ASSESSING THE FUNCTION OF EBV-SPECIFIC CD4^{+} T cells

BY

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ABSTRACT

$CD4^+$ T cells play a key role in orchestrating an efficient immune response to viral infection; yet due to a variety of factors remain relatively poorly studied. However, recent advancements in MHC class II tetramer technology are allowing single cell analysis of $CD4^+$ T cell responses to be studied for the first time. We sought to take advantage of these reagents in the context of the oncogenic herpesvirus EBV, in order to establish the cytokine profiles and cytotoxic potential of $CD4^+$ T cells specific for a variety of viral epitopes. Such knowledge would help with the improvement of adoptive T cell therapies for EBV-related malignancies that have been shown to be more efficacious with higher proportions of $CD4^+$ T cells.

Using flow cytometry we found that the EBV-specific $CD4^+$ T cells are capable of responding to EBV-infected cells *ex vivo*. Using MHC class II tetramers we have demonstrated that responding cells were a heterogeneous population expressing the T_h1 associated transcription factor T-bet and were polyfunctional producing IFNγ, TNFα or a combination of both. In addition we found that unmanipulated $CD4^+$ T cells specific for EBV-derived epitopes possess cytotoxic potential *ex vivo*.

These results may help to improve immune therapy for EBV-associated cancers.

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1. INTRODUCTION

1.1 Epstein-Barr virus

The Epstein Barr virus (EBV) is an oncogenic human gammaherpesvirus that is widespread throughout the world. Infection is usually asymptomatic if it occurs during childhood, however seroconversion can cause a self-limiting illness, infectious mononucleosis in adolescence, characterised by fever and malaise. Thereafter, in the majority of immunocompetent individuals, the virus persists for life, largely asymptomatically, as it is kept under control by the host's T cell immune system.

Virus transfer occurs orally through saliva and while EBV is B cell tropic, it is also capable of infecting epithelial cells and in rare cases T and natural killer cells. The early stages of infection are not well understood but involve lytic effect of permissive cells in the oropharynx. However, once the virus has passed through the epithelial layer, infection of locally infiltrating B cells has been well described and occurs through the respective interaction between the viral glycoproteins gp350 and gp42 with CD21 and MHC class II present on B lymphocytes (Young, Rickinson, 2004).

During this time, EBV establishes a latent growth-transforming infection in B cells by expressing a set of ten latent proteins comprised of six EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and –LP), three latent membrane proteins (LMPs 1, 2A and 2B) and BHRF1 (Young, Rickinson, 2004; Kelly *et al.*, 2009). The EBV lytic and latent proteins are highly immunogenic to the immune system and primary infection is eventually brought under control by a strong T cell response. Some EBV infected B cells down regulate gene expression to avoid T cell destruction and thus a reservoir of genome positive but antigen negative B cells is established. The periodic reactivation of such cells coupled with newly infected B cells maintains this population throughout the life of the host.

1.2 T cell response to EBV

In the case of EBV, the identifiable clinical syndrome of infectious mononucleosis has enabled researchers to study the immune response to primary infection and to follow patients through recovery and into asymptomatic carriers of EBV. Thus, this is a particularly good model to study the immune response to viruses. The initial response is characterised by a large expansion of EBV-specific $CD8^+$ T cells and as a result, the $CD8^+$ T cell response has been well studied. With the use of MHC class I tetramer staining, it has been shown that this response is skewed towards proteins from the immediate early (IE) and early (E) phases of the lytic virus replication cycle (Pudney *et al.*, 2005, Abbott *et al*., 2013). Responses to single epitopes derived from lytic proteins can account for up to 40% of the total $CD8^+$ T cell population and it has also been observed that following primary infection, responses against proteins from the latent cycle account for up to 5% of circulating $CD8^+$ T cells. The dominant responses are generally against epitopes drawn from the EBNA3 family of proteins and to a lesser extent LMP2 (reviewed in Hislop *et al*., 2007). During resolution of the acute disease, most of the T cells undergo apoptosis, leaving a small proportion in the memory population that control the persistent infection.

Until recently, there has been a disparity in knowledge of the $CD4^+$ T cells response to EBV compared to $CD8^+$ T cells. A lack of information regarding individual epitope responses, the lower frequency of virus specific $CD4^+$ T cells in the blood and a paucity in reagents such as MHC class II tetramers to enable the analysis of epitope specific cells $CD4^+$ T cells, have all caused difficulty in analysing the $CD4^+$ T cell response to EBV. However recent elucidation of the epitopes recognised by EBV-specific $CD4^+$ T cells (Long *et al.*, 2005, 2011) and the improved reproducibility of testing performed with MHC class II tetramers (Long *et al.*, 2013) have meant that some of these experimental and technological issues have been overcome. Recent studies show that $CD4^+$ T cells against lytic and latent proteins (except EBNA1) are expanded in the blood of IM patients, but to a lesser extent than their $CD8^+$ counterparts

(Long *et al.*, 2013). Furthermore, $CD4^+$ T cell responses are maintained in memory at low frequency but are spread throughout immediate early, early and late proteins of the lytic cycle as well as across the latent EBNA proteins (Long *et al.*, 2011). Thus, individual $CD4^+$ responses are lower than $CD8^+$ responses but are spread over a broader repertoire.

1.3 EBV associated malignancies and immunotherapy

The balance between the virus and the immune system allows most individuals to stay asymptomatic, but the virus does have oncogenic potential. Therefore, despite the T cell surveillance, EBV is associated with a number of haematological and epithelial malignancies including Burkitt's lymphoma (BL) Hodgkin's lymphoma (HL), nasopharyngeal carcinoma (NPC), some gastric carcinomas, diffuse large B-cell lymphoma (DLBCL), NK/T cell lymphoma and, in individuals undergoing therapeutic immunosuppression, post-transplant lymphoproliferative disease (PTLD).

The expression of viral proteins by these cancerous cells make them ideal candidates for T-cell based immunotherapy as they can be directly recognised and killed by cytotoxic T cells specific for such antigens. In particular the use of adoptive transfer of polyclonal EBV-specific T cells has proved to be a highly successful way of treating PTLD.

At present, PTLD is the only EBV related malignancy that is treated with infusion of EBV specific CTLs. These cytotoxic lymphocytes are generated by stimulation with *in vitro* generated autologous lymphoblastoid cell lines (LCLs). These EBV-transformed cells express the full range of EBV latent proteins and resemble tumour cells of PTLD. In a recent trial, EBV-specific CTL infusions were administered as a prophylactic to 101 patients who had undergone hematopoietic stem cell transplantation (HSCT) and who were deemed to be at high risk of developing PTLD. Remarkably, none of these patients have since developed the disease. Furthermore, 11 of 13 patients who received EBV-CTLs following clinical

diagnosis of EBV-positive PTLD achieved a complete response (Heslop *et al.*, 2010). However immunosuppressed patients undergoing solid organ transplant (SOT) only responded in approximately 50% of cases (Haque *et al.*, 2007). In HSCT, CTLs generated from the allograft have not only been shown to persist in the circulating memory pool but also to expand in response to antigen challenge. This is in contrast to infusion of patient derived EBV-specific CTLs following SOT as they struggle to persist in the presence of continuous immunosuppression.

It has been thought that the particular success of this treatment was due to the high proportion of cytotoxic $CD8^+$ T cells in the EBV-specific preparations. Recent evidence however suggests that responses are greatly improved with a higher percentage of $CD4^+$ T helper cells (Haque *et al.*, 2007). Unlike $CD8^+$ T cells whose specificity is conferred through MHC class I recognition, $CD4^+$ T cells recognise antigens bound to MHC class II molecules which predominantly arise from the extracellular processing pathway of antigen presenting cells. Nonetheless, it has been shown that in some cases epitopes can gain direct access to the MHC class II processing pathway (Leung *et al.*, 2010; Nedjic *et al.*, 2009). In addition, like $CD8^+$ T cells, $CD4^+$ T cells are also capable of functioning as effectors through cytotoxicity in a peptide-specific and MHC class II restricted manner (Suni *et al.*, 2001). In the context of viral infections and cancers occurring in MHC class II positive cells (such as EBV and its associated malignancies), $CD4^+$ T cells therefore represent an interesting area to study for therapeutic applications.

Despite the positive correlation between frequency of EBV specific $CD4^+$ T cells in CTL infusions and the clinical outcome, we do not currently know anything about their functional characteristics or which epitopes have the greatest clinical effect.

1.4 CD4$^+$ T cells in immunity and infection

CD4$^+$ T cells play a central role in immunity and responding to viral infections by orchestrating the response of multiple wings of the immune system. CD4$^+$ T cells interact with CD8$^+$ T cells by secreting IL-2 and promote differentiation as well as enhancing both the primary and memory antiviral responses of cytotoxic CD8$^+$ T cells. They also play a vital role in stimulating antigen presenting cells by secreting IFNγ but also through the binding of CD40 to its associated ligand that is present on the surface of activated CD4$^+$ T cells. These signals enhance antigen processing and presentation on the surface of macrophages and dendritic cells while also boosting antibody mediated immune response by increasing memory B cell and plasma cell differentiation (Fig 1.1).

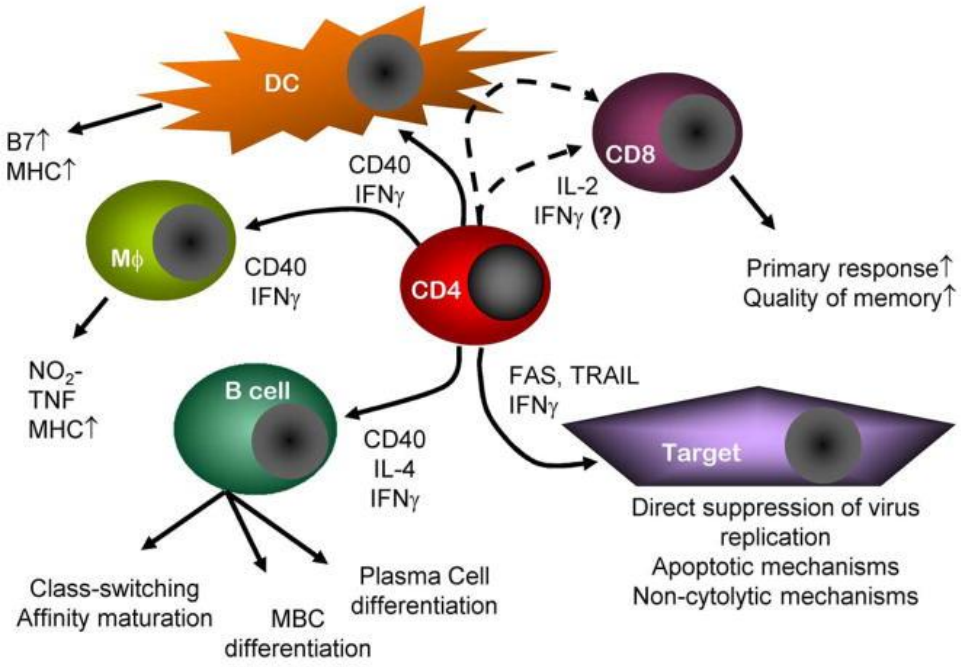


Figure 1.1 CD4$^+$ T cells play a central role in the immune system response to viral infections by orchestrating the activity of cytotoxic CD8$^+$ T cells, dendritic cells, macrophages and B cells. (Whitmire, 2011)

The example of HIV infection gives the clearest indication of $CD4^+$ T cells' critical importance as disease progression to AIDS occurs when $CD4^+$ T cells are at extremely low levels and are therefore unable to orchestrate an efficient immune response to opportunistic infections.

For all the complex functions that $CD4^+$ T cells carry out, they can differentiate into a number of different subsets. The differentiation from a naïve $CD4^+$ T cell into a subpopulation occurs once an antigen in complex with MHC class II molecules is recognised by its cognate T-cell receptor (TCR). The lineage a cell is subsequently differentiates to is influenced by the resulting strength of the TCR signal as well as cytokines present in the extracellular environment and the type of antigen presenting cell (ligation of different types of co-stimulatory molecules) (Murphy, Reiner, 2002).

The T_h1 and T_h2 subtypes are perhaps the most extensively studied effector helper cells and are heavily associated with the expression of the transcription factors T-bet and GATA3 respectively. Beyond these two classical subsets, which are the main effector subsets involved in host immunity against viral/microbial infections and extracellular parasites respectively, a number of new lineages are now know to also play role in the adaptive immune system. T_h9 and the closely related T_h17 and T_h22 effector subsets possess a variety of different functions ranging from defence against parasitic infections (T_h9) to anti-microbial immunity at mucosal sites (T_h17) and wound healing (T_h22). Much like the T_h1 and T_h2 subsets, each lineage also secretes specific cytokines: IL-9 for T_h9, IL-17and IL-22 for T_h17, IL-22 and TNFα for T_h22 cells (reviewed by Luckheeram, 2012) (Fig1.2). Differentiation to a particular effector subtype was previously thought to be an irreversible process however research now suggests that $CD4^+$ T cells are capable of remaining plastic (Murphy, Stockinger, 2010). The exact mechanisms underlying this ability to change subtype is not well understood, nonetheless factors such as the differentiation state and epigenetic changes are thought to influence this plasticity (Murphy *et al.*, 1996; Hirahara *et al.*, 2011). Furthermore, recent experimental evidence has demonstrated that cytokines secreted by the

different subsets are able to affect the characteristic profile of helper cells. It has been shown that transforming growth factor-β, a cytokine predominantly associated with promoting the differentiation of naïve $CD4^+$ T cells into T_h17 or T_{reg} subsets, can cause the "reprogramming" of T_h2 cells to an IL-9 secreting profile associated with the T_h9 subset (Veldhoen *et al.*, 2008). Moreover the localised presence of IL-6, IL-23 and TGF-β and interaction between the transcription factors FoxP3 and RORγt play a siginificant role in the interconversion that can occur between the regulatory and T_H17 subsets (reviewed by Tato and Cua, 2008).

In addition to the "helper" subsets, $CD4^+$ T cells can also differentiate into subtypes that play a role in the regulation of the immune system. Regulatory T cells (T_{regs}) and follicular helper cells (T_{FH}), as their names suggest, regulate the immune response and help in the maintenance of germinal centres (stimulating antibody production by B cells) respectively. T_{regs} exert their role by secreting immunosuppressive cytokines such as IL-10 and TGFβ and are characterised by the presence of the FoxP3 transcription factor and high expression of CD25 on the cell surface (Vignali 2008). Meanwhile T_{FH} cells migrate to B cell follicles due to the expression of the homing receptor CXCR5 and signal to plasma cells and memory B cells through the secretion of CD40L, IL-4 and IL-21 (Crotty, 2011) (Fig 1.2).

The majority of research into anti-viral $CD4^+$ T cells has focused on looking at the secretion of IFNγ by responding cells however mounting evidence suggests that polyfunctional $CD4^+$ T cells that produce multiple cytokines and perform multiple functions are of vital importance in controlling viral infections as they can exert more effective control (Makedonas, Betts, 2006). In patients who have undergone liver transplant, presence of polyfunctional $CD4^+$ T cells specific for the herpesvirus CMV, protect against high-level replication of the virus (Nebbia *et al.*, 2011). Additionally, studies have recently shown that subjects with higher proportions of polyfunctional T cells are more able to spontaneously control HIV infection (Van Braeckel *et al.*, 2012). In the context of EBV, it has been shown that in patients suffering from PTLD,

frequency of polyfunctional T cells specific for EBV were significantly decreased compared to asymptomatic long term carriers of the virus (Ning *et al.*, 2011).

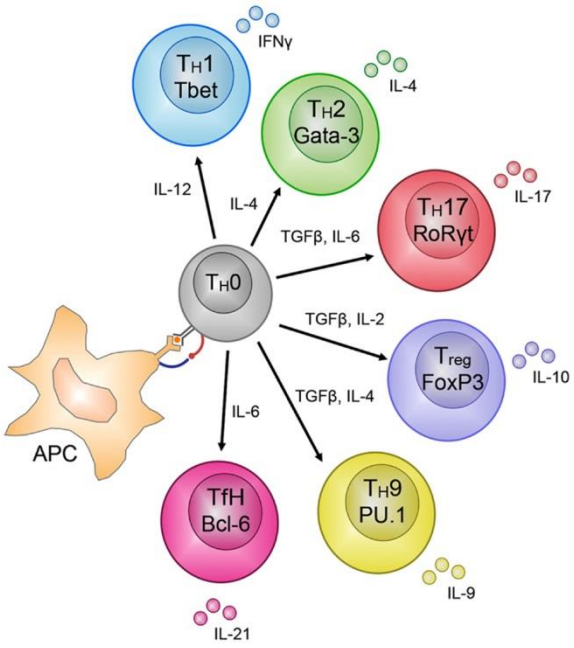
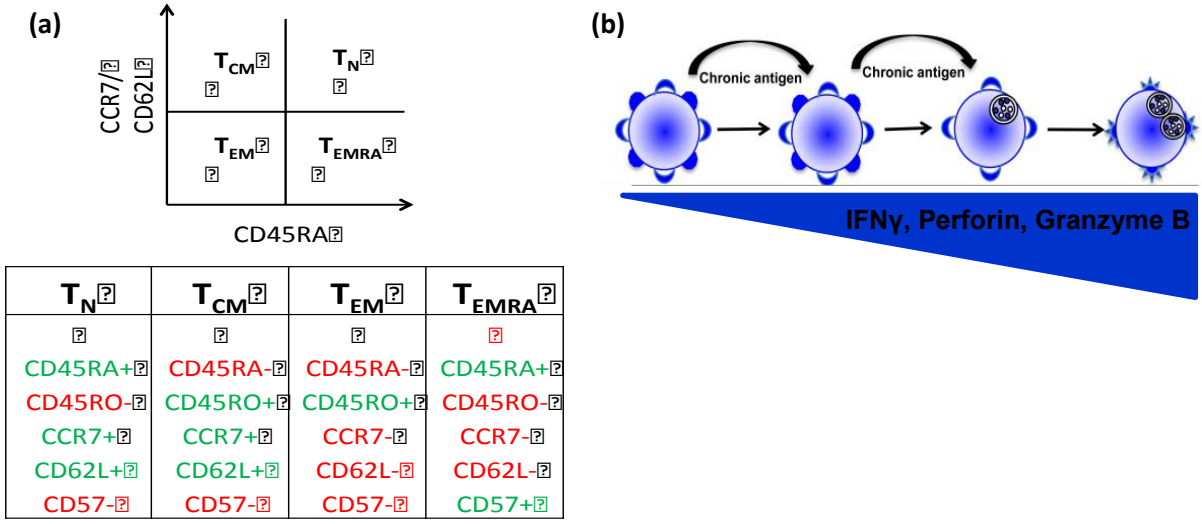


Figure 1.2. Naïve $CD4^+$ T cells can differentiate into a variety of different subsets associated with expression of transcription factors and production of cytokines. (Russ 2013)

Along with secretion of effector cytokines, $CD4^+$ T cells can possess cytotoxic abilities. Although it was originally thought that the capacity to kill antigen expressing targets was only a feature of $CD8^+$ T cells, it is now clear that in certain circumstances $CD4^+$ T cells can also exhibit cytotoxic function on MHC class II positive cells expressing their cognate antigen. The polarizing conditions that determine $CD4^+$ subtype differentiation also influences their cytotoxic potential. Thus, research has shown that T_h1 effectors exhibit high levels of granzyme B whereas T_h2 effectors (and to an extent T_h17 cells) possess much lower levels and are therefore thought to be less able to lyse target cells (Brown *et al.*, 2009).

Induction of lysis of cells targeted by CD4 T cells is mediated through apoptotic pathways and can occur through a number of distinct mechanisms. Perforin and granzyme B are released by cytotoxic $CD4^+$ T cells and while the former creates pores within the plasma membrane of a target cell, granzyme B can enter a cell to cleaves and activate pro-apoptotic proteins. Additionally, binding of Fas receptor on target cells to its cognate ligand FasL on the surface of the T cell or binding of the TNF related apoptosis inducing ligand (TRAIL) present on the T cell to death receptors can initiate signal transduction pathways of apoptosis (reviewed by Brown, 2010; Herbeuval *et al.*, 2005).

The mechanism employed by a cytotoxic $CD4^+$ T cell is linked with the differentiation status of $CD4^+$ T cells. Indeed $CD4^+$ T cells express cell surface receptors depending on their activation and migration state. Naïve T cells (T_N) express high levels of CD45RA and the lymphoid homing markers CD62L and CCR7, however following activation (contact with an antigen presenting cell), the phenotype changes. Expression of CD45RA switches to its isoform CD45RO and cells can be subdivided into either $CCR7^+$ $CD62L^{hi}$ central memory cells (T_{CM}) that will migrate to the lymphoid tissue or, $CCR7^-$ $CD62L^{low}$ effector memory cells (T_{EM}) that will produce cytokines such as IFNγ and IL-4 (Fig 1.3.a). The latter are also know in some circumstances to express high levels of perforin and granzyme (Appay *et al.* 2002). An additional effector memory population that is positive for CD45RA, called T_{EMRA}, exists and represent the most differentiated type of $CD4^+$ T cell. It is however believed that in the case of chronic infections such as EBV, where T cells are constantly being exposed to antigens, a population of terminally differentiated effector cells with cytotoxic potential is present in the blood (Fig1.3.b). This particular phenotype is also what is acquired by cultured $CD4^+$ T cell clones over time.



T_N	T_{CM}	T_{EM}	T_{EMRA}
CD45RA+	CD45RA-	CD45RA-	CD45RA+
CD45RO-	CD45RO+	CD45RO+	CD45RO-
CCR7+	CCR7+	CCR7-	CCR7-
CD62L+	CD62L+	CD62L-	CD62L-
CD57-	CD57-	CD57-	CD57+

Figure 1.3 **(a)** Memory phenotype of $CD4^+$ T cells is associated with, but not exclusively linked to, cell surface expression of, the differentiation marker CD45RA and the lymphoid homing marker CCR7. **(b)** Model for cytolytic $CD4^+$ T cells with increasing cytotoxic potential and cytokine productions in chronic infections through repeated challenge with antigen. (Adapted from Brown, 2010).

1.5 Therapeutic use of $CD4^+$ T cells

There is therefore potential for $CD4^+$ T cells to be used therapeutically, particularly in the case of EBV associated malignancies where the target B cell constitutively express MHC class II molecules on the surface. In the case of Burkitt's lymphoma, tumours are deficient in class I antigen processing thereby allowing them to evade recognition by $CD8^+$ T cells. However, they retain a degree of class II function and as a result can be targeted by $CD4^+$ T cells (God, Haque, 2010).

Recently, infusion of an expanded $CD4^+$ T cell clone specific for a melanoma associated antigen into a patient with refractory metastatic melanoma mediated a durable clinical remission (Hunder *et al.*, 2008). This demonstrated the potential for cytotoxic $CD4^+$ T cells to be used in cancer immunotherapy in general and not necessarily restricted to EBV associated cancers.

In the context of this virus it is now known what epitope responses are present in the circulating blood of healthy donors, but most *ex vivo* studies have been limited to the analysis the production of IFNγ and or IL-4 (Bickham *et al.*, 2001; Long *et al.*, 2011). However, phenotyping of EBV specific $CD4^+$ T cells by looking at the expression of transcription factors has not been performed alongside cytokine profile analysis.

In addition to analysing cytokine production, studies have shown that *in vitro* isolated EBV-specific $CD4^+$ T cells clones can kill EBV infected cells (Long *et al.*, 2005; Munz *et al.*, 2000) and preferentially use perforin/granzyme dependent mechanism (Haigh *et al.*, 2008; Khanolkar *et al.*, 2001). However, as mentioned previously, such $CD4^+$ T cell clones kept in culture need to be constantly challenged with antigen and therefore adopt a late differentiated memory phenotype which is associated with a higher presence of cytotoxic molecules and increased production of cytokines. Circulating EBV specific $CD4^+$ T cells are predominantly central or effector memory cells and it has yet to be studied whether these are primed for direct cytotoxic capacity. Such studies would enable us to confirm whether cytotoxic EBV-specific cells are present in the circulation or whether what has been previously observed is in fact an artefact of *in vitro* culture of $CD4^+$ T cell clones. Assessing the frequency of un-manipulated EBV specific cells expressing perforin and granzyme as well as their ability to directly recognise and kill virus infected cells would further enhance our understanding of the importance of these cytotoxic $CD4^+$ T cells in controlling the virus.

Understanding the functional characteristics of $CD4^+$ T cells that are important in controlling EBV in healthy donors will help recognize the type of T cells that may be important therapeutically. As a result this could help enhance the efficacy of adoptive T cell therapy and potentially identify $CD4^+$ T cells that would assist in induction of therapeutic vaccines for EBV associated diseases.

1.6 Aims

The aims of this project were therefore:

1. To design and optimise panels of antibodies to analyse transcription factor expression, cytokine production, memory phenotype and cytotoxic potential of EBV-specific $CD4^+$ T cells.
2. To use these panels to investigate the cytokine profiles and effector subsets of $CD4^+$ T cells responding to autologous EBV-transformed LCLs *ex vivo*.
3. To use novel MHC class II EBV tetramers to analyse the cytokine profiles, transcription factor expression and cytotoxic potential of individual $CD4^+$ T cells responding to EBV-derived-peptides at an epitope level.

2. MATERIALS AND METHODS

2.1 Ethic statements and donors

All experiments were approved by the South Birmingham Local Research Ethics Committee (07/Q2702/94) and all donors provided written consent for the collection of blood samples and their subsequent analysis. The cohort consisted of 9 EBV seropositive donors whose HLA II type included DR7 (HLA DRB1*07), DR52b (HLA DRB3*02), DR51 (HLA DRB5*01) and DR17 (HLA DRB81*0301) or combinations thereof.

2.2 Cell preparations

PBMCs from healthy EBV seropositive donors were separated by Ficoll-Hypaque centrifugation into RPMI 1640 (Sigma) containing 100IU/mL penicillin and 100µg/mL streptomycin (standard medium). Cells were either used immediately or frozen for later use.

Depletion of $CD8^+$ T cells was achieved by washing cells in RPMI 1640 containing no serum and incubating them in a 15mL tube with CD8 Dynabeads (Life Technologies) (approximately 4 beads per $CD8^+$ T cell) for 30 mins at 4°C on a rotator. $CD8^+$ cells were removed by placing the tube in a magnet for 2mins and harvesting the $CD4^+$ T cell enriched supernatant.

2.3 Lymphoblastoid cell line (LCL) generation

PBMC pellets were resuspended in supernatant from the Marmoset B95.8 producer line filtered through a 0.45µm filter. Cells were left at 37˚C overnight before washing twice in RPMI and resuspending in standard medium supplemented with 1µg/ml cyclosporine A. Cells were maintained in RPMI with 20% FCS for 2 weeks and in RPMI 8% FCS thereafter.

2.4 Intracellular cytokine staining following staphylococcus enterotoxin B (SEB) or LCL stimulation or EBV peptide stimulation

a. *Cell stimulation*

Approximately $1x10^6$ (SEB stimulation) or $2x10^6$ (LCL and peptide stimulations) $CD8^+$ depleted T cells were placed into tubes for each experimental condition. Tubes for unstained control and appropriate compensation tubes were also prepared. Cells were either, stimulated with the superantigen SEB at a final concentration of 0.2µg/mL, or incubated with autologous LCLs at a ratio of 1:1, or incubated with EBV peptides (Alta Bioscience, see Table 2 for sequences) at concentrations ranging from 5µg/mL to 0.005µg/mL, or left unstimulated and placed at 37°C, 5% CO_2. All cells were gently resuspended every 15 mins for the first hour, after which Brefeldin A (BFA) (final concentration 0.2µg/mL) was added and the tubes placed back at 37°C overnight.

b. Viability and Surface Staining

The following day cells were washed in PBS using a standard centrifugation (2000rpm, 5mins) and stained with a 1:100 dilution of live/dead fixable aqua cell stain kit (Invitrogen) for 20 minutes in the dark at room temperature. After washing away excess stain with PBS, cells were stained for surface markers using various combinations, as described in the results for each experiment, of pre-determined saturating concentrations of the following antibodies: CD3 (AmCyan –conjugated, clone SK7, BD), CD4 (ECD-conjugated, clone SFCI12T4D11, Beckman Coulter), CD14 (clone HCD14) and CD19 (clone HIB19) (both Pacific Blue-conjugated, both Biolegend) on ice for 20 minutes.

c. ICS for cytokines and transcription factor expression

After a wash with PBS, cells were fixed by incubation with Fixation concentrate (Foxp3 / Transcription Factor Staining Buffer Set, eBioscience) for 30 mins on ice and subsequently permeabilized by performing two washes with Permeabilization buffer (same kit). Relevant

volumes and combinations of the following antibodies were added to the experimental tubes (Table 1) which were left at room temperature for 30 mins : IFNγ (FITC- labelled, clone 4S.B3), IFNγ (Alex Fluor 700-labelles, same clone) TNFα (PE-Cy7, clone MAb11), IL-10 (PE-Cy7, clone JES3-9D7), IL-2 (Alexa Fluor 700, clone MQ1-17H12), IL-2 (PerCP-Cy5.5-labelled, same clone) IL-21 (APC, clone 3A3-N2), GM-CSF (PerCP-Cy5.5, clone BVD2-21C11) (all Biolegend), IL-4 (PE-Cy7, clone 8D4-8), T-bet (eFluor 660, clone 4B10), FoxP3 (APC, clone 236A/E7) (all eBioscience), GATA3 (Alex Fluor 488, clone L50-823, BD).

Cells were analysed on an LSRII flow cytometer (Beckman Coulter) and data was processed using FlowJo software (Treestar, OR, USA).

2.5 *EBV MHC class II tetramer staining*

MHC class II tetramers representing the HLA allele – EBV epitope combination shown in Table 2. (manufactured by "The tetramer core facility of the Benaroya Research Institute", Seattle, WA) were tested against appropriate donor PBMCs based on their HLA-DR type. To perform the staining, CD8 depleted PBMCs were washed in human serum followed by addition of relevant PE labelled tetramers, or no tetramer for control tubes, and were incubated for 1 hour at 37°C with gentle resuspension every 15 mins. Vital dye and surface staining were performed as described in section 3, and cells were fixed in 4% paraformaldehyde before analysis on the LSR II.

2.6 Combination of MHC II Tetramer and intracellular cytokine staining following peptide stimulation

CD8 depleted PBMCs from DR7- or DR52b-positive donors were stimulated with 0.005µg/mL of the PRS peptide for 4 hours, with addition of BFA after the first hour of incubation. Following one wash in human serum, MHC II tetramer staining was conducted as detailed in section 5, followed by viability, surface and ICS for cytokines and transcription factors as

described in section 4. Cells were analysed on an LSRII flow cytometer (Beckman Coulter) and data was processed using FlowJo software (Treestar, OR, USA).

2.7 EBV specific CD4$^+$ T cell phenotyping

Following MHC class II tetramer and vital dye staining, CD8 depleted PBMCs were surface stained using saturating concentrations of CD3, CD4, CD14, CD19 (same as above), CD45RA (Alexa Fluor 700 labelled, clone HI100, Biolegend) and CCR7 (Brilliant Violet 650 labelled, clone 3D12, BD) on ice for 20 mins. Subsequent to a wash with PBS, cells were fixed by incubation with 2% PFA for 20 minutes at room temperature. Following successive washes with PBS/2% BSA and saponin buffer (PBS, 0.5% FCS, 0.5% w/v saponin), cells were left to permeabilize for 20 minutes at room temperature. Experiment tubes were then incubated with Perforin (PE-Cy7 labelled, clone dG9, ebioscience) and Granzyme-B (Alexa Fluor 647 labelled, clone GB11, Biolegend) for 30 minutes at room temperature. Finally, cells were washed with PBS/2 % BSA and resuspended in 2% PFA before analysis on LSRII.

Table 1. MHC II tetramer reagents

Virus phase	Protein	Coordinates	Epitope	MHC II restriction
Latent	EBNA1 (E1)	474-493	SNPKFENIAEGLRVLLARSH	DRB5*01:01 (DR51)
Latent	EBNA2 (E2)	276-295	PRSPTVFYNIPPMPLPPSQL	DRB1*07:01 (DR7)
Latent	EBNA2 (E2)	276-295	PRSPTVFYNIPPMPLPPSQL	DRB3*02:02 (DR52b)
Latent	EBNA2 (E2)	301-320	PAQPPPGVINDQQLHHLPSG	DRB1*03:01 (DR17)
Lytic	BMRF1	136-150	VKLTMEYDDKVSKSH	DRB1*03:01 (DR17)

3. RESULTS

3.1 Optimisation of antibody panels for CD4$^+$ T cell phenotype and function identification

To enable characterization of the phenotype and functional capacity of EBV specific CD4$^+$ T cells in the blood of healthy seropositive donors, several antibody panels were set up. Using these panels the aim was to assess expression of specific transcription factors associated with various CD4$^+$ T cell subsets within the EBV-specific populations, and their production of a range of cytokines when challenged with antigen. Additionally we also wished to measure levels of proteins associated with cytotoxic function and correlate those with memory status of the cells by assessing expression of phenotypic markers on the cell surface of virus specific CD4$^+$ T cells.

In order to achieve these aims, initial experiments to optimise the concentrations of antibodies present in these panels, that had not previously been used in the laboratory, were carried out. All new antibodies were tested at a number of different concentrations, guided by the manufacturer's recommended dilution, on CD8$^+$ depleted PBMCs of healthy donors. All tests were performed alongside surface staining of CD3 AmCyan, CD4 ECD, CD14 and CD19 Pacific Blue (B cell and macrophage surface markers respectively) as well as vital dye (also on Pacific Blue) to allow the analysis of experimental antibodies binding to viable CD3$^+$ CD4$^+$ cells.

An example of the gating strategy for viable CD3$^+$ CD4$^+$ cells is shown in Fig 3.1.a. Firstly, lymphocytes were selected using the forward and side scatter (FSC and SSC) plot. Within this population, viable, CD14$^-$ CD19$^-$ cells negative for Pacific Blue staining were isolated (middle plot) and CD3$^+$ CD4$^+$ cells within this gate were selected for subsequent analysis (right plot).

Unless stated otherwise, this gating strategy applied throughout this study and the selected $CD3^+$ $CD4^+$ double positive population was further investigated for the characteristics of interest in each experiment as described.

Firstly, surface antibodies to be used for phenotyping and compensation that had not previously been used in the laboratory were titrated in order to determine optimal working concentrations based on clear defined populations of positive cells and limited background staining of negative cell populations. As shown in Fig 3.1.b, the mean fluorescence intensities (MFI) of cells positive for CD4 when stained with a Brilliant Violet 650 labelled antibody (left plot), do not noticeably change between the manufacturers recommended dilution of 1:10 and 1:50. Therefore the latter was chosen as the most appropriate for these experiments as the antibody is still in excess at this dilution. Staining with CCR7 (also with a Brilliant Violet 650 conjugated antibody, middle plot) and CD127 with a PerCP-Cy5.5 labelled antibody (left plot) show different MFI of staining based on the concentration used. From the respective histograms it was determined that dilutions of 1:20 for CCR7 and 1:20 for CD127 be used for this study, as clear populations of cells could be distinguished. An analogous approach was used for all other previously un-validated surface antibodies.

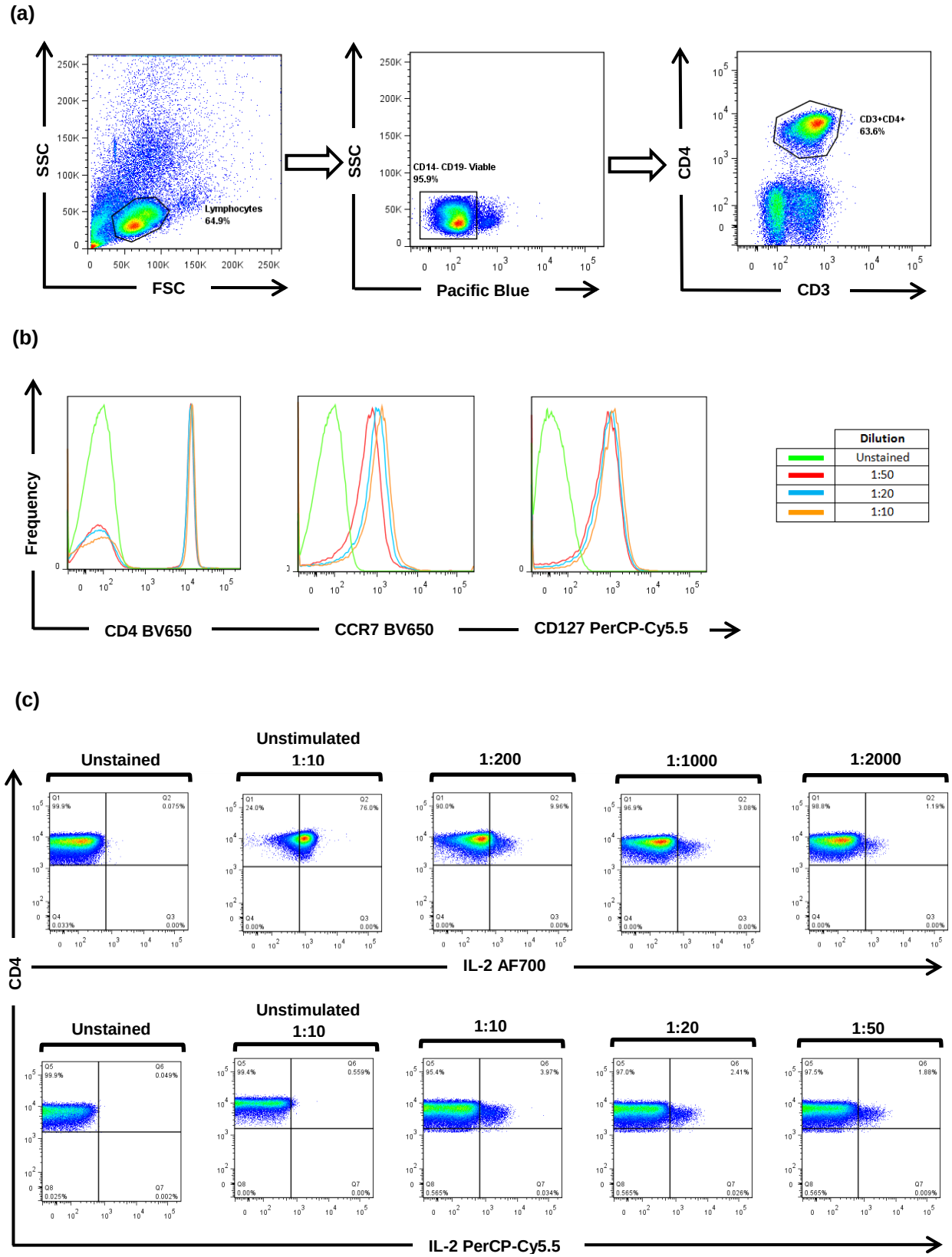
Optimisation of antibodies to be used for intracellular cytokine staining was carried out by stimulating $CD8^+$ depleted PBMCs from healthy donors with the superantigen SEB and incubating in the presence of Brefeldin A to inhibit protein transport to the Golgi apparatus. Following viability and surface staining with CD3, CD4, CD14 and CD19; cells were fixed and permeabilised as described in the methods. Different concentrations, using the manufacturers' recommendations as a guide as for titration of surface anitbodies, were tested on all new antibodies and two representative results are shown in Fig 3.1.c. Gating was set on the unstained control in order to assess whether background staining at the varying concentrations caused a shift in the MFI of the entire population. Unstimulated controls were stained with the manufacturers' recommended concentration which was the

lowest dilution in each case. Where possible, isotype control antibodies were used to confirm no non-specific binding of antibodies (data not shown).

The results of titrating the Alexa Fluor 700 labelled IL-2 antibody (top plots) demonstrate that using the recommended dilution of 1:10 and even 1:200 dilution, lead to a shift of the MFI of the negative population, which would result in difficulty in determining positive populations. However, further dilutions of 1:1000 and 1:2000 were tested and the former was chosen as the optimal dilution for our studies, as the positive population could be clearly distinguished without a shift in the total cell MFI. Conversely, staining with the recommended 1:10 dilution with the PerCP-Cy5.5 labelled IL-2 antibody (bottom plots) allowed clear visualisation of IL-2 positive cells, with negative cells showing similar MFI to the unstained populations. In addition titrating the antibody down to 1:20 and 1:50 dilutions resulted in lower numbers of IL-2^+ cells being detected indicating the antibody was no longer in excess, and positive cells could be missed

Fig 3.1.d summarises the finalised antibody panels used, with the dilutions used for each antibody based on a reaction volume of 50µL. All antibodies optimised during this study are identified with an asterisk.

FIGURE3.1


(a)
SSC
FSC
Lymphocytes
64.9%
SSC
Pacific Blue
CD14- CD19- Viable
95.9%
CD4
CD3
CD3+CD4+
63.6%
(b)
Frequency
CD4 BV650
CCR7 BV650
CD127 PerCP-Cy5.5
Dilution
Unstained
1:50
1:20
1:10
(c)
Unstained
Unstimulated
1:10
1:200
1:1000
1:2000
CD4
IL-2 AF700
Unstained
Unstimulated
1:10
1:10
1:20
1:50
IL-2 PerCP-Cy5.5

Panel	Antigen	Fluorochrome	Dilution
Surface markers	CD3	AmCyan	1:20
	CD4	ECD	1:50
	CD14	Pacific Blue	1:25
	CD19	Pacific Blue	1:20

Panel	Antigen	Fluorochrome	Dilution
Phenotype	CD45RA	AF700	1:50
	CCR7	BV650	1:20*
Cytotoxicity	Perforin	PE-Cy7	1:25
	Granzyme B	AF647 (APC)	1:25

Panel	Antigen	Fluorochrome	Dilution
T_h1	T-bet	eFluor660 (APC)	1:25
	IFNγ	FITC	1:25
	IL-2	AF700	1:1000*
	TNFα	PE-Cy7	1:100*
	GM-CSF	PerCP-Cy5.5	1:25*
T_h2	GATA3	AF488 (FITC)	1:2.5
	IL-4	PE-Cy7	1:50*
	IL-2	AF700 or PerCP-Cy5.5	1:1000 or 1:10*
	GM-CSF	PerCP-Cy5.5	1:25*
	IL-21	APC	1:50
	IFNγ	AF700	1:200
T_{reg}	CD25	AF700	1:100*
	CD127	PerCP-Cy5.5	1:10*
	FoxP3	APC	1:20
	IL-10	PE-Cy7	1:25
	IFNγ	FITC	1:25

Figure 3.1 **Gating strategy and antibody titration**

(a) Gating strategy for all flow cytometry analysis. CD8 depleted PBMCs were gated on lymphocytes (based on forward and side scatter), distinguished for negative staining for CD14, CD19 and viability dye on Pacific Blue (dump channel). Surface and intracellular staining of $CD4^+$ T cells was analysed by gating on double positive CD3 and CD4 cells. **(b)** Histograms representing MFI of PBMCs stained with increasing dilutions of either: CD4 BV650 (right hand side), CCR7 BV650 (middle) or CD127 PerCP-Cy5.5 (left hand side). **(c)** Intracellular cytokine staining of PBMCs with decreasing concentrations of IL-2 AF700 (top) and IL-2 PerCP-Cy5.5 (bottom). **(d)** Final subsets and optimised dilutions for all panels. * indicates antibodies titrated during the project.

3.2 Lineage and cytokine profile of EBV specific CD4$^+$ T cells

It is now accepted that CD4$^+$ T cells can directly recognise and function in response to MHC class II positive target cells expressing their cognate antigen. Studies looking at the ability of CD4$^+$ T cells to respond to EBV infected cells *ex vivo* have previously focused on the quantification of one or two cytokines (principally IFNγ and IL-4) yet recent studies suggest that CD4$^+$ T cell responses to viral infections are heterogeneous and contain polyfunctional T cells. To further investigate the profile of CD4$^+$ T cells responding to EBV infected cells, CD8$^+$ depleted PBMCs from six EBV seropositive donors were stimulated with their autologous EBV-transformed lymphoblastoid cell lines (LCLs) overnight in the presence of Brefeldin A. Three of the donor LCLs were not readily available and were therefore generated during this study by infecting donor PBMCs with supernatant from B95.8 virus producer line in the presence of cyclosporin A to abrogate the T cell response. Following the overnight incubation with LCL, viability and surface staining for CD3, CD4, CD14 and CD19 was performed as described earlier. Cells were subsequently fixed and permeabilised to enable intracellular staining for T_h1, T_h2 and T_{reg} T cell subset associated transcription factors and cytokines as described in Fig 3.1.d

For this series of experiments, following gating on lymphocytes and viable CD14$^-$ CD19$^-$ cells, PBMCs were gated on CD3$^+$ rather than CD3$^+$ CD4$^+$ as shown in Fig 3.2.a. This is because expression of CD4 can be down regulated following T cell activation and therefore using the strategy shown in Fig 3.1.a (right plot) might lead to responding cells being lost from the analysis.

In order to study the response of T_h1 type CD4$^+$ lymphocytes, following the surface staining described above, cells were stained for the transcription factor T-bet along with the cytokines IFNγ, TNFα, IL-2 and GM-CSF. Analysis of these cells was performed by gating on the CD4$^+$ T-bet$^+$ cells shown in Fig 3.2.b (top plot, top right quadrant Q2). As can be seen from this

plot, the vast majority of $CD4^+$ T cells in the peripheral blood are positive for T-bet and approximately 80% of the $CD4^+$ enriched PBMC population shown here are $CD4^+$ T cells expressing the T_h1 associated transcription factor. Subsequently the percentage of LCL stimulated cells producing T_h1 like cytokines within the total $CD4^+$ population (left hand plots) and T-bet^+ $CD4^+$ population (right hand plots) were analysed.

Low background frequencies of TNFα and GM-CSF producing cells were observed in the unstimulated populations of both the total CD4 lymphocytes and T-bet^+ gated cells. Similar low numbers of IFNγ and IL-2 producing cells were also observed in the total $CD4^+$ population but these did not appear to be amongst the T-bet^+ population.

LCL stimulation resulted in the total frequency of IFNγ producing cells increasing from 0.095% to 0.480% in the total $CD4^+$ population and from 0.050% to 0.045% in the T-bet^+ population. Similarly, the percentage of cells producing TNFα increased from 0.579% to 0.791% and from 0.570% to 0.741% for the total $CD4^+$ and T-bet^+ populations respectively. However, while the percentage of cells producing IFNγ alone increased by approximately 0.1%, the frequency of cells producing just TNFα decreased slightly and the vast majority of cells responding to the presence of LCLs produced both IFNγ and TNFα. This population was not observed in the unstimulated populations but accounts for 0.278% of total $CD4^+$ cells and 0.268% of T-bet^+ LCL stimulated cells. Interestingly percentages of cells producing IL-2 remained unchanged between the unstimulated and stimulated cells in both the total $CD4^+$ population and the T-bet^+ population and no production of GM-CSF was detected. These results show that $CD4^+$ cells activated by EBV infected cells, which express the T_h1 associated transcription factor secrete IFNγ alone or a combination of IFNγ and TNFα.

Control of viral infections is most commonly associated with the T_h1 subset of $CD4^+$ T due to the secretion of its associated anti-viral cytokine IFNγ, however studies have suggested that some T cells responding to LCLs are capable of releasing the T_h2 associated cytokine IL-4.

Thus, in the second panel, cells were stained for the T_h2 associated transcription factor GATA3 and associated cytokines IL-4 and IL-21. Levels of IFNγ and IL-2 were also assessed in this panel for comparison purposes. The frequency of T_h2 $CD4^+$ lymphocytes within the total $CD4^+$ T cell enriched population was analysed by staining cells for the transcription factor GATA3, a representative example of which is shown in Fig 3.2.c. In this donor the percentage of peripheral cells expressing GATA3 was 2.02%. As a result, the cytokine profiles of fewer cells could be analysed compared to the T_h1 subset, however in a parallel way to the previous panel, the unstimulated and LCL stimulated cells of the $CD4^+$ $GATA3^+$ population (right plots) was compared to the total $CD4^+$ population (left plots). In the representative example shown here, the frequency of IL-4 producing cells in the unstimulated total $CD4^+$ population was 2.59% but noticeably this was only enriched to 4.99% when the $GATA3^+$ $CD4^+$ T cells were were analysed. Interestingly, following stimulation the frequency of IL-4 producing cells did not increase either in the total $CD4^+$ T cell population, or in the $GATA3^+$ cells. Similarly, the GATA3 positive cells did not produce IL-21 or IL-2 upon stimulation. Interestingly, the percentage of IFNγ producing cells in the total $CD4^+$ population increases from 0.03% to 0.33% after stimulation, confirming the result seen in Fig 3.2.b. Notably, none of the $GATA3^+$ $CD4^+$ T cells produced IFNγ in response to stimulation demonstrating that production of this cytokine does not occur when the T_h2 transcription factor is expressed. Overall, these results suggest that the GATA3 positive T_h2 subset is not activated by exposure to EBV infected cells.

The final panel was set up in order to establish whether any $CD4^+$ T cells amongst the population activated by autologous LCLs possessed the characteristics of a regulatory T cell. To achieve this, cells were stained for the T_{reg} associated transcription factor FoxP3 and for the immunosuppressive cytokine IL-10. Again, IFNγ production was also measured for comparison. In order to look at the response of T_{regs} to EBV infected PBMCs, we gated on

$CD4^+$ cells positive for the transcription factor FoxP3, as shown in Fig 3.2.d (top plot). T lymphocytes expressing the FoxP3 transcription factor in the donor shown represented 3.7% of the total circulating population, in line with published normal ranges (appropriate ref). The cytokine profile of the resulting $FoxP3^+$ $CD4^+$ cells (shown in the right hand plots) would be compared to that of the total $CD4^+$ population (left hand plots). A low percentage of unstimulated cells in the total $CD4^+$ population produced IL-10 and IFNγ but this did not appear to be directly associated with expression of FoxP3. Following LCL stimulation, the percentage of cell producing IL-10 did not change dramatically in either the total $CD4^+$ population or the $FoxP3^+$ $CD4^+$ populations. However, in line with the data presented for the T_h1 and T_h2 panels (shown in Fig 3.2.b and 3.2.c) the percentage of IFNγ producing cells increased in the total $CD4^+$ population from 0.07% to 0.41%. Perhaps more striking was that LCL stimulation provoked a response in the $FoxP3^+$ population. Thus, 0.86% of cells positive for the T_{reg} associated transcription factor appeared to produce IFNγ in reponse to LCL stimulation, a cytokine whose expression has not routinely been linked with this type of regulatory T cell. The results obtained by staining with this third panel of antibodies show that a small percentage of FoxP3 expressing $CD4^+$ T cells may be able to respond to autologous LCL stimulation by production of IFNγ and not of the more commonly T_{reg} associated cytokine IL-10.

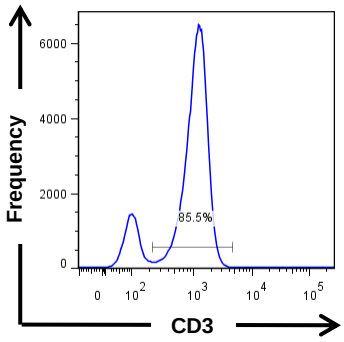
As mentioned previously, analysis of cytokine production by $CD4^+$ T cells activated by EBV infected cells has mainly been measured through the secretion of IFNγ. This cytokine is associated with anti-viral responses and is known to stimulate cytotoxic $CD8^+$ T cells and phagocytic macrophages. The anti-IFNγ antibody was included in all three of the panels tested, acting as an internal control and production of IFNγ was always observed in the total $CD4^+$ population after incubation with LCLs (Fig 3.2.b, 3.2.c and 3.2.d). In order to confirm the transcription factors expressed in the $CD4^+$ T cells that were producing IFNγ following

LCL stimulation, in the final analyses the gating strategy was reversed. Thus, expression of T-bet, GATA3 and FoxP3 was analysed in $CD4^+$ T cells producing IFNγ. Fig 3.2.e shows a representation of the IFNγ producing cells between unstimulated and LCL stimulated conditions (top). The frequencies of T-bet, GATA3 or FoxP3 positive cells within the IFNγ-producing cells was subsequently assessed (bottom). The staining shows that the vast majority (over 80% in all donors) of IFNγ producing cells are of a T_h1 lineage, expressing T-bet. The T_h2 GATA3 subset does not produce IFNγ and a small proportion of cytokine secreting cells express FoxP3. The latter result confirming the detection of IFNγ observed when gating on $FoxP3^+$ cells (Fig 3.2.d).

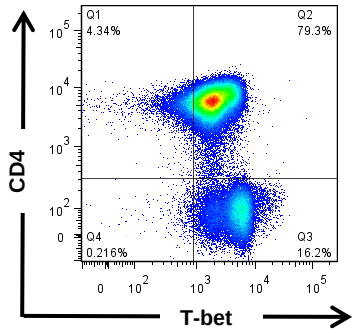
In summary, this data demonstrate that $CD4^+$ T cells can directly respond to EBV infected LCLs *ex vivo* by producing effector cytokines. Furthermore, the vast majority of responding $CD4^+$ T cells are of a T_h1-like phenotype, expressing T-bet and producing IFNγ and TNFα, with a minority population of cells expressing the regulatory T cell associated transcription factor FoxP3.

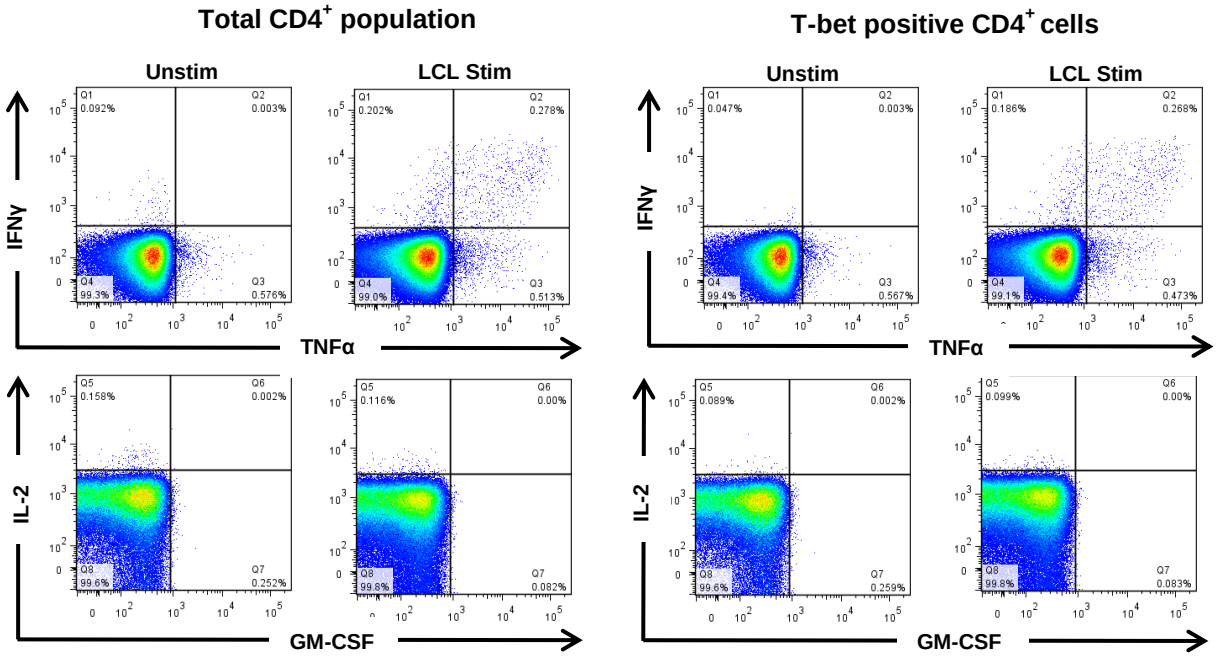
FIGURE 3.2

(a)


Frequency
CD3
85.5%

(b)


CD4
T-bet
Q1 4.34%
Q2 79.3%
Q4 0.216%
Q3 16.2%


Total CD4+ population
T-bet positive CD4+ cells
Unstim
LCL Stim
IFNγ
TNFα
IL-2
GM-CSF

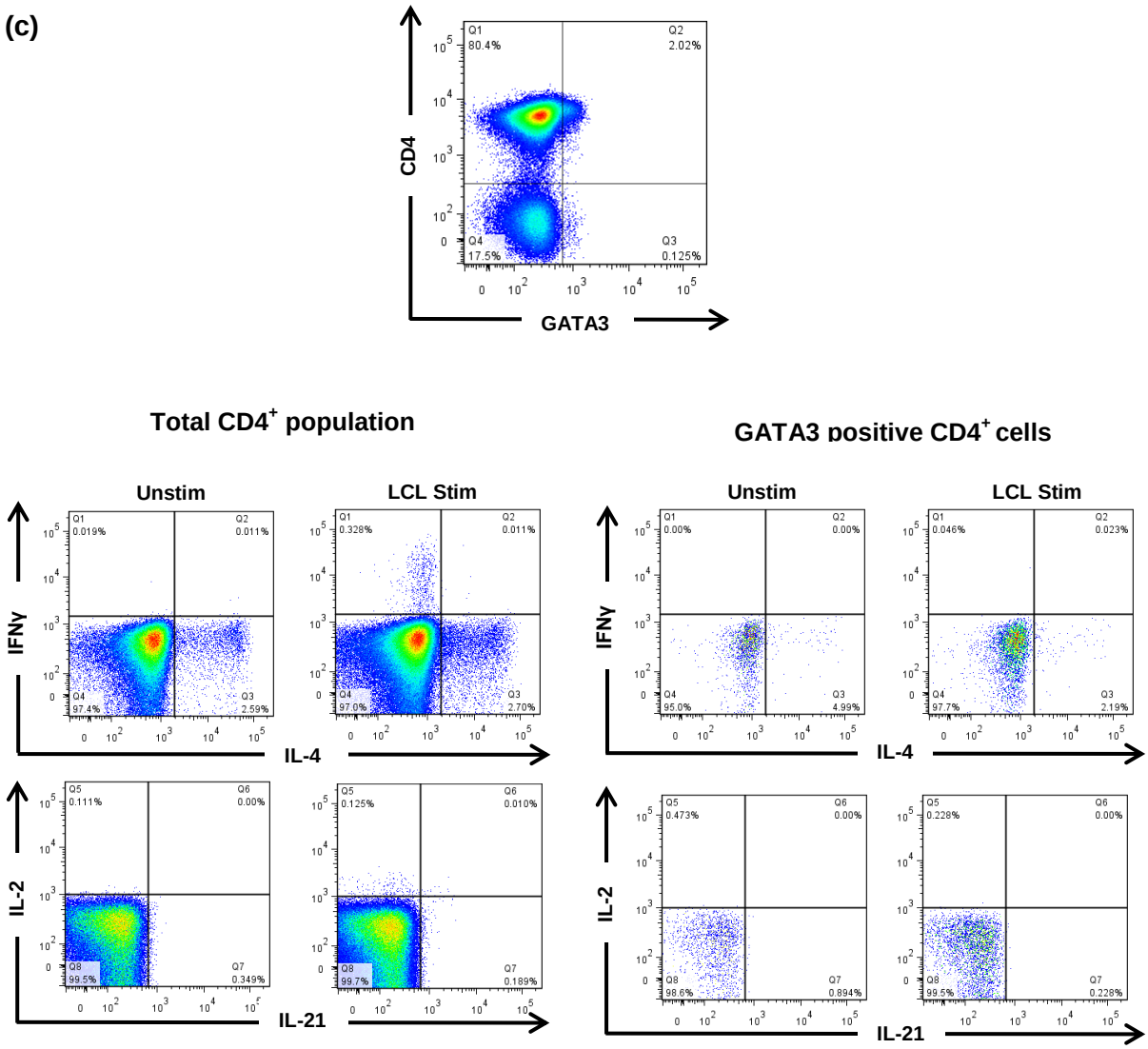
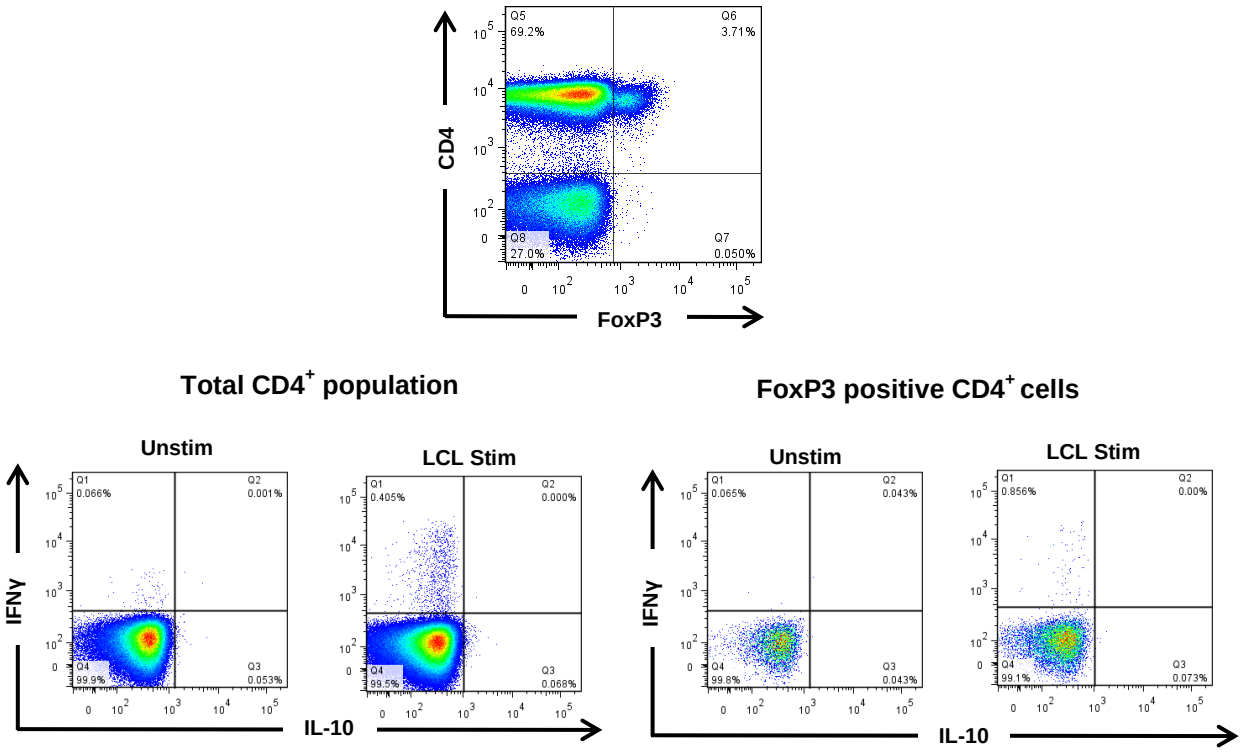


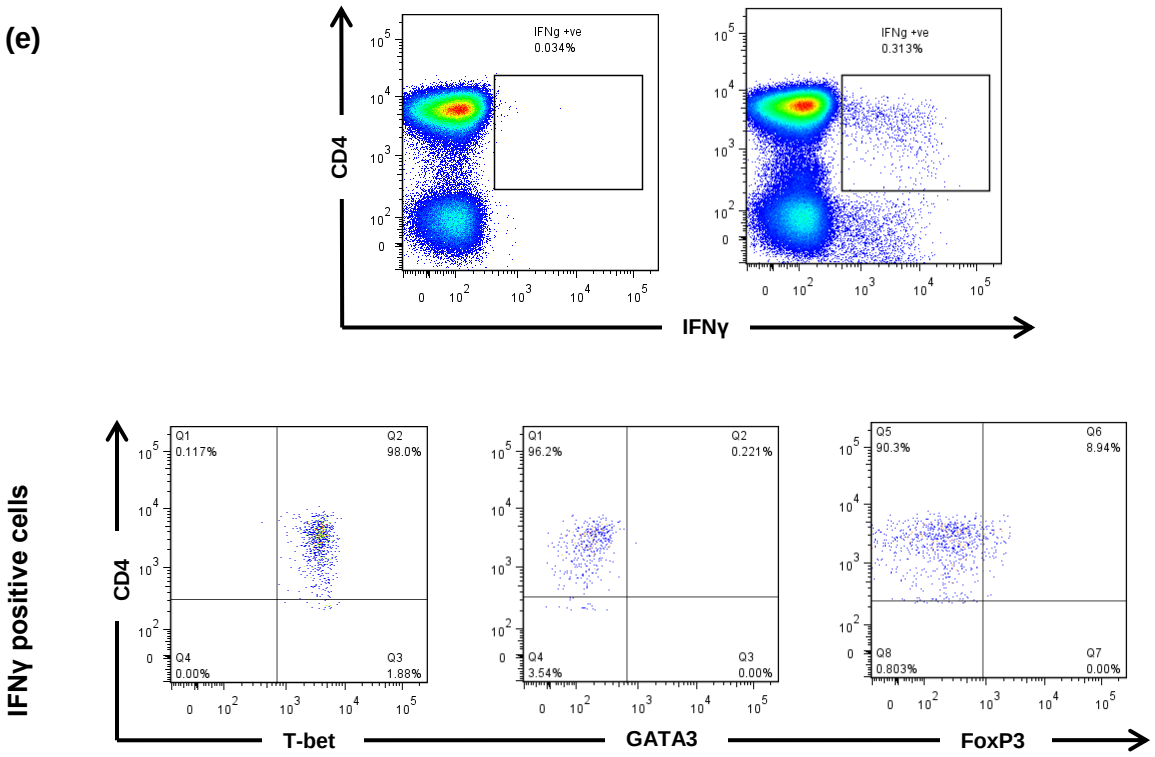
Figure 3.2 **CD4$^+$ T cell response to autologous LCL stimulation**

(a) Analysis of T cells responding to LCL stimulus done by initially gating on CD3$^+$ T cells to take into account downregulation of CD4 following activation. Expression of T_h1 **(b)**, T_h2 **(c)** and T_{reg} **(d)** associated transcription factors and cytokines analysed in total CD4$^+$ population and transcription factor positive CD4$^+$ T cells. For each panel, the top plot shows the expression of transcription factor in the total CD4$^+$ population. Production of cytokines in the total CD4$^+$ T cell population (left hand side) and transcription factor expressing CD4$^+$ T cells (right hand side) in unstimulated (left plots) and LCL stimulated (right plots) are shown. **(e)** Expression of T-bet (left hand), GATA3 (middle) and FoxP3 (right hand) shown in CD4$^+$ T cells producing IFNγ following LCL stimulation.

(d)


Q5
69.2%
Q6
3.71%
Q8
27.0%
Q7
0.050%
CD4
FoxP3
Total CD4+ population
FoxP3 positive CD4+ cells
Unstim
LCL Stim
Unstim
LCL Stim
Q1
0.066%
Q2
0.001%
Q4
99.9%
Q3
0.053%
Q1
0.405%
Q2
0.000%
Q4
99.5%
Q3
0.068%
Q1
0.065%
Q2
0.043%
Q4
99.8%
Q3
0.043%
Q1
0.856%
Q2
0.00%
Q4
99.1%
Q3
0.073%
IFNγ
IL-10
IFNγ
IL-10

(e)


IFNg +ve
0.034%
IFNg +ve
0.313%
CD4
IFNγ
IFNγ positive cells
Q1
0.117%
Q2
98.0%
Q4
0.00%
Q3
1.88%
Q1
96.2%
Q2
0.221%
Q4
3.54%
Q3
0.00%
Q5
90.3%
Q6
8.94%
Q8
0.803%
Q7
0.00%
CD4
T-bet
GATA3
FoxP3

3.3 Frequency of CD4$^+$ EBV epitope MHC class II tetramer responses in healthy seropositive individuals

Having identified the profile of transcription factors and cytokine expression in CD4$^+$ T cells responding to EBV infected cells, we wanted to compare the response of CD4$^+$ cells specific for individual epitopes derived from latent and lytic EBV proteins. These experiments used novel EBV-peptide:MHC class II tetramer reagents, to analyse the functional and phenotypical characteristics of EBV specific T cells at the epitope level for the first time. A previous study has demonstrated the specificity and reproducibilityof these novel MHC class II reagents (Long 2013), but also that CD4$^+$ T cells specific for a given EBV epitope can be present at very low frequencies. In order to analyse the highest number of CD4$^+$ T cells specific for lytic and latent epitopes, donors with the largest response needed to be identified.

The five MHC class II tetramers used in this work are listed in Table 1 and each represent a different MHC class II allele-EBV epitope combination. The first tetramer tested contained an EBNA1 epitope (SNP) restricted to the DRB5*01:01 (DR51) allele. Two tetramers were directed against the same PRS epitope derived from the latent protein EBNA2 but presented through two distinct MHC II restricting alleles: DRB1*07:01 (DR7) and DRB3*02:02 (DR52b). Finally, two DRB1*03:01 (DR17) restricted tetramers against an EBNA2 derived epitope (PAQ) and an epitope the lytic protein BMRF1 (VKL) were also tested.

CD8$^+$ depleted PBMCs from donors expressing one or more of the HLA-DR alleles of interest were stained with relevant tetramer followed by viability and surface staining for CD3, CD4, CD14 and CD19. Gating was done on CD3$^+$ CD4$^+$ cells as shown in Fig 3.1.a followed by assessment of tetramer-PE positive cells within the CD3$^+$ CD4$^+$ population.

Representative responses from donor PBMCs stained with for each EBV peptide:MHC II tetramer are shown in Fig 3.3.a. PBMCs stained with surface antibodies and no tetramer are shown as controls (left hand plots) alongside responses following incubation with tetramer

(right hand plots). No background levels of PE positive cells in the absence of the tetramer were detectable for all five EBV epitopes. Amongst the results shown, the response to the SNP DR51 restricted tetramer was the highest 0.128%, while percentages of PRS^+ cells be it DR7 or DR52b restricted were similar in the two donors shown at 0.041 and 0.043% respectively. The third EBV EBNA2 derived tetramer, PAQ, however gave the lowest response of 0.023%. The frequency of $CD4^+$ T cells specific for the epitope derived from the lytic protein BMRF1 was 0.041%.

A summary of the frequencies of epitope-specific T cells obtained by MHC class II tetramer staining across all donors tested is shown in Fig 3.3.b.

Frequencies of SNP-specific cells reached the highest values of the epitopes tested here but also gave the most diverse response ranging from 0.128% to 0.054% between 4 donors.

The response to the PRS tetramers was broadly similar across the DR7 and DR52b restricted tetramers and was seen to be just below 0.05% for all except one donor. $CD4^+$ T cells specific for the other EBNA2 epitope PAQ were lower ranging from 0.07% to 0.26%. Finally, the response to the VKL tetramer was comparable between the three donors ranging from 0.041 to 0.058%. Although these responses are low, they are based on analysis of up to 1×10^6 cells and were reproducible in further experiments.

The positive results obtained for EBV peptide:MHC II tetramers across all donors show that in circulating cells, $CD4^+$ T lymphocytes specific for both lytic and latent derived epitopes are present.

FIGURE 3.3

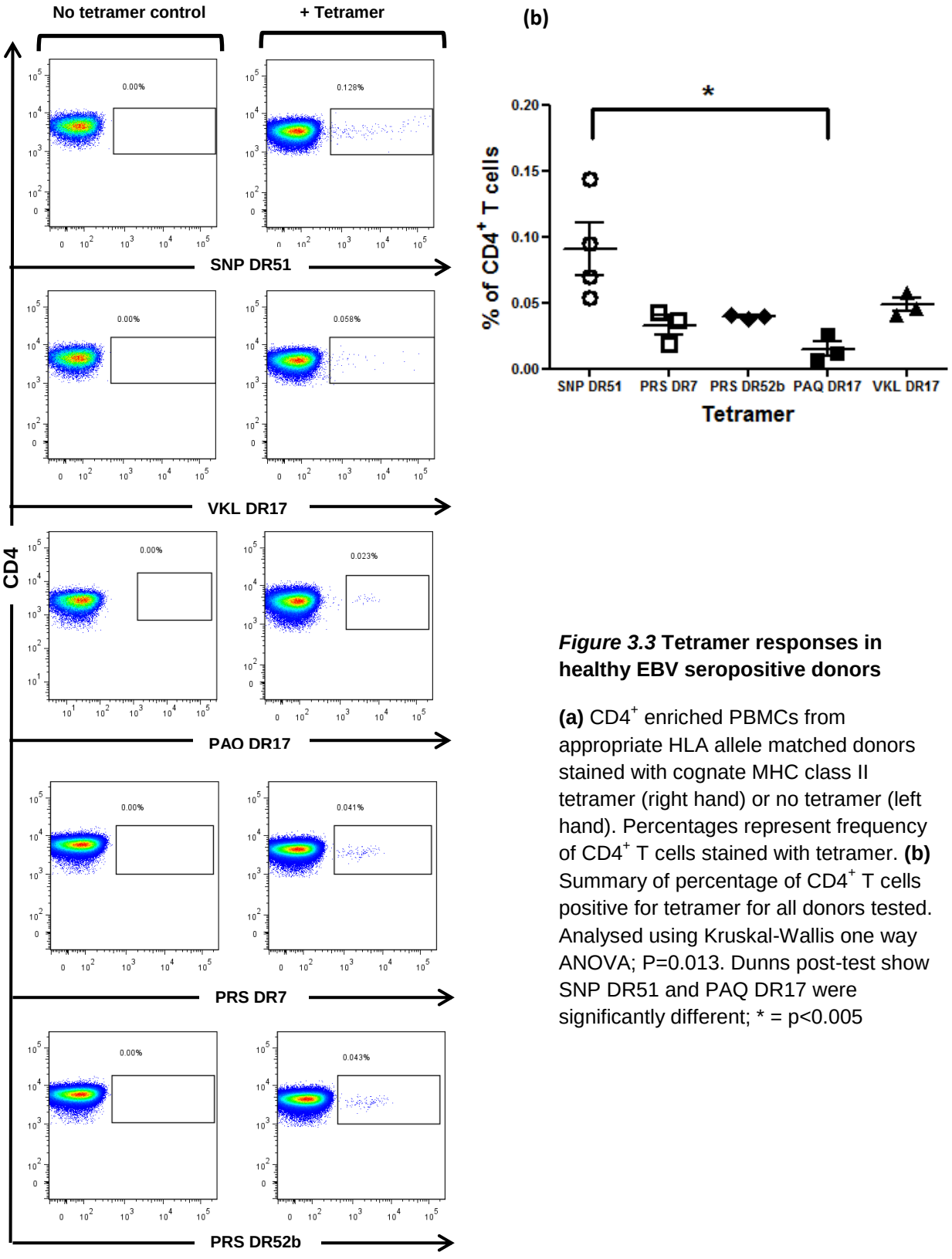


Figure 3.3 **Tetramer responses in healthy EBV seropositive donors**

(a) $CD4^+$ enriched PBMCs from appropriate HLA allele matched donors stained with cognate MHC class II tetramer (right hand) or no tetramer (left hand). Percentages represent frequency of $CD4^+$ T cells stained with tetramer. **(b)** Summary of percentage of $CD4^+$ T cells positive for tetramer for all donors tested. Analysed using Kruskal-Wallis one way ANOVA; P=0.013. Dunns post-test show SNP DR51 and PAQ DR17 were significantly different; * = p<0.005

3.4 Lineage and cytokine profile of EBV epitope specific CD4$^+$T cells in healthy seropositive individuals

Having identified donors with the largest frequency responses to EBV epitopes, we then wanted to investigate the functional characteristics of epitope specific CD4$^+$ T cells *ex vivo* using MHC class II tetramers. This required the combination of stimulation with an EBV peptide and staining with the peptide specific MHC class II tetramer. This is a technically challenging experiment as the target for the both the peptide and tetramer will be the T cell receptor. Thus TCR down-regulation following peptide stimulation may prevent tetramer binding. The stimulation protocol used for this study had previously been optimised in the laboratory to measure IFNγ release from PRS (EBNA2) specific T cells. Therefore, initial experiments to analyse the full functional profile of EBV epitope-specific CD4$^+$ T cells focused on the PRS epitope, restricted by the DR7 and DR52b HLA-DR alleles.

CD4$^+$ enriched PBMCs from donors chosen for their high frequency of PRS specific CD4+ T cells were stimulated with PRS peptide (sequence in table 2.) in the presence of Brefeldin A for four hours. Viability and surface staining for CD3, CD4, CD14 and CD19 was then performed prior to cells being fixed and permeabilised. Intracellular transcription factor and cytokines associated with the T_h1, T_h2 and T_{reg} T cell subsets were stained for using the panels in Table 1 and example results are shown in Fig 3.4.b, c and d.

The total CD3$^+$ CD4$^+$ population was gated on as shown in Fig 3.1.a and cells positive for the PRS tetramer were analysed as shown in Fig 3.4.a. In the first panel, cells were stained for the T_h1 associated transcription factor T-bet along with IFNγ, TNFα, IL-2 and GM-CSF. Fig 3.2.b shows a representation of the results obtained from one example donor. The percentage of T-bet$^+$ cells in the total CD4$^+$ population is 76%, in line with the results in Fig 3.2.b. Interestingly this percentage rises to 88.9% and 82.4% in the unstimulated and

stimulated PRS^+ populations respectively (top plots), indicating a higher proportion of PRS specific T cells express this transcription factor than in the $CD4^+$ population as a whole . Background levels of IFNγ secretion in the unstimulated PRS population were negligible however 49.1% of the tetramer positive cells did produce TNFα (middle plots), possibly due to activation of the cells caused by binding of the tetramer. Stimulation with the PRS peptide provoked an increase in IFNγ producing cells from 0.9% to 44.5% and an increase in the total frequency of TNFα producing cells to 78.7% amongst the epitope specific T cells. Remarkably, the vast majority of IFNγ producing cells were also positive for TNFα, yet 36.1% of cells produced the latter cytokine alone. Interestingly19.4% of PRS tetramer positive cells in this donor produced neither IFNγ nor TNFα upon stimulation. Background levels of both GM-CSF and IL-2 were very low in the unstimulated PRS^+ population and the frequency of cells producing either cytokine did not significantly increase following incubation with the EBNA2 derived peptide (bottom plots).

Fig 3.2.c shows cells from the same experiment stained with antibodies within the second panel, used for T_h2 subset identification. Staining for the associated transcription factor GATA3 (top plot) along with the cytokines IL-4 and IL-21 (bottom plot) were performed. In this donor the $GATA3^+$ cells accounted for 2% of the total $CD4^+$ population but no expression was seen in the PRS tetramer positive populations. No IL-21 production was seen in the unstimulated or PRS stimulated populations however a small increase in frequency of IL-4 producing cells from 2.82% to 7.23% was observed. Nevertheless, due to the small number of cells being analysed here, further analysis is required to confirm this result.

As with the PBMCs stimulated with autologous LCLs, we also wanted to investigate the possibility of the presence of EBV epitope specific regulatory T cells. We therefore stained cells for FoxP3, IL-10 and IFNγ expression before and after PRS stimulation. The percentage

of FoxP3$^+$ cells accounts for 4.41% of the total CD4$^+$ population as shown in Fig 3.4.d (top plots), again in line with the percentage of FoxP3 positive cells sen in total CD4$^+$ population in Fig 3.2.d . The cells positive for the PRS tetramer however did not appear to also be positive for the T_{reg} associated transcription factor. Similarly production of the regulatory cytokine IL-10 was not detected in unstimulated or stimulated populations of PRS tet$^+$ cells (bottom plots). The frequency of IFNγ producing cells did nonetheless increase from 0% to 46.9% following stimulation with the PRS peptide confirming the result observed in Fig 3.4.b.

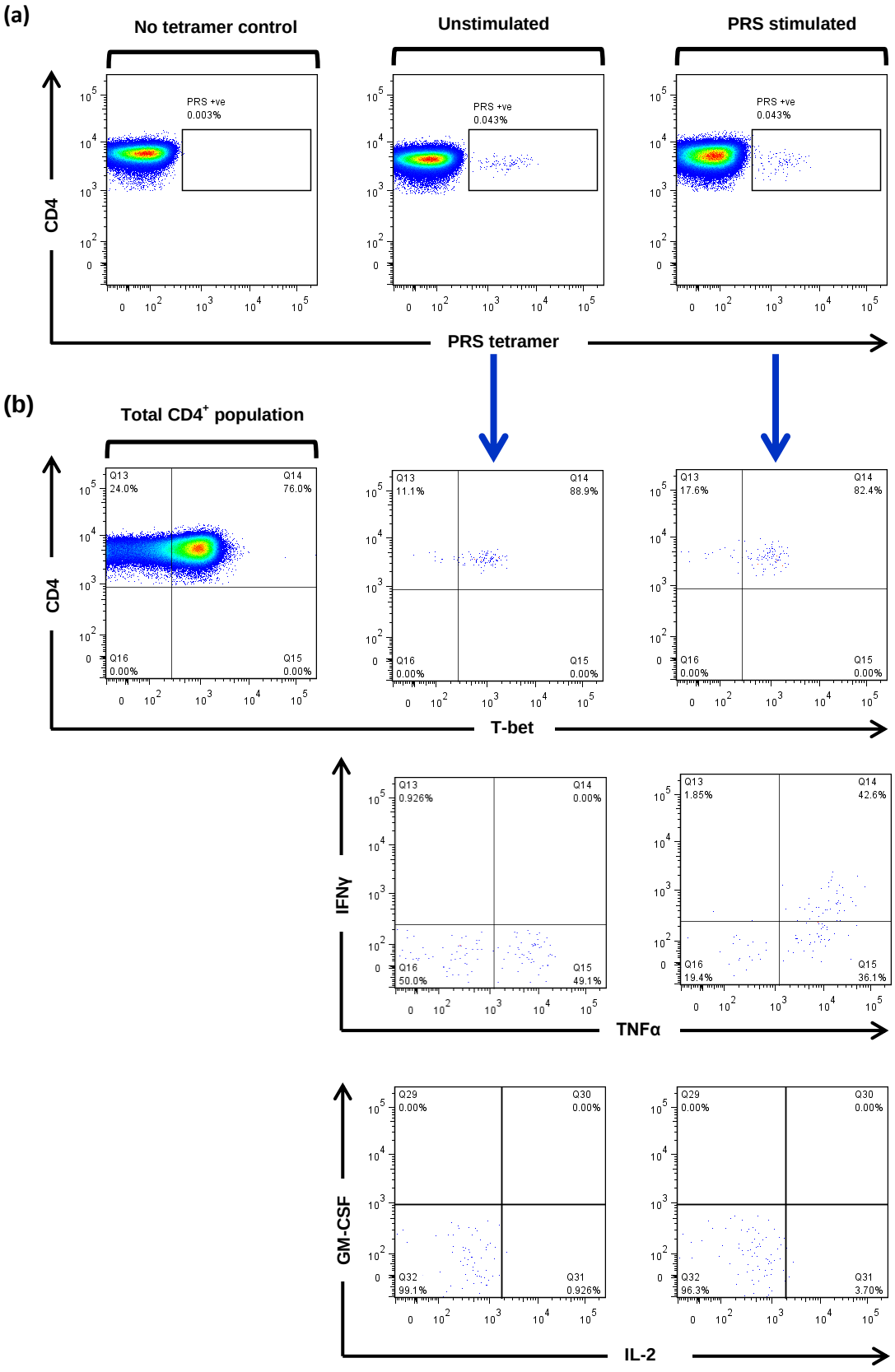
A summary figure of the variations in cytokine expression from the six donors tested is shown in Fig 3.4.e. In all donors, T-bet was the principal transcription factor expressed in the PRS tetramer positive cells, with no GATA3-positive cells and very few FoxP3-positive cells detected. Significant increases between the unstimulated and stimulated populations of PRS specific CD4$^+$ T cells in IFNγ and TNFα expression was seen across all donors. However little or none of the other cytokines appeared to be produced in response to incubation with the PRS peptide.

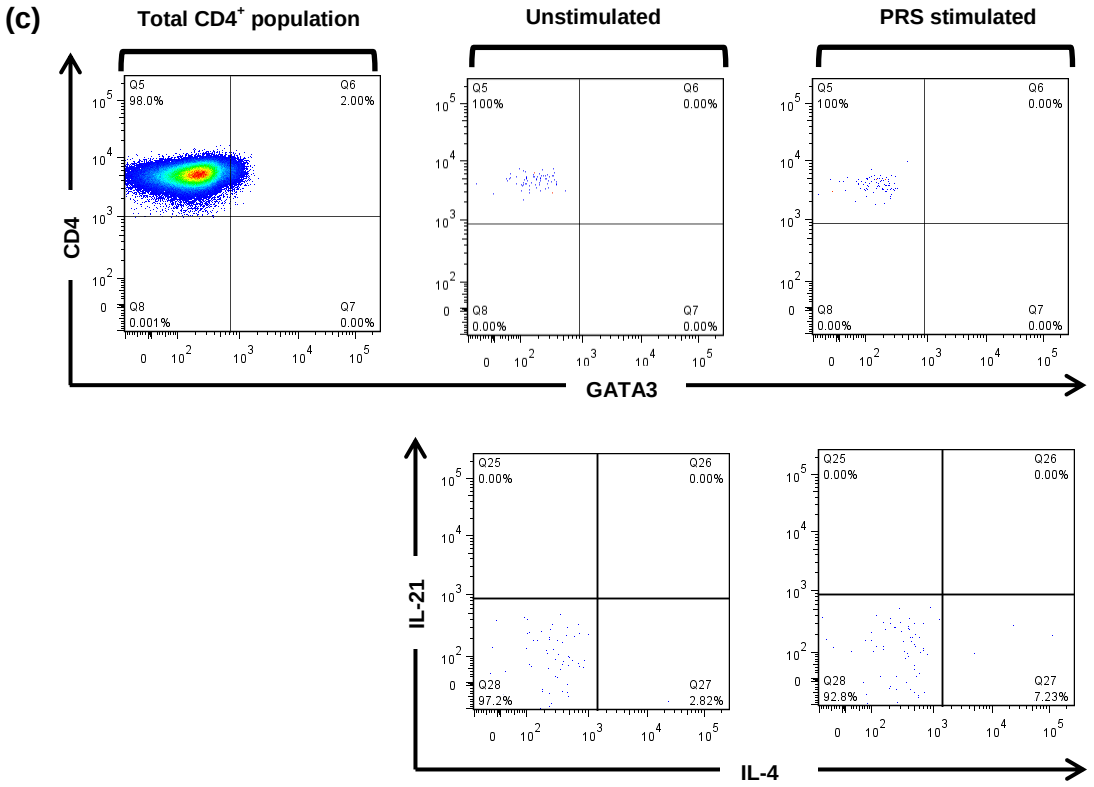
In addition to analysing the response of PRS specific CD4$^+$ T cells we also sought to examine transcription factor and cytokine profiles of CD4$^+$ T cells specific for other EBV derived antigens to determine whether transcription factor and cytokine profiles seen in the context of PRS were consistent across CD4$^+$ T cells specific for different spitopes/stages of EBV cycle. Donors with the largest frequency response were chosen to optimise the stimulation and the aim was to find a peptide concentration where stimulation would induce cytokine production but leave sufficient TCR on the cell surface for MHC II tetramer binding . CD4$^+$ enriched PBMCs were stimulated with different concentrations of peptide in the presence of Brefeldin A and subsequently stained with the relevant MHC class II tetramer. Following viability and surface staining as described above, cells were fixed, permeabilised and stained for IFNγ and TNFα. An example of the results obtained for stimulation with the

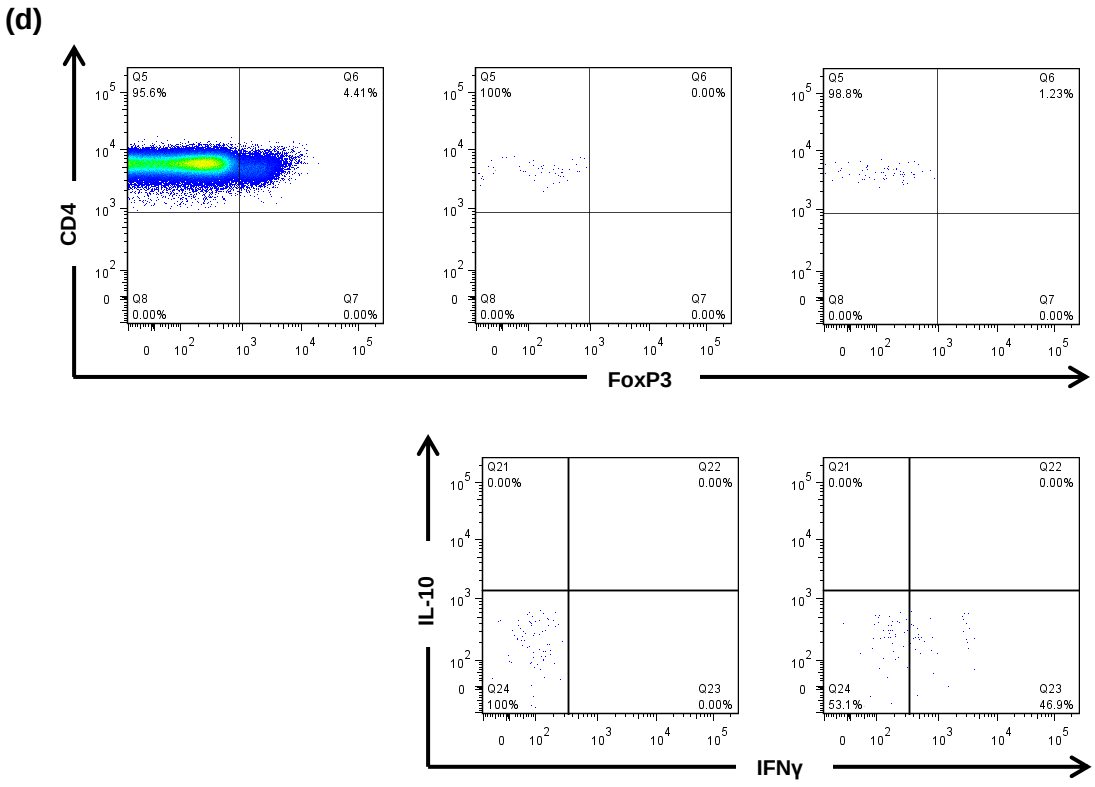
SNP peptide are shown in Fig 3.4.f. Despite using peptide concentrations 100 to 500 fold higher than what had previously been established as optimal concentration of PRS peptide, staining with the MHC class II tetramer was still possible, indicating that T cell receptors were not saturated following stimulation (top plots). We did nonetheless observe a decrease in SNP tetramer binding as the concentration of peptide increased. Dropping from 0.227% SNP tetramer positive cells when using 0.5µg/mL compared to 0.162% with 2.5µg/mL. In order to assess cytokine production from SNP specific $CD4^+$ T cells following stimulation, we analysed their production of IFNγ and TNFα. The percentage of IFNγ producing cells did not increase over background levels irrespective of the concentration of SNP peptide used (middle plots). No cells dually positive for SNP MHC class II tetramer and IFNγ were seen. Background levels of TNFα production were similar to what had been previously observed when studying PRS specific $CD4^+$ T cells and the frequency of cells producing this cytokine did increase with higher concentrations of SNP peptide (bottom plots). However while the proportion of $CD4^+$ T cells positive for TNFα went from 0.367% when stimulated with 0.5µg/mL of peptide to 0.463% when stimulated with 2.5µg/mL, no significant number of cells were simultaneously positive for the SNP tetramer.

We also tried to establish a protocol for the VKL peptide and tetramer without success. Further optimisation is required before comparison of T cells specific for different EBV eptiopes can be done.

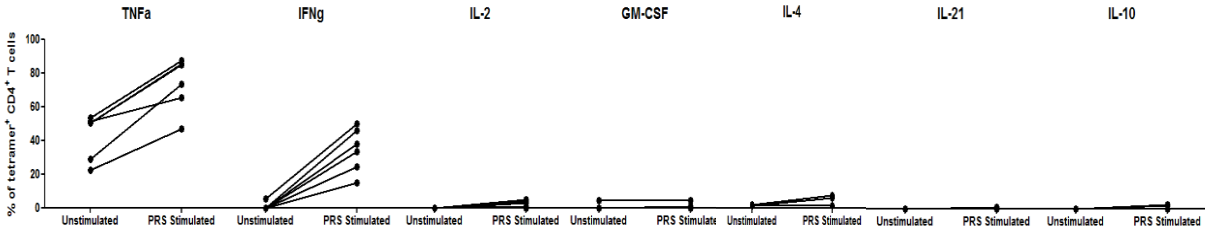
FIGURE 3.4


(a)
No tetramer control
Unstimulated
PRS stimulated
PRS +ve
0.003%
PRS +ve
0.043%
PRS +ve
0.043%
CD4
PRS tetramer
(b)
Total CD4+ population
Q13
24.0%
Q14
76.0%
Q16
0.00%
Q15
0.00%
Q13
11.1%
Q14
88.9%
Q16
0.00%
Q15
0.00%
Q13
17.6%
Q14
82.4%
Q16
0.00%
Q15
0.00%
CD4
T-bet
Q13
0.926%
Q14
0.00%
Q16
50.0%
Q15
49.1%
Q13
1.85%
Q14
42.6%
Q16
19.4%
Q15
36.1%
IFNγ
TNFα
Q29
0.00%
Q30
0.00%
Q32
99.1%
Q31
0.926%
Q29
0.00%
Q30
0.00%
Q32
96.3%
Q31
3.70%
GM-CSF
IL-2


(c)
Total CD4+ population
Unstimulated
PRS stimulated
CD4
GATA3
Q5 98.0%
Q6 2.00%
Q8 0.001%
Q7 0.00%
Q5 100%
Q6 0.00%
Q8 0.00%
Q7 0.00%
Q5 100%
Q6 0.00%
Q8 0.00%
Q7 0.00%
IL-21
IL-4
Q25 0.00%
Q26 0.00%
Q28 97.2%
Q27 2.82%
Q25 0.00%
Q26 0.00%
Q28 92.8%
Q27 7.23%


(d)
CD4
FoxP3
Q5 95.6%
Q6 4.41%
Q8 0.00%
Q7 0.00%
Q5 100%
Q6 0.00%
Q8 0.00%
Q7 0.00%
Q5 98.8%
Q6 1.23%
Q8 0.00%
Q7 0.00%
IL-10
IFNγ
Q21 0.00%
Q22 0.00%
Q24 100%
Q23 0.00%
Q21 0.00%
Q22 0.00%
Q24 53.1%
Q23 46.9%

(e)



(f)

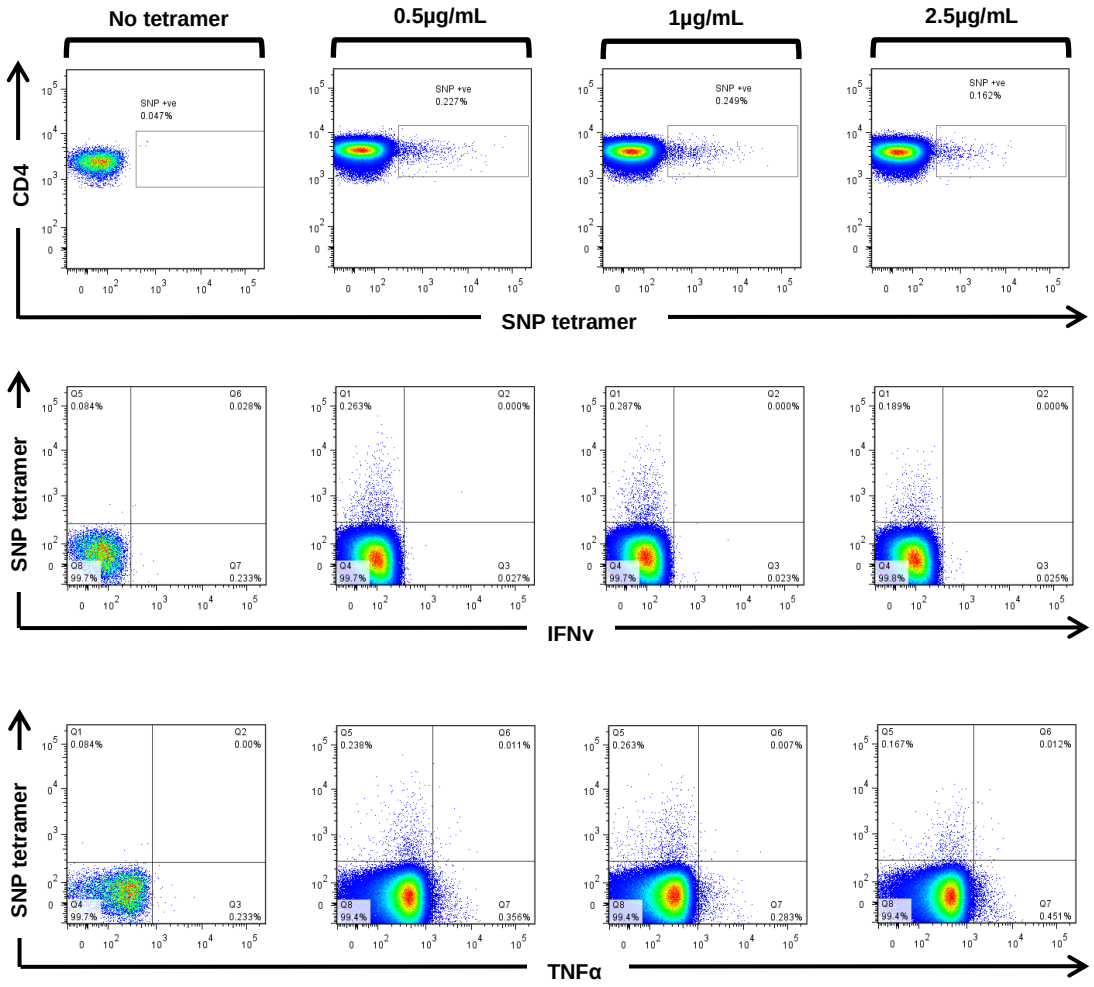


Figure 3.4 **Intracellular staining of PRS tetramer positive CD4$^+$ T cells following EBV peptide stimulation**

(a) Analysis of expression of transcription factors and cytokines performed on CD4$^+$ T cells positive for PRS tetramer staining. Expression of T_h1 **(b)**, T_h2 **(c)** and T_{reg} **(d)** associated transcription factors and cytokines analysed in CD4$^+$ PRS tetramer$^+$ unstimulated and PRS stimulated populations. For each panel top left plot shows expression of transcription factor in total CD4+ population. Production of cytokines in unstimulated (middle plot) and PRS stimulated (right hand plots) are shown. **(e)** Summary of change in cytokine production following stimulation with PRS in the six donors tested. **(f)** Staining of SNP tetramer (top plots), IFNγ (middle plots) and TNFα (bottom plots) in the total CD4$^+$ population following incubation with increasing concentrations of SNP peptide.

3.5 Cytotoxic potential and phenotype of EBV epitope specific CD4$^+$ memory T cell population

In the final set of experiments, we wanted to analyse the cyotoxic potential of EBV-specific cells *ex vivo*. Several previous studies performed *in vitro* have shown that CD4$^+$ T cell clones specific for EBV antigens are capable of directly killing infected cells. Expression of high levels of cytotoxic molecules required for cytolytic activity in CD4$^+$ T cells has been associated with late differentiated cells of a CD45RA$^+$ CCR7$^-$ phenotype. Since long-term culture of T cells drives differentiation, it is unclear whether such killing activity is an inherent property of circulating virus-specific CD4$^+$ T cells or an artefact of *in vitro* culture. The presence of cells with cytotoxic potential *ex* vivo has not yet been systematically assessed. In addition, analysis of the phenotype of EBV specific CD4$^+$ memory cells in the blood of healthy carriers has previously shown that the majority lay in the earlier differentiation states of CD45RA$^-$ CCR7$^-$ effector memory (EM) or CD45RA$^-$ CCR7$^+$ central memory (CM) compartments (Fig 1.3). Furthermore, studies performed at the epitope level on MHC II tetramer-binding cells have confirmed this, and shown that majority of CD4$^+$ memory T cells have an effector or central memory phenotype. We therefore sought to use our MHC II reagents to identify the phenotype of EBV epitope specific CD4$^+$ memory T cells and their expression of the cytotoxic molecules Perforin and Granzyme B in the CD4$^+$ memory T cell subsets.

To achieve this, CD4$^+$ enriched PBMCs from donors expressing the DR7 or DR52b alleles were stained with the appropriate PRS MHC class II tetramer followed by viability and surface staining for CD3, CD4, CD14, CD19, CD45RA and CCR7. Cells were subsequently fixed in paraformaldehyde and permeabilised using saponin before, intracellular staining for Perforin and Granzyme B was performed.

Gating was done on $CD3^+$ $CD4^+$ as show in Fig 3.1.a, followed by assessment of tetramer-PE cells within this population (Fig 3.5.a). As shown in Fig 3.5.a, this representative donor has 0.060% of peripheral $CD4^+$ T cells specific for PRS.

Cytotoxic potential of PRS specific $CD4^+$ T cells was assessed by analysing the presence of Perforin and Granzyme B. As shown in Fig 3.5.b (left plot), only 0.228% of this donor's $CD4^+$ T cell repertoire expresses Granzyme B alone and 0.138% exclusively expressing Perforin. $CD4^+$ T cells positive for both cytotoxic molecules accounted for 0.152% of the total $CD4^+$ population. Within the PRS specific $CD4^+$ population, no cells expressing Perforin alone were observed, nonetheless 7.95% were positive for Perforin and Granzyme B and an equal percentage were positive for just Granzyme B.

A summary graph of the mean frequencies of expression of Perforin and Granzyme obtained in the five donors tested is shown in Fig 3.5.c. These initial results show that EBV specific cells can possess cytotoxic potential through the presence of Perforin, Granzyme B or a combination of the two. Further analysis on larger responses needs to be performed in order to confirm these early findings.

The distribution of $CD4^+$ memory T cell populations in the total $CD4^+$ population and in the PRS-specific cells was achieved by looking at expression levels of the differentiation marker CD45RA and the lymphoid homing marker CCR7. The example in Fig 3.5.d (left plot) shows that 50.2% of the $CD4^+$ population in this donor is made up of naïve T cells (T_N) displaying a $CD45RA^+$ $CCR7^+$ phenotype. The $CD45RA^-$ $CCR7^+$ central memory (T_{CM}) and $CD45RA^-$ $CCR7^-$ effector memory (T_{EM}) cells represent 35.4% and 12.6% of the total CD4 T lymphocyte population respectively. The terminally differentiated effector cells (T_{EMRA}) positive for CD45RA but not CCR7 only account for 1.79% of the total $CD4^+$ cells analysed in this donor. When the phenotype of PRS specific $CD4^+$ T cells (Fig 3.5.d, right plot) was compared, we observed that the percentage of T_N was significantly lower at 5.68%. The T_{CM}

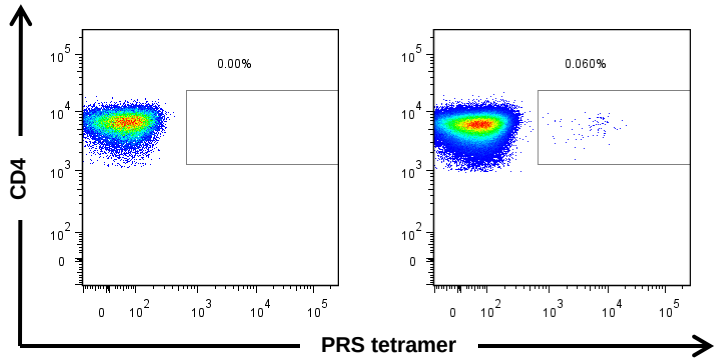
and T_{EM} populations however were higher at 59.1% and 23.9%% respectively, demonstrating that the vast majority of tetramer-binding cells in the blood are antigen experienced, in line with the previous observations. The frequency of T_{EMRA} cells was also more abundant in the epitope specific $CD4^+$ population of this donor at 11.4%, compared to 1.79%.

The graph shown in Fig 3.5.e represents the mean frequencies of each memory T cell subsets within the $CD4^+$ PRS specific cells across the five donors tested. As for the representative example shown, in all donors the majority of EBV epitope specific cells lay in the effector populations negative for CD45RA with a small majority being in the central $CCR7^+$ population. Only a small percentage can be categorized as $CD45RA^+$. The $CCR7^+$ naïve and $CCR7^-$ terminally differentiated subsets appear to be present at similar levels.

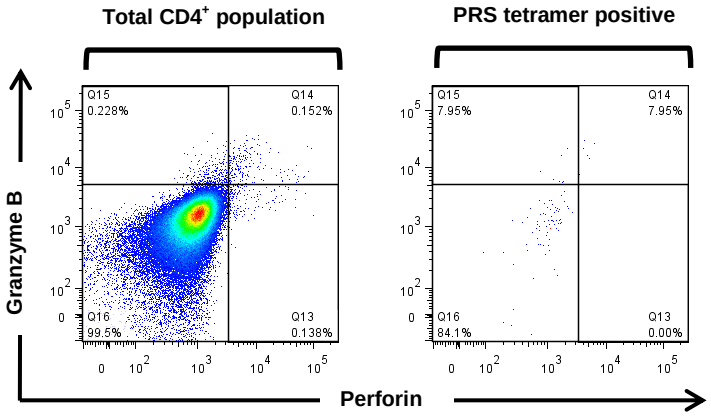
These initial tests have support previous findings that at an epitope level the majority of EBV specific $CD4^+$ T cells possess either a central or memory phenotype with a small percentage of naïve and terminally differentiated cells. Preliminary investigation into the presence of cytotoxic molecules has shown that some EBV specific $CD4^+$ T cells, at least, may be primed for immediate cytotoxic function against cells expressing their cognate antigen, however, these are present at low levels in the circulating blood of healthy donors.

FIGURE 3.5

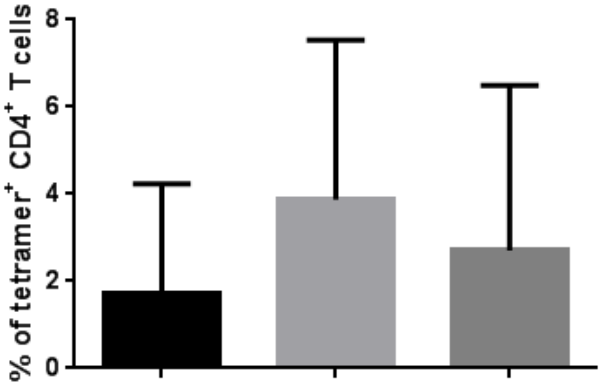
(a)



(b)



(c)



Perforin	**+**	**-**	**+**
Granzyme B	**-**	**+**	**+**

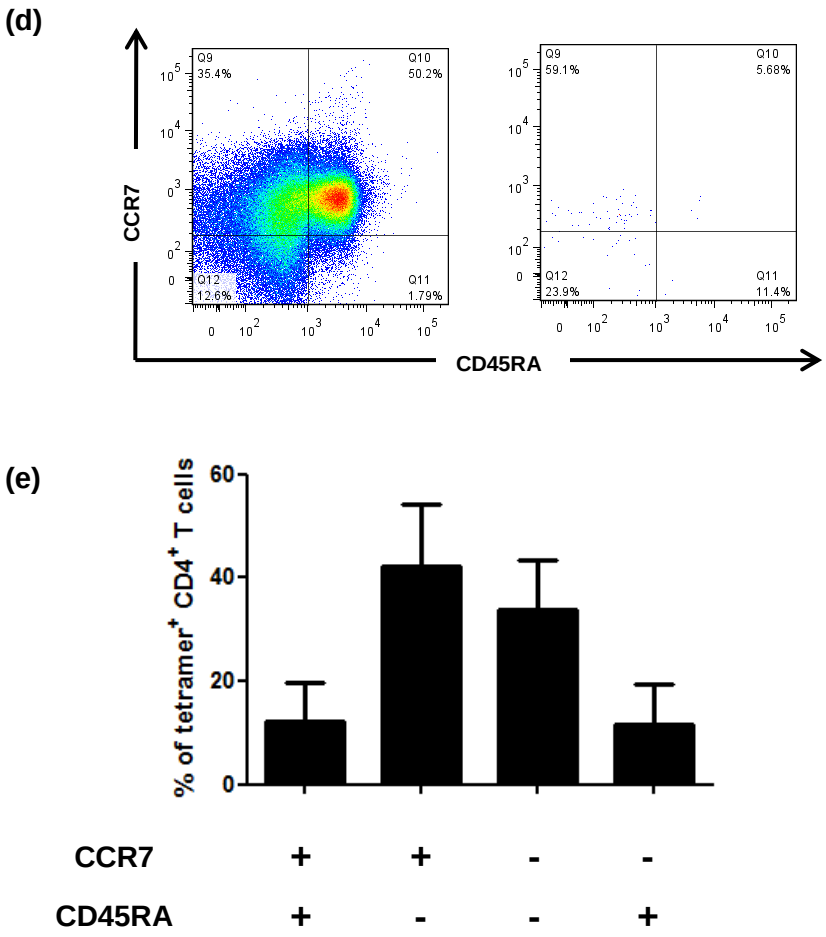


Figure 3.5 **Cytotoxic potential and phenotype of EBV MHC II tetramer specific CD4 populations**

(a) $CD4^+$ T cells positive for PRS tetramer were analysed for their cytotoxic potential and memory phenotype. **(b)** Percentage of total $CD4^+$ cells staining for perforin and Granzyme B (left hand) and in the PRS tetramer positive cells (right hand). **(c)** Summary of tetramer positive $CD4^+$ cells staining for perforin and Granzyme in five donors tested. **(d)** Percentage of cells stained for CCR7 and CD45RA in the total $CD4^+$ population (left hand) and in the PRS tetramer positive population (right hand). **(e)** Summary of tetramer positive $CD4^+$ T cells staining for the memory phenotype markers CCR7 and CD45RA in the five donors tested.

4. DISCUSSION

The Epstein Barr virus is a B cell tropic agent that is estimated to infect approximately 90% of the worldwide population. Despite the virus being tightly controlled by a strong T cell response, it is associated with a number of cancers. One such malignancy is post-transplant lymphoproliferative disease (PTLD) that is susceptible to develop in immunosuppressed transplant patients. Recently, transfer of EBV specific T cells has been shown to be not only an effective treatment option for PTLD but also a highly successful prophylactic for patients at risk of developing this malady. Within this context it has been observed that a better clinical outcome is linked to a higher proportion of $CD4^+$ T cells in the EBV specific preparations (Haque *et al.*, 2007). Direct effector function of T cells has principally been associated with $CD8^+$ T cells and the recognition of MHC class I expressing cells however it is now accepted that $CD4^+$ T cells are also able to exert such functions on MHC class II positive cells.

There is precedent for using $CD4^+$ T cells in a therapeutic setting as a recent trial demonstrated that a melanoma patient achieved durable remission following infusion of a tumour antigen specific $CD4^+$ T cell preparation (Hunder *et al*., 2008). Furthermore, a higher percentage of $CD4^+$ T cells in EBV specific cytotoxic T cells administered for the treatment of PTLD is associated with a better clinical response (Haque *et al*., 2007). There is therefore potential for EBV specific $CD4^+$ T cells to be use therapeutically for virus associated diseases. At present however, little is known about the functional characteristics of $CD4^+$ T cells that control the persistent infection of the virus in healthy individuals. Understanding what types of T cells respond to EBV infected cells may be important in to help improve future therapies for not only PTLD but other cancers characterised by uncontrolled proliferation of MHC class II positive cells.

The recent advances in MHC class II tetramer technology has enabled the analysis of $CD4^+$ T cells on a single cell basis and will allow further knowledge of $CD4^+$ T cell function to be gained at the epitope level.

Most studies analysing the ability of $CD4^+$ T cells to directly recognise EBV infected cells have used *in vitro* isolated and expanded T cell clones. These have generally relied on assays that measure IFNγ (Amyes *et al.*, 2003) however T cells producing IL-4 (Bickham *et al.*, 2001) have also been observed. Such findings could however be artefacts of culturing T cell clones. In addition studies analysing the response of EBV specific $CD4^+$ T cells *ex vivo* have been performed using overlapping peptide pools which do not necessarily correspond with the physiological amounts of antigen presented to T cells (Ning *et al.*, 2011). The secretion of these cytokines has led to the categorisation of EBV specific $CD4^+$ T cells into certain T helper cell subsets; however analysis of the expression of transcription factors in responding T cells has not yet been performed. Assessing the presence of transcription factors along with cytokines would enable the confirmation of the type of $CD4^+$ T cell responding to EBV stimulus. It is also known that $CD4^+$ T cells are a heterogeneous population and polyfunctional T cells secreting multiple cytokines are important in conferring T-cell immunity in human virus infections (Harari *et al.*, 2006).

We sought to gain a better understanding of EBV specific $CD4^+$ T cells following activation by an EBV infected cell by analysing the expression of transcription factors and production of a range of cytokines. We successfully optimised a multitude of antibody panels that subsequently allowed us to measure the functional characteristics of interest.

We therefore analysed the expression of transcription factors and cytokines associated with the T_h1, T_h2 and T_{reg} $CD4^+$ T cell subsets following stimulation with autologous LCLs. In all of the panels tested here, we observed a $CD4^+$ T cell response to autologous LCLs by secretion of IFNγ. The cells producing the anti-viral cytokine accounted for between 0.3 and 0.5% of

the total $CD4^+$ population depending on the donor. This is in line with what is known about frequency of individual responses that range between 0.008 and 0.040% (Long *et al.* 2013) and the broad repertoire of epitopes recognised by $CD4^+$ T cells (Long *et al.*, 2011). Furthermore, a similar percentage of IFNγ producing $CD4^+$ T cells within the total $CD4^+$ populations was observed for a related herpesvirus, CMV (Gamadia *et al.*, 2003). IFNγ is a cytokine predominantly associated with T_h1 type T helper cells that express the transcription factor T-bet. Although cells responding to stimulation with EBV infected cells have been shown to produce IFNγ it was assumed that they were of a T_h1 lineage without analysing expression of the associated transcription factor. With our antibody panels we were able to confirm this this by observing that approximately 90% of cells producing this cytokine also expressed the transcription factor T-bet. The high proportion of T_h1 cells is to be expected as this subset is the host immunity effectors against intracellular viral pathogens. The secretion of IFNγ by this type of $CD4^+$ T cell can activate phagocytic activity of macrophages and up regulates the expression of class I and II MHC functions to enhance the activity of cytotoxic T cells.

Perhaps unsurprisingly we did not observe any $CD4^+$ T cells positive for the T_h2 associated transcription factor GATA3 producing IFNγ as this subset is associated with immune response to large extracellular pathogens. In contrast to previous studies we also did not observe any production of IL-4 in response to LCL stimulation. We did however detect a small percentage of cells expressing the T_{reg} associated transcription factor FoxP3. Expression of cytokines more commonly associated with this subset such as IL-10 was not observed but studies have suggested that in a polarizing T_h1 response regulatory T cells can be induced into producing IFNγ. In addition, such T_h1-like T_{reg} cells express the transcription factor T-bet alongside FoxP3 (Koch *et al.*, 2009). In our experiments we did not seek to analyse the co-expression of T-bet and FoxP3, however given the dominant T_h1 response

observed following LCL stimulation it would perhaps not be surprising to observe a suppressive subset directed against responding cells.

Responses from un-manipulated $CD4^+$ T cells were clearly seen in the circulating blood of all the healthy donors demonstrating that they are directly functional in short term assays against autologous LCLs (Long *et al.*, 2005; Munz *et al.*, 2000). Furthermore, responses were obtained with physiological level of naturally processed antigen present on the surface of virus infected cells as opposed to saturating concentrations of peptide pools (Ning *et al*., 2011). These results suggest that circulating EBV specific $CD4^+$ T cells may have similar direct function against virus infected B cells *in vivo* and thus are able to control infection.

Our results show that $CD4^+$ T cells responding *ex vivo* to autologous LCLs express the T_h1 associated transcription factor T-bet alongside IFNγ and TNFα predominantly as polyfunctional cells (Fig 3.2.b). However, we did not observe significant levels of IL-2 production. This was unexpected as IL-2 can be used as a marker for T cell activation and stimulates the clonal expansion of $CD8^+$ T cells while also stimulating proliferation of $CD4^+$ T cells and has been detected in previous studies (Ning *et al.*, 2011). This finding was nonetheless consistent throughout the donors and did not vary depending on the antibody used. In addition we did not observe any cells responding to EBV infected cells by producing IL-4 as had been previously reported (Bickham *et al.*, 2001).

The assays analysing the $CD4^+$ T cells following LCL stimulation have shown the profiles of the total EBV specific responses, however they do not give information regarding individual epitope specificities. The responses of $CD8^+$ T cells to epitopes derived from lytic and latent antigens have been well studied and we therefore wanted to investigate profiles of EBV specific $CD4^+$ T cells. To achieve this we took advantage of novel MHC class II tetramers to look for the first time at the responses at the epitope level. We initially performed experiments with the EBNA2 derived peptide PRS (as the assay had previously been

optimised) before attempting to look at other $CD4^+$ T cells specific for lytic and latent derived epitopes.

Further examination of EBV specific $CD4^+$ T cells using MHC class II tetramers revealed that cells specific for an epitope derived from the latent protein EBNA2 respond in a similar way to cells that have been stimulated by autologous LCLs. Indeed the majority of responding cells secreted IFNγ, TNFα, or both. Interestingly, there were background levels of TNFα observed in all donors but we did notice a dose dependent response when different dilutions of the tetramer were tested (data not shown) indicating that production of cytokines was not due to activation of the TCR upon binding of the MHC class II reagent as has been reported for MHC class I tetramers (. Furthermore, similar background levels were observed in the LCL stimulation experiments.

Nevertheless it is important to note that not all cells positive for the cognate receptor to the PRS MHC class II tetramer responded to the peptide and produced cytokines. This further supports previous observations that even though these $CD4^+$ T cells are evidently antigen experienced, they are capable of reverting to a central memory phenotype. Thus, not all EBV-specific cells are primed and immediately available as anti-viral effectors. In addition $CD4^+$ T cells for a given epitope are likely to be a polyclonal population and therefore some may have low avidity TCR that were not activated by low concentrations of peptide.

We intended to further confirm the functional characteristics observed with PRS specific $CD4^+$ cells by analysing the profiles of $CD4^+$ T cells directed against other lytic and latent protein derived antigens. However, multiple tests performed with increasing peptide concentrations and longer incubation times did not result in cells responding by producing IFNγ and TNFα as had previously been seen with the PRS peptide and therefore further optimisation is required. A potential alternative test would be to look at the $CD4^+$ T cell response to LCLs pulsed with the peptide of interest. Indeed, EBV specific $CD4^+$ T cell

clones have a higher capacity to directly recognise and kill LCLs when the target transformed cell line has been “primed” with peptides prior to incubation with the effector T cells (Long *et al.*, 2005).

Using MHC class II tetramer we were able to demonstrate that epitope specific $CD4^+$ T cells can act as direct effectors *ex vivo* and we were interested in analysing whether this function was further extended to the ability to kill infected cells. Studies have shown that cytotoxic $CD4^+$ T cells are present in low numbers in the circulating blood of healthy donors but are expanded in patients suffering from chronic viral infections such as HIV (Appay *et al.*, 2002). They are characterised by the presence of perforin and display a distinct surface phenotype associated with end stage differentiation. Furthermore, it was observed that $CD4^+$/perforin$^+$ cells produced IFNγ and TNFα. Previous work performed with EBV specific $CD4^+$ T cell clones has suggested that cytotoxic function is present (Long *et al.*, 2005; Munz *et al.*, 2000) however this may be an artefact of *in vitro* culture (Fleischer *et al.*, 1984). Indeed,*in vitro* culture may drive $CD4^+$ T cells to more differentiated phenotype associated with cytotoxic capacity (Hintzen *et al.*, 1993).

By combining MHC class II staining along with perforin and granzyme staining we were able to analyse whether cells stained for the tetramer possessed cytotoxic potential. Additionally we included memory phenotype markers in order to assess the differentiation status of any cells that were positive for perforin or granzyme B.

Within the the PRS tetramer specific population tested we did detect cells positive for perforin, granzyme B or a combination of both, however due to the small number of events analysed it was not possible to accurately determine the memory phenotype of these cells. The frequency of perforin and granzyme B expressing cells in the total $CD4^+$ population was very low as has been previously reported (Appay *et al.*, 2002). We did observe much higher levels of perforin and granzyme expressing $CD4^+$ T cells in the total $CD4^+$ T cell population in

one donor however we did not detect any tetramer positive cells with cytotoxic potential. Our initial tests suggest that EBV specific $CD4^+$ T cells with cytotoxic potential are present at higher proportions in the blood of healthy donors. The repeated exposure of lymphocytes to EBV derived antigens in the circulating blood could increase the cytotoxic potential of EBV-specific $CD4^+$ T cells as has been seen in the context of other chronic viral infections such as HIV.

The memory phenotype of the total tetramer positive population was similar to what had previously been seen despite using a distinct panel of antibodies and different donors (Long *et al.*, 2013). The selection of the PRS tetramer may not have been the most appropriate for this assay as the response is low and using the SNP tetramer, which has a larger response in healthy donors, could have led to more events being analysed. These experiments do not require additional optimisation as peptide stimulation is not required and analysis was not performed due to time limitations.

Presence of molecules associated with cytotoxicity does not necessarily result in cells killing when challenged with their cognate antigen. Thus, in addition to using flow cytometry analysis to assess cytotoxic potential we also performed one preliminary killing assay using a population of PRS specific $CD4^+$ T cells enriched directly *ex vivo*. This purified population did appear to preferentially kill autologous LCLs pulsed with PRS peptide compared to an HLA mismatched LCL. Nonetheless cell numbers used in the assay were low and improving the yield and purity of PRS specific cells from donor PBMCs would be essential in order to confirm the initial observation.

Overall, this study has shown for the first time that $CD4^+$ T cells can have effector function directly *ex vivo* by responding to autologous LCLs by secreting the anti-viral cytokine IFNγ along with the inflammatory cytokine TNFα. We also confirmed that these cells were

predominantly of a T_h1 subtype. Using MHC class II tetramers enabled us to analyse the responses at the epitope level and assays stimulating $CD4^+$ T cells with an EBNA2 derived peptide also showed the majority of $CD4^+$ T cells specific for the peptide expressed the T_h1 associated transcription factor T-bet. Furthermore, tetramer positive cells that responded to incubation with the peptide produced IFNγ and TNFα as was seen during stimulation with autologous LCLs. We also observed that unmanipulated $CD4^+$ T cells analysed *ex vivo* possess cytotoxic potential and that such cells are specific for EBV derived antigens.

Further work is however needed to analyse cytokine profiles across a range of epitopes from lytic and latent proteins. Moreover our initial observations that some EBV specific $CD4^+$ T cells possess cytotoxic potential needs to be confirmed for other epitopes. Ultimately we would like to be able to set up a killing assay with enriched EBV specific $CD4^+$ T cells to examine whether they are indeed able to kill EBV infected cells.

The work performed here is important in the context of enhancing adoptive T cell therapies currently used for the EBV-related malignancy PTLD. By improving knowledge of the subtypes of $CD4^+$ T cells responding to EBV in healthy donors, we will be able to preferentially stimulate those that can efficiently act directly on virus infected cells. Recently, peptide-selected T cells have been administered to patients suffering from lymphoproliferative diseases (Moosman *et al.*, 2010; Icheva *et al.*, 2013), and this approach could be further improved with knowledge of the $CD4^+$ T cell epitopes that respond to antigenic stimulation in healthy donors.

5. REFERENCES

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