murine models and human cancer cell targets (Dudeley et al 1999, Zeh et al 1999).

However, these results cannot be solely explained by the correlation observed with T cell clonal functional avidities. Evidence of this has been reported through clinical trials, in which high avidity T cells, specific for the tumour associated antigen, Her-2/neu+ were unable to recognise tumour cells despite showing response towards peptide loaded targets (Zaks et al 1998, Knutson et al 2002). These conflicting results emphasise the complexity of the relationship between avidity and target cell recognition, with many other presently unknown underlying factors in need of investigation.

The level of LCL recognition may in fact be influenced by the degree of PRS epitope presentation on the autologous unmanipulated LCL surface. This could therefore be again linked to efficiency of intracellular processing of the cognate PRS peptide by the target antigen presenting cell. However, further investigations would be required to assess this.

To prevent bias arising due to this, within this study we conducted the assays on all clones in parallel, and performed three replicates to generate a mean. Furthermore, all assays used the same number of autologous HLA matched LCL and T cells per well, and further equal numbers of control HLA mismatched LCLs.

The observed ability to successfully recognise LCL targets, which represent EBV infected B cell blasts, provides support for the possibility that EBV epitope specific CD4+ T cells, circulating within the peripheral blood of seropositive carriers, may display comparable properties, *in vivo* and could be further investigated for other direct effector mechanisms and functional relationships. The relative sensitivity of a T cell towards an antigen target, thus may influence its subsequent ability to recognise cancer cells, emphasising the importance of high avidity T cells in anti-tumour immunity and subsequent selection for adoptive immunotherapeutic applications.

4.4 TCRVβ usage influences on T cell function

Our main motivation for this work was to address the potential relationship between Vβ usage and the function of epitope specific CD4+ T cell clones. The ability to determine TCRs from defined clonal populations, conferring the greatest functional output with regards to activation, proliferation, and target cell lysis is of key importance for future therapeutic application to be successful. However, the biological impact of TCR repertoire usage within epitope specific T cell populations, is still yet to be investigated fully.

From our analysis, when comparing clones of the same Vβ usage, for example clone 99 and clone 53 derived from donor 1, using Vβ1, these displayed a high functional avidity

of 5nM and percentage LCL recognition efficiencies of 21% and 27%. However, other clones from donor 1 also using Vβ1, had much lower functional avidities and percentage LCL recognition efficiencies. For example, clone 83 using Vβ1 had a functional avidity of 30nM and an LCL recognition efficiency of 18%. Furthermore, within donor 1 high avidities and LCL recognition efficiencies were apparent in clones using differing Vβs including clone 2 and 21 which both showed usage of Vβ21.3. These findings are interesting, as despite the differences in Vβ expression between clones from the same individual, structural conservation of defined TRBV genes and CDR3 motifs between clones of similar functional capacity may still have been apparent. TCR repertoire diversity is now known to contribute significantly towards immune defence mechanisms; providing a large initial pool of clonal populations, with differing TCRVβ from which the most efficient T cells can then be selected for (InYong Song et al 2017). Thus, this would imply that within epitope specific CD4+ T cell populations, the combination of overall diversity and finite structural similarities in specific clonal populations is of key importance in an efficient and robust immune response.

To determine whether fine differences in the CDR3 binding region of the TCRs were responsible for the observed differing functional properties of the PRS-specific CD4+ T cell clones, we went on to sequence the alpha and beta chains of the TCRs from the clones isolated from donor 1 that used Vβ1 and Vβ21.3. Structural and sequence analysis has reported, in the context of chronic CMV infection in healthy individuals, extreme conservation of T cell receptor usage in both TCRα and TCRβ chains of epitope specific cytotoxic CD4+ T cell clones displaying high functional avidity, similarly to that seen in cytotoxic CD8+ responses (Chrompton et al 2008). This

suggests that during chronic viral infections, clonal selection occurs upon initial epitope recognition. Subsequent repeated exposure of lymphocytes to the same antigen derived epitope may thus result in immunodominance of specific TCR gene sequences conferring cells of the greatest cytotoxic potential.

From our own analysis performed, despite successful amplification of PCR products for both TCRα and TCRβ chains of all clones assessed, the sequencing data was inconclusive. This may have been due to the presence of other cells carrying none specific TCRs, for example if the T cell clones were not truly monoclonal or if feeder cells were still remaining within the cultures after re-stimulation. Unfortunately, due to the limited time availability and substantial cost of TCR isolation and sequencing, subsequent TCRseq replicates could not be performed and it was not possible to draw definite conclusions. A full analysis of whether there are similarities in the CDR3 motifs of the TCRs of the clones with similar functional parameters and the same/different Vβ usage remains to be performed.

There have been earlier attempts to characterise the function of epitope-specific CD4+ T cells with distinct TCR usage, however this analysis was based on the functional interactions of T cell receptors contacting different residues located within the target peptide, and thus binding interactions rather than differences in TCR sequence (Pu et al 2002). Nevertheless, these investigations do open up the possibility to explore influences other than the TCR structural components, such as the conformational recognition of peptide by TCR contact residues. Further detailed structural information is therefore required to determine if initial TCR-pMHC binding interactions, and the components within them, do in fact have any significant influence on the functioning of an activated T cell.

More recently, a study investigating influenza virus epitope specific CD8+ T cell immune responses has shown that a highly diverse repertoire, including both dominant public TCRs and a variety of private structural CDR3 components, may be key to successful antigen recognition and effective immune responses. These diverse TCR patterns have been suggested to be crucial to protect against individual clonal loss, thereby ensuring the maintenance of an antigen-specific effector memory T cell pool (InYoung Song et al 2017). Based on these findings it was proposed that differing sequences may consequentially activate downstream effector mechanisms differently, resulting in heterogenous functioning of different T cells (InYoung Song et al 2017). Recent work has attempted to build on these ideas integrating information about TCR sequence specificity with multiparametric phenotypic analysis of bulk T cells at a single cell level, in the context of cancers such as colorectal carcinoma (Han et al 2014). It was reported that similarities were apparent in cells displaying the same TCR sequence and transcription factor regulating cellular differential status. However, there was no indication of TCR structure influencing immune response efficiency, sensitivity, or target cell elimination. Further to this, it did not have the availability of MHC tetramer reagents and thus identification of epitope specificity and HLA restriction was not achieved.

Gros and colleagues additionally reported within melanoma patients tumour-infiltrating PD-1 neoantigen specific CD8+ T cell populations proved a valuable source in identification of TCRs with therapeutic potential (Gros et al 2014). Despite these conclusions made, to what extent these TCRs represented T cells of the best avidity was not fully clarified.

To date no conclusive evidence has been made into the influences of TCRVβ usage on cytotoxic capacity of anti-viral and anti-tumour T cell immune responses. From our knowledge this is the first exploration of the TCRVβ usage of human CD4+ T cell clones of known epitope specificity and MHCII restriction isolated, *ex vivo*. The biological basis for this distinct TCR repertoire usage and its association with cellular functional output still remains unclear. Thus, deeper insights into the mechanisms regulating this, are required to improve understanding of T cell immunology and further help to identify optimal TCRs for future targeted immunotherapies.

4.5 Future work

To generate more in depth knowledge regarding the link between TCR usage and functioning of PRS specific CD4+ T cells further work is needed. Unfortunately, the TCR sequencing data produced from this study was inconclusive. With additional troubleshooting, both the alpha and beta chain gene sequences could be successfully identified meaning more structural comparisons between the PRS specific clones could be made. In the context of viral infection and cancer, TCR sequencing has recently provided useful insights into the T cell repertoire within both diseased and healthy individuals. Previous studies have reported homology of conserved TRAV, TRBV genes and amino acid CDR3 motifs, between HLA matched healthy viral carriers and cancer patients, targeting the same peptide epitope (Chrompton et al 2008). Identification of public T cell clonotypes, shared between HLA matched healthy and diseased individuals, is essential in the development of biomarkers and diagnostics for various infectious and neoplastic diseases. As a technique, antigen receptor profiling further provides the potential to develop immunotherapies utilised in the reconstitution of the immune responses within affected patients, without causing adverse effects such

as GvHD. TCR gene sequences conferring the best functional properties, could thus be isolated and assessed for potential gene modification of subsequent transfused T cell populations.

The initial monomeric engagement between the TCR and its pMHC complex plays a critical role in determining the activity of a T cell. The binding property of TCR affinity, associated with this interaction, has been correlated to more efficient cellular activation by lower concentrations of cognate peptide and an increase in T cell functional capacity (Holler et al 2003). This has been assessed through measuring the dissociation constant rate (K_{off}) of soluble TCRs through surface plasmon resonance (SPR). SPR is an effective biosensor tool for quantitative characterisation and real-time information on the course of reversible interactions between biological macromolecules.

In the context of viral infection, interactions with longer dissociation kinetics/slower koff rates have been previously reported to confer significantly better protection than T cell populations with fast koff rates (Nauerth et al 2013). However, this rate needs to be fast enough to prevent detrimental effects on subsequent serial triggering of multiple other TCRs to a single pMHC. Thus, being able to pinpoint optimal dissociation time for maximal T cell activation, is needed to decipher the exact relationship between the kinetics of TCR-pMHC binding interactions and functional output of a cell.

There is some evidence achieved through thermodynamic analysis, to further suggest that conformational plasticity, during TCR-pMHC initial binding, may also be a factor determining the strength of this TCR binding parameter and overall T cell activity (Krogsgaard et al 2003)

What still remains unclear is why physiological TCR affinities have evolved to be relatively low. It has been proposed that the immune system sacrifices TCRs of high affinity, which could mediate greater functional activity in order to maintain a TCR repertoire diverse enough to combat a wide range of different potential antigens preventing clonal deletion of both epitope specific CD4+ and CD8+ T cells. Ultimately, future work is required to underpin the exact causative agent promoting higher affinity TCRs from these complex binding interactions.

Once the TCRs have been isolated from our PRS-specific T cell clones, similar evaluation of these TCR-pMHC binding kinetics, on a single cell level, could be achieved in order to provide further information regarding the influence of TCR binding affinity to the overall difference in function of CD4+ T cell clones with different TCRVβ usage and functional avidities.

The direct effector mechanisms of epitope specific T cells are paramount for establishment of an effective immune response. Phenotypical characterisation of total CD4+ T cell populations has reported low frequencies of cells expressing many of the surface markers required for cytotoxic killing (Appay et al 2002). However, the presence of such molecules does not always result in the induction of killing mechanisms after cells are challenged with their cognate antigen. The use of killing assays to evaluate this further, has shown promising results. In the context of EBV, previous studies have investigated the cytotoxic potential of EBV-specific CD4+ T cells and their ability to inhibit target cell outgrowth, *in vitro* (Munz et al 2000, Nikiforow et al 2003, Long et al 2005). It has been reported that the inhibition of LCL growth correlates with cytotoxic activity, and CD4+ T cell clones with high LCL recognition efficiency show the greatest cytotoxic killing. Thus, it would be interesting to assess the

cytotoxic capacity of our PRS-specific clones of known Vβ usage, to determine whether this also correlates with their functional avidity and LCL recognition efficiency. This would enable determination of individual clonotypes with the most therapeutic relevance based on direct elimination of EBV infected cells and inhibition of cancer cell outgrowth. These features within anti-viral and anti-tumour immune responses are paramount for subsequent adoptive immunotherapeutic strategies to be effective.

4.6 Conclusions

In summary, our results have demonstrated that HLADR7 restricted CD4 T cell clones specific for the immunodominant EBNA2, PRS epitope comprise TCRs with a number different TCR usages, including common use of distinct Vβs. We have further shown through functional analysis of clones with known Vβ usage, that a range of functional avidities are apparent among the clones of the same and different Vβ usages, within different healthy individuals. Those T cells exhibiting better functional avidity for their cognate peptide similarly display higher levels of percentage recognition efficiency of autologous EBV-infected LCL targets. The finding thus indicate that distinct TCR usage may contribute to differences in T cell function, and that TCRs used therapeutically should be carefully selected based on their preferential properties. This study should enable similar analysis on other viral systems and lead to research in identifying immunodominant TCR sequences and motifs of epitope specific T cell clonotypic populations inducing the most robust immune responses. Similarly, identifying the mechanisms by which epitopes of interest access the MHC class II pathway in infected cells and are processed and presented to CD4 T cells, to allow

efficient recognition, binding and high avidity responses, represents a significant priority for ongoing future work.

The work performed here provides a useful model for harnessing and amplifying epitope specific CD4+ T cell immune responses. From a clinical standpoint, this has important implications for the development of effective and viable TCR gene transfer immunotherapies, targeting a variety of cancers presenting antigens through MHC class II, such as the EBV associated malignancy; PTLD.

5 REFERENCES

Abbot RJ, Quin LL, Leese AM, Scholes AP, and Rickinson AB (2013) CD8+ T cell responses to lytic Epstein-Barr virus infection: late antigen specificities as subdominant components of the total response. *J Immunol.*191(11):5398–5409.

Allison TJ & Garboczi DN (2002) Structure of γδ T cell receptors and their recognition of non-peptide antigens. *Molecular Immunology*.38(14):1051-1061.

Almeida JR, Sauce D, Price DA, Papagno L, Shin SY, Moris A (2009) Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity. *Blood.*113(25):6351–60.

Appay V, Zaunders JJ, Papagno JJ, Sutton J, Jaramillo A, Waters A, Easterbrook P, Grey P, Smith D, McMichael AJ, Cooper DA, Rowland-Jones SL and Kelleher AD (2002) Characterization of CD4+ CTLs Ex Vivo. *J Immunol.*168(11):5954-5958.

Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P et al (1999) A direct estimate of the human alphabeta T cell receptor diversity. *Science.*286(5441):958-61.

Artyomov MN, Lis M, Devadas S, Davis MM, Chakraborty AK (2010) CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery. *Proc Natl Acad Sci* USA.107:16916–16921

Babcock GJ, Decker LL, Freeman RB, and Thorley-Lawson DA (1999) Epstein-Barr Virus–Infected Resting Memory B Cells, Not Proliferating Lymphoblasts, Accumulate in the Peripheral Blood of Immunosuppressed Patients. *J Exp Med.*190(4):567-576.

Banchereau J & Steinman RM (1998) Dendritic cells and the control of immunity. *Nature.*392(6673):245-252.

Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science.*285(5428):727-729.

Bendle GM, Linnemann C, Hooijkaas AI, Bies L, de Witte MA, Jorritsma A, Kaiser AD, Pouw N, Debets R, Kieback E, Uckert W, Song JY, Haanen JB, Schumacher TN (2010) Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy.*Nat Med.*16(5):565-70.

Betts MR et al (2006) HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T-cells. *Blood*. 107(12):4781–4789.

Billeskov R, Wang Y, Solaymani-Mohammadi S, Frey B, Kulkarni S, Andersen P, Agger EM, Sui Y, Berzofsky JA (2017) Low Antigen Dose in Adjuvant-Based Vaccination Selectively Induces CD4 T Cells with Enhanced Functional Avidity and Protective Efficacy. *J Immunol.*198(9):3494-3506

Blair GE & Cook GP (2008) Cancer and the immune system: an overview. *Oncogene.*27:5868.

Blum JS, Wearsch PA, and Cresswell P (2013) Pathways of Antigen Processing. *Annu Rev Immunol*.31:443-473.

Bollard CM, Aguilar L, Straathof KC, Gahn B, Huls MH, Rousseau A, Sixbey J, Gresik MV, Carrum G, Hudson M, Dilloo D, Gee A, Brenner MK, Rooney CM, Heslop HE (2004) Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease.*J Exp Med.*200(12):1623-33.

Burrows SR, Silins SL, Moss DJ, Khanna R, Misko S, and Argaet VP (1995) T Cell Receptor Repertoire for a Viral Epitope in Humans Is Diversified by Tolerance to a Background Major Histocompatibility Complex Antigen. *J Exp Med*.182(6):1703-1715.

Callan MF, Tan L, Annels N, Ogg GS, Wilson JD, O'Callaghan CA, Steven N, McMichael AJ, Rickinson AB (1998) Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo. *J Exp Med*.187(9):1395-402.

Choo SY (2007) The HLA System: Genetics, Immunology, Clinical Testing, and Clinical Implications. *Yonsei Med J*.48(1):11-23.

Cresswell P (1996) Invariant Chain Structure and MHC Class II Function.*Cell*.84(4):505-50.

Crompton L, Khan N, Khanna R, Nayak L and Moss P.A.H (2008) CD4+ T cells specific for glycoprotein B from cytomegalovirus exhibit extreme conservation of T-cell receptor usage between different individuals. *Blood*.111(4):2053-2061.

Dudlely EC, Petrie HT, Shah LM, Owen MJ, and Hayday AC (1994) T Cell Receptor β Chain Gene Rearrangement and Selection during Thymocyte Development in Adult Mice.*Immunity*.1(2):83-93.

Dudley ME, Nishimura MI, Holt AK, Rosenberg SA (1999) Antitumor immunization with a minimal peptide epitope (G9-209-2M) leads to a functionally heterogeneous CTL response. JImmunother. 22(4):288-298

Dunmire SK, Hogguist KA and Balfour HH (2015) Infectious mononucleosis. *Clinical & Translational Immunology*.4:33.

Fehlings M, Chakarov S, Simoni Y, Sivasankar B, Ginhoux F, Newell EW (2018) Multiplex peptide-MHC tetramer staining using mass cytometry for deep analysis of the influenza-specific T-cell response in mice. *J Immunol Methods*. 453:30-36.

Garcia KC, Degano, M., Stanfield RL, Brunmark A, Jackson MR, Peterson PA, Teyton L, and Wilson IA (1996) An alphabeta T cell receptor structure at 2.5 angstoms and its orientation in the TCR-MHC complex. *Science*.274(5285):209-220.

Golubovskaya V and Wu L Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy (2016) *Cancers*.8(3):36.

Gottschalk S, Rooney CM, Heslop HE (2005) Post-transplant lymphoproliferative disorders. *Annu Rev Med*.56:29-44.

Gros A, Robbins PF, Yao X, Li YF, Turcotte S, Tran E, Wunderlich JR, Mixon A, Farid S, Dudley ME, Hanada K, Almeida JR, Darko S, Douek DC, Yang JC, Rosenberg SA. (2014) PD-1 identifies the patient-specific CD8+ tumor-reactive repertoire infiltrating human tumors. *J. Clin. Invest*. 124:2246–2259

Grywalska E, Rolinski J (2015) Epstein-Barr virus-associated lymphomas. *Semin Oncol*. 42(2):291-303.

Han A, Glanville J, Hannsmann L, Davis MM (2014) Linking T-cell receptor sequence to functional phenotype at the single-cell level. *Nat Biotechnol*.32(7): 684–692.

Haque T, Wilkie GM, Jones MM, Higgins CD, Urquhart G, Wingate P, Burns D, McAulay K, Turner M, Bellamy C, Amlot PL, Kelly D, MacGilchrist A, Gandhi MK, Swerdlow AJ, Crawford DH (2007) Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood*.110(4):1123-31.

Henle W, Henle G, Anderson J, Ernberg I, Klein G, Horwitz CA, Marklund G, Rymo L, Wellinder C, and Straus SE (1987) Antibody responses to Epstein-Barr virus-determined nuclear antigen (EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection. *Proc Natl. Acad. Sci. USA*.84:570-574.

Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, Bollard CM, Liu H, Wu MF, Rochester RJ, Amrolia PJ, Hurwitz JL, Brenner MK and Rooney CM (2010) Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood*.115(5): 925–935.

Hislop AD, Taylor GS, Sauce D, Rickinson AB (2007) Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol*.25:587-617.

Holler PD and Kranz DM (2003) Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. *Immunity*.18(2):255–264.

Iwasaki A, Medzhitov R (2004) Toll-like receptor control of the adaptive immune responses. *Nature Immunology*.5(10):987-995.

Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS et al (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood*.114(3): 535-546.

Jurgans LA, Khanna R, Weber J and Orentas R (2006) Transduction of Primary Lymphocytes with Epstein-Barr Virus (EBV) Latent Membrane Protein-Specific T-Cell Receptor Induces Lysis of Virus-Infected Cells: A Novel Strategy for the Treatment of Hodgkin's Disease and Nasopharyngeal Carcinoma. *J Clin Immunol*.26(1):22-32.

Kang MS and Kieff E (2015) Epstein–Barr virus latent genes. *Exp Mol Med*.47(1): e131.

Kempkes B, Robertson ES (2015) Epstein-Barr virus latency: current and future perspectives. *Curr Opin Virol*.14:138-44.

Khanna R, Burrows SR, Thomson SA, Moss DJ, Cresswell P, Poulsen LM, Cooper L (1997) Class I processing-defective Burkitt's lymphoma cells are recognized efficiently by CD4+ EBV-specific CTLs. *J Immunol*.158(8):3619-25.

Kim JH, Kim WS, Hong JY, Ryu KJ, Kim SJ, and Park C (2017) Epstein-Barr virus EBNA2 directs doxorubicin resistance of B cell lymphoma through CCL3 and CCL4-mediated activation of NF-κB and Btk. *Oncotarget*.8(3): 5361–5370.

Klarenbeek PL, Remmerswaal EMB, ten Berge JM, Doorenspleet ME, van Schaik BDC, Esveldt REE, Koch SD, Brinke A ten, van Kampen AHC, Bemelman FJ, Tak PP, Baas F, Vries N, and van Lier RAW (2012) Deep Sequencing of Antiviral T-Cell Responses to HCMV and EBV in Humans Reveals a Stable Repertoire That Is Maintained for Many Years. *PLoS Pathog*.8(9):e1002889.

Knutson KL, Schiffman K, Cheever MA, Disis ML (2002) Immunization of cancer patients with a HER-2/neu, HLA-A2 peptide, p369- 377, results in short-lived peptide-specific immunity. *ClinCancer Res*.8 (5):1014-1018

Krogsgaard M, Prado N, Adams EJ, He XL, Chow DC, Wilson DB, Garcia KC, Davis MM (2003) Evidence that structural rearrangements and/or flexibility during TCR binding can contribute to T cell activation. Mol Cell.12:1367–1378.

Kupper R (2003) B cells under the influence: transformation of B cells by Epstein-Barr virus. *Nature Reviews Immunology*.3(10):801-812.

Lawson T (2001) Epstein-Barr virus: exploiting the immune system. *Nat Rev Immunol*.1(1):75-82.

Laydon DJ, Bangham C, and Asquith B (2015) Estimating T-cell repertoire diversity: limitations of classical estimators and a new approach. *Philos Trans R Soc Lond B Biol Sci*.370:20140291.

Leen AM, Bollard CM, Mendizabal AM, Shpall EJ, Szabolcs P, Antin JH, Kapoor N, Pai SY, Rowley SD, Kebriaei P, Dey BR, Grilley BJ, Gee AP, Brenner MK, Rooney CM, Heslop HE (2013) Multicenter study of banked third-party virus-specific T cells to treat severe viral infections after hematopoietic stem cell transplantation. *Blood*.121(26):5113-23.

Lim A, Trautmann L, Peyrat MA, Couedel C,Davodeau F, Romagne F, Kourilsky P, Bonneville M (2000) Frequent Contribution of T Cell Clonotypes with Public TCR Features to the Chronic Response Against a Dominant EBV-Derived Epitope: Application to Direct Detection of Their Molecular Imprint on the Human Peripheral T Cell Repertoire. *J Immunol*.165(4):2001-2011.

Liu Y and Zeng G (2013) Cancer and Innate Immune System Interactions: translational Potentials for Cancer Immunotherapy. *J Immunother*.35(4):299-308.

Long HM, Chagoury OL, Leese AM, Ryan GB, James E, Morton LT, Abbott RJ, Sabbah S, Kwok W, Rickinson AB (2013) MHC II tetramers visualize human CD4+ T cell responses to Epstein-Barr virus infection and demonstrate atypical kinetics of the nuclear antigen EBNA1 response. *J Exp Med*.210(5):933-49.

Long HM, Haigh TA, Gudgeon NH, Leen AM, Tsang CW, Brooks J, Landais E, Houssaint E, Lee SP, Rickinson AB, Taylor GS (2005) CD4+ T-cell responses to Epstein-Barr virus (EBV) latent-cycle antigens and the recognition of EBV-transformed lymphoblastoid cell lines. *J Virol*.79(8):4896-907.

Long HM, Leese AM, Chagoury OL, Connerty SR, Quarcoopome J, Quinn LL, Shannon-Lowe C, Rickinson AB (2011) Cytotoxic CD4+ T cell responses to EBV contrast with CD8 responses in breadth of lytic cycle antigen choice and in lytic cycle recognition. *J Immunol*.187(1):92-101.

Lowe CS, Rickinson AB, and Bell AI (2017) Epstein–Barr virus-associated lymphomas. *Philos Trans R Soc Lond B Biol Sci*.19(1732) 20160271.

Makedonas G & Betts MR (2006) Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection. *Springer Seminars in Immunopathology*.28(3):209-219.

Mansilla-Soto J and Cortes P (2003) VDJ Recombination Artemis and Its In Vivo Role in Hairpin Opening. *J Exp Med*.197(5):543-547.

Mansour ZA, Nelson BP, and Evens AM (2016) Post-Transplant Lymphoproliferative Disease (PTLD): Risk Factors, Diagnosis, and Current Treatment Strategies. *Curr Hematol Malig Rep*. 8(3):173–183.

McBlane JF, Gent VDC, Ramsden DA, (1995) Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. *Cell*.83(3):387-395.

Mogensen TH (2009) Pathogen Recognition and Inflammatory signaling in Innate Immune Defenses. *Clinical Micorbiol Reviews*.22(2):240-273.

Münz C, Bickham KL, Subklewe M, Tsang ML, Chahroudi A, Kurilla MG, Zhang D, O'Donnell M, and Steinman RM (2000) Human Cd4+ T Lymphocytes Consistently Respond to the Latent Epstein-Barr Virus Nuclear Antigen Ebna1. *J Exp Med*.191(10):1649-1660.

Murphy K, Travers P, Walport, M, & Janeway, C (2012) *Janeways Immunology* (8th ed) New York: Garland Science.

Nauerth M, Weißbrich B, Knall R, Franz T, Dössinger G, Bet J, Paszkiewicz PJ, Pfeifer L, Bunse M, Uckert W, Holtappels R, Gillert-Marien D, Neuenhahn M, Krackhardt A, Reddehase MJ, Riddell SR, Busch DH. (2013) TCR-ligand koff rate correlates with the protective capacity of antigen-specific CD8+ T cells for adoptive transfer. *Sci Transl Med.*5(192):192ra87

Neefies J, Jongsma ML, Paul P, Bakke O (2011) Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol.*11(12)823-836.

Nielsen LK, Clements CS, Brooks AG, Purcell AW (2002) The 1.5 Å Crystal Structure of a Highly Selected Antiviral T Cell Receptor Provides Evidence for a Structural Basis of Immunodominance.*Cell.*10(11):1521-1532.

Nikiforow S, Bottomly K, Miller G, Münz C (2003) Cytolytic CD4(+)-T-cell clones reactive to EBNA1 inhibit Epstein-Barr virus-induced B-cell proliferation. *J Virol.*77(22):12088-104.

O'Reilly RJ, Prockop S, Hasan AN, Koehne G, and Doubrovina E (2016) Virus-Specific T-cell banks for "Off the shelf" adoptive therapy of refractory infections.*Bone Marrow Transplant*.51(9):1163-1172.

Oertel SH, Verschuuren E, Reinke P, Zeidler K, Papp-Váry M, Babel N, Trappe RU, Jonas S, Hummel M, Anagnostopoulos I, Dörken B, Riess HB (2005) Effect of anti-CD 20 antibody rituximab in patients with post-transplant lymphoproliferative disorder (PTLD). *Am J Transplant*.5(12):2901-6.

Omiya R, Buteau C, Kobayashi H, Paya CV, Celis E (2002) Inhibition of EBV-induced lymphoproliferation by CD4(+) T cells specific for an MHC class II promiscuous epitope.*J Immunol.*169(4):2172-9.

Ostrand-Rosenberg S (2008) Immune surveillance: a balance between protumor and antitumor immunity. Current Opinion in Genetics & Development. *Curr Opin Genet Dev*.18(1):11-8.

Papadopoulos EB, Ladanyi M, Emanuel D, Mackinnon S, Boulad F, Carabasi MH, Castro-Malaspina H, Childs BH, Gillio AP, Small TN, et al. (1994) Infusions of Donor Leukocytes to treat Epstein-barr virus- associated lymphoproliferative disorder after allogeneic bone marrow transplantation.*N Engl J Med.*330(17):1185-91.

Price AH, Luftig MA (2015) To Be or Not IIb: A Multi-Step Process for Epstein-Barr Virus Latency Establishment and Consequences for B Cell Tumorigenesis. *PLoS Pathog*.11(3):1-7.

Pu Z, Carrero JA, Unanue ER (2002) Distinct recognition by two subsets of T cells of an MHC class II-peptide complex. *Proc Natl Acad Sci U S A*. 99(13):8844-9.

Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT2, Lacey SF2,5, Badros AZ, Garfall A, Weiss B, Finklestein J, Kulikovskaya I, Sinha SK, Kronsberg S, Gupta M, Bond S, Melchiori L, Brewer JE, Bennett AD, Gerry AB, Pumphrey NJ, Williams D, Tayton-Martin HK, Ribeiro L, Holdich T, Yanovich S, Hardy N, Yared J, Kerr N, Philip S, Westphal S, Siegel DL, Levine BL, Jakobsen BK, Kalos M, June CH (2015) NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med.*21(8):914-921.

Rickinson AB, Long HM, Palendira U, Münz C, Hislop AD (2014) Cellular immune controls over Epstein-Barr virus infection: new lessons from the clinic and the laboratory. *Trends Immunol*.35(4):159-69.

Rothschild SI, Thommen DS, Moersig W, Müller P, Zippelius A (2015) Cancer immunology–development of novel anti-cancer therapies. *Swiss Med Wkly.*145:14066.

Rowe M and Zuo J (2010) Immune responses to Epstein–Barr virus: molecular interactions in the virus evasion of CD8+ T cell immunity.*Microbes Infect*.12(3):173-181.

Sandberg JK, Fast NM, Nixon DF (2001) Functional heterogeneity of cytokines and cytolytic effector molecules in human CD8+ T lymphocytes. *J Immunol.*167(1):181–187.

Schmidt J, Dojcinovic D, Guillaume P, and Luescher I (2013) Analysis, Isolation, and Activation of Antigen-Specific CD4+ and CD8+ T Cells by Soluble MHC-Peptide Complexes. *Frontiers in Immunology*.4:218.

Song Y, Gil A, Mishra R, Ghersi D, Selin LK & Stern LJ (2017) Broad TCR repertoire and diverse structural solutions for recognition of an immunodominant CD8+ T cell epitope. *Nature Structural & Molecular Biology*.24(4):395-406.

Steven NM, Leese AM, Annels NE, Lee SP, Rickinson AB (1996) Epitope focusing in the primary cytotoxic T cell response to Epstein-Barr virus and its relationship to T cell memory. *J Exp Med*. 184(5):1801-13.

Steven NM, Annels NE, Kumar A, Leese AM, Kurilla MG, Rickinson AB (1997) Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T cell response.*J Exp Med*.185(9):1605-17.

Swain SL, McKinstry KK, Strutt TM (2012) Expanding roles for $CD4^+$ T cells in immunity to viruses. *Nat Rev Immunol*.12(2)136-148.

Takeuchi A, Saito T (2017) CD4 CTL, a Cytotoxic Subset of CD4+ T Cells, Their Differentiation and Function. *Front Immunol*.8:194.

Tauber A (1992) The birth of immunology: III. The fate of the phagocytosis theory. *Cellular Immunology.* 139 (2): 505-530.

Taylor GS, Long HM, Brooks JM, Rickinson AB, Hislop AD (2015) The immunology of Epstein-Barr virus-induced disease. *Annual Reviews Immunology.*33:787-821.

Taylor GS, Long HM, Haigh TA, Larsen M, Brooks J, Rickinson AB. (2006) A Role for Intercellular Antigen Transfer in the Recognition of EBV-Transformed B Cell Lines by EBV Nuclear Antigen-Specific CD4+ T Cells. *J Immunol.*177(6):3746-3756.

Tembhare P, Constance M, Yuan CM, Liqiang Xi L, Morris JC, Liewehr D, Venzon D, Janik JE, Raffeld M, Stetler-Stevenson M (2011) Flow Cytometric Immunophenotypic Assessment of T-Cell Clonality by Vβ Repertoire Analysis: Detection of T-Cell Clonality at Diagnosis and Monitoring of Minimal Residual Disease Following Therapy. *American Journal of Clinical Pathology.*135(6):890–900.

Thorley-Lawson DA (2016) EBV Persistence-Introducing the Virus. *Curr Top Microbiol Immunol.*390(1):151-209.

Thorsby E & Lie BA (2005) HLA associated genetic predisposition to autoimmune diseases: Genes involved and possible mechanisms. *Transplant Immunology.*14(3):175-182.

Thomas S, Xue SA, Bangham CRM, Jakobsen BK, Morris EC and Stauss HJ (2011) Human T cells expressing affinity-matured TCR display accelerated responses but fail to recognize low density of MHC-peptide antigen. *Blood.* 118:319-329

Ting JPY and Trowsdale J (2002) Genetic Control of MHC Class II Expression. *Cell.*109(2):21-33.

Vahedi G, Takahashi H, Nakayamada S, Sun HW, Sartorelli V, Kanno Y, O'Shea JJ (2012) STATs Shape the Active Enhancer Landscape of T Cell Population.*Cell*.151(21):981-993.

Van De Wiele CJ, Marino JH, Murray BW, Vo SS, Whetsell ME and Teague TK (2004) Thymocytes between the β-Selection and Positive Selection Checkpoints Are Nonresponsive to IL-7 as Assessed by STAT-5 Phosphorylation. *The Journal of Immunology*.172.(7):4235-4244.

Van Gent DC, Ramsden DA, Gellert M (1996) The RAG1 and RAG2 Proteins Establish the 12/23 Rule in V(D)J Recombination.*Cell*.85(1):107-113.

Vanhoutte VJ, McAulay KA, McCarrell E, Turner M, Crawford DH and Haque T (2009) Cytolytic mechanisms and T-cell receptor Vb usage by ex vivo generated Epstein–Barr virus-specific cytotoxic T lymphocytes. *Immunology*.127(4):577-586.

Vickers MA, Wilkie GM, Robinson N, Rivera N, Haque T, Crawford DH, Barry J, Fraser N, Turner DM, Robertson V, Dyer P, Flanagan P, Newlands HR, Campbell J, Turner ML (2014) Establishment and operation of a Good Manufacturing Practice-compliant allogeneic Epstein-Barr virus (EBV)-specific cytotoxic cell bank for the treatment of EBV-associated lymphoproliferative disease. *Br J Haematol*.167(3):402–410.

Viganò S, Utzschneider DT, Perreau M, Pantaleo G, Zehn D, and Harari A (2012) Functional Avidity: A Measure to Predict the Efficacy of Effector T Cells? *Journal of Immunology Research*.7:1-14.

Wang CY, Fang YX, Chen GH, Jia HJ, Zeng S et al (2017) Analysis of the CDR3 length repertoire and the diversity of T cell receptor α and β chains in swine CD4+ and CD8+ T lymphocytes. *Mol Med Rep*.16(1):75-86.

Warren RL, Freeman JD, Zeng T, Choe G, Munro S, Moore R, Webb JR, and Holt RA. (2011) Exhaustive T-cell repertoire sequencing of human peripheral blood samples reveals signatures of antigen selection and a directly measured repertoire size of at least 1 million clonotypes. *Genome Res.*21(5):790–797.

Watts C (2004) The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules.*Nature Immunology*.5(7):685-692.

Williams H and Crawford DH (2006) Epstein-Barr virus: the impact of scientific advances on clinical practice. *Blood.*107(3):862-869.

Wherry E.J (2011) T cell exhaustion. *Nat Immunol*.12(6): 492-499.

Yao X, Lu YC, Parker LL, Li YF, El-Gamil M, Black MA, Xu H, Feldman SA, van der Bruggen P, Rosenberg SA, Robbins PF (2016) Isolation and Characterization of an HLA-DPB1*04: 01-restricted MAGE-A3 T-Cell Receptor for Cancer Immunotherapy. *J Immunother*.39(5):191-201.

Young LS, Murray PG (2003) Epstein-Barr virus and oncogenesis: from latent genes to tumours. *Oncogene*.22(33):5108-5121.

Zaks TZ, Rosenberg SA (1998) Immunization with a peptide epitope (p369-377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu+ tumors. *Cancer Res.* 58 (21): 4902-4908

Zeh HJIII, Perry-Lalley D, Dudley ME, Rosenberg SA, Yang JC (1999) High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. JImmunol.162(2):989-994

Zheung Y, Parsonage G, Zhuang X, Machado LR, James CH, Salman A, Searle PF, Hu EP i,3 Chan ATC, Lee SP (2015) Human Leukocyte Antigen (HLA) A*1101-restricted Epstein-Barr Virus-specific T-cell Receptor Gene Transfer to Target Nasopharyngeal Carcinoma. *Cancer Immunol Res.*3(10): 1138–1147.