PROJECT 2 : PHENOTYPIC AND FUNCTIONAL ANALYSIS OF EBV-SPECIFIC CD4+ T CELLS IN BLOOD AND TONSILS'

BY

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Abstract (not to exceed 200 words - any continuation sheets must contain the author's full name and full title of the thesis):

CD4+ T cells play a central role in the immune response and their role in the control of EBV infection has become of increasing interest in recent years. Advances in class II tetramer technology has allowed the discovery of a unique phenotype of EBV-specific CD4+ T cells in IM donor blood and throughout IM resolution, cells begin to show an up regulation of lymphoid homing markers, CCR7 and CD62L. This suggests EBV-specific CD4+ T cells may be homing to the tonsil, the potential organ harboring EBV. For the first time, EBV-specific CD4+ T cell frequencies and their phenotype can be analyzed using an internally optimized class II tetramer staining protocol.

Here, we have shown an enrichment of EBV-specific CD4+ T cells in the tonsil of healthy carriers compared to the blood. These cells share the same phenotype as the total CD4+ T cell population in both blood and tonsil preparations. We have discovered a novel phenotype of EBV-specific CD4+ T cells in which the majority show CCR7+CD45RA+ expression, characteristic of a naïve population. These cells are clearly not naïve and the necessity to explore new memory markers to characterize EBV-specific CD4+ T cells is imminent.

This project shows exciting results indicating a clear importance of CD4+ T cells in the control of EBV infection and with the use of class II tetramers will eventually enable their exploitation as immunotherapeutic options for disease.

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1.1 *CD4+ T cell lineages and function*.

CD4+ T cells act as part of the adaptive immune response which provides specific immunity against pathogens. T cells detect the presence of pathogens through their T cell receptor by engaging with peptide, derived from the pathogen, which is presented on major histocompatibility complex (MHC) molecules. CD4+ T cells recognise peptide in the context of MHC class II, in complexes which generally arise from the extracellular processing pathway of an antigen presenting cell (APC). CD4+ T cells, commonly known as T helper cells (Th), are the orchestrators of the adaptive immune response and interact with B cells to aid in antibody production and produce cytokines that maintain CD8+ T cell responses (figure1.1). Unlike CD8+ T cells, CD4+ T cells differentiate into a variety of lineages dependant on the cytokine milieu present during naïve CD4+ T cell activation by its cognate antigen. Initially, CD4+ T cells were separated into just two subtypes, Th1 and Th2, however recently further subsets have been identified such as Th17, inducible Treg (iTreg) and T follicular helper cells (Tfh). Tfh's, characterised by surface expression of CXCR5 and CD278^{hi}, are only found in the germinal centres of lymph nodes and have a prominent role in B cell class switching and hypermutation (Crotty 2011). It is currently unknown whether this CD4+ cell represents a distinct lineage or if they arise following further differentiation of cells of the Th1, Th2 or Th17 lineages. However, each lineage is characterised by expression of 'master regulator' transcription factors that lead to production of different arrays of cytokines (Zhu, Yamane et al. 2010).

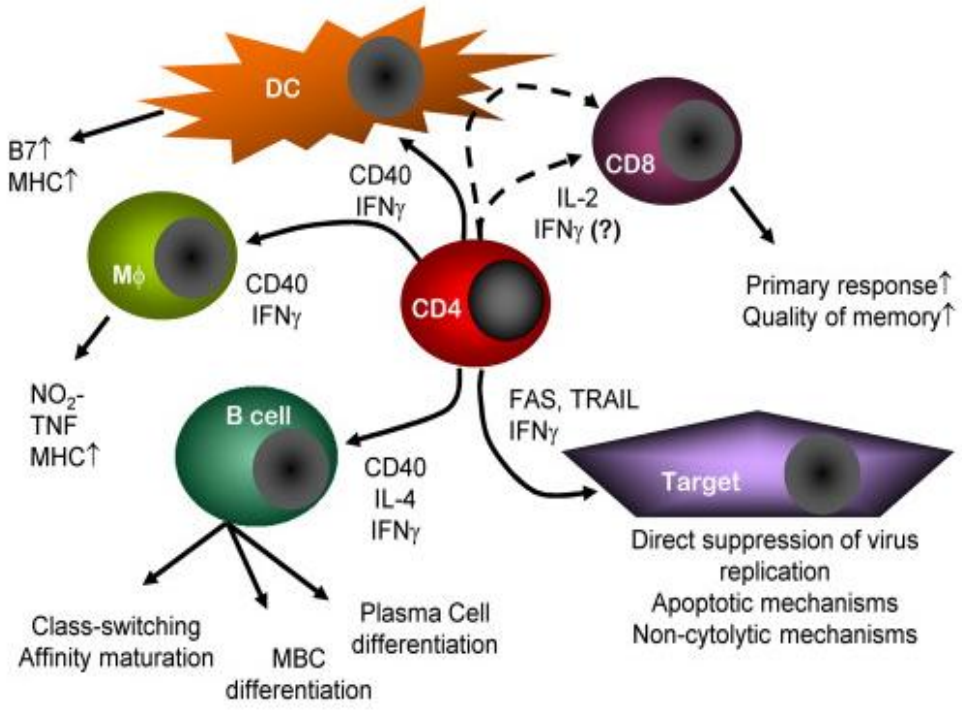

Figure 1.1 CD4+ T cell central role in the immune response. CD4+ T cells interact directly with antigen presenting cells to enhance their ability to present viral antigen to T cells. They engage B cells encouraging hypermutation and class switching resulting in memory B cells (MBC) and plasma cells. CD4+ T cells can directly suppress virus infection in MHCII+ target cells and are essential in the maintenance of CD8+ T cells and formation of CD8+ T cell memory.(Whitmire 2011)

1.2 CD4+ T cell importance in immunity and infection

The multiple subsets of CD4+ T cells are fundamental in controlling the immune response to infection. Studies have shown that in the absence of a functional CD4+ T cell response, antibody production halts and CD8+ T cell memory development is impaired (Whitmire 2011). This reliance of the immune system on CD4+ T cells is apparent in many viral infections for example in HIV infection, progression to AIDS only occurs when CD4+ T cell responses fall to a critically low frequency. Individuals undergoing transplantation showed better control against reactivation of CMV (Sester, Sester et al. 2001) and EBV (Haque, Wilkie et al. 2007) infections if virus-specific CD4+ T cells were present. The discovery that some CD4+ T cells had cytotoxic capacity opens up the possibility of direct targeting of MHC class II-positive cells. Thus CD4+ T cells are truly a diverse lineage of cells with many different capabilities, in both viral infection as well as the

targeting of tumours (Elkington and Khanna 2005; Long, Haigh et al. 2005; Quezada, Simpson et al. 2010). Direct recognition of infected cells by CD4+ T cells has been targeted by viruses as an immune evasion strategy by the down regulation of MHC class II or even by dampening the CD4+ T cell response by producing viral homologues or suppressive cytokines (Hegde, Tomazin et al. 2002). This suggested it is in the interest of viruses to avoid CD4+ T cells highlighting an importance of CD4+ T cells in viral immunity.

1.3 ***Phenotypic markers of CD4+ T cells.***

Naïve CD4+ T cells circulate through the lymphatic system until they encounter a dendritic cell presenting their cognate antigen. To enable homing to secondary lymphoid tissue, naïve CD4+ T cells express high levels of the chemokine receptor CCR7, and also high levels of the adhesion molecule CD62L, allowing for the attachment of T cells to high endothelial venules and migration of T cell from the blood into the lymph nodes (Sallusto, Lenig et al. 1999).

Upon antigen engagement, T cells down-regulate CCR7 expression and up regulate other chemokine receptors allowing them to leave the lymphatic system and travel to the site of infection. An activated T cell also shows an up regulation of markers characteristic of an activated cell. T cells switch from CD45RA expression to its isoform CD45RO upon recent stimulation of cells along with CD38, also a marker of recent activation and are shown to be highly expressed in acute viral infection (Hislop, Kuo et al. 2005). Co-stimulation markers, CD27 and CD28, can also determine stages of differentiation of CD4+ T cells as co-stimulation is lost in T cell differentiation. Unlike CD8+ T cells, CD27 is down regulated first, and the memory population shows a terminally differentiated, CD27-CD28-, phenotype (Yue, Kovacs et al. 2004).

During resolution of infection and contraction of the T cell response, CD4+ memory T cells are formed. Memory T cells have improved cytokine production and proliferate quicker upon reencounter with its antigen (Stockinger, Kassiotis et al. 2004). Lanzavecchia's group in 1999,

showed two subsets of T lymphocytes representing either a central memory (Tcm) pool or an effecter memory (Tem) pool (Sallusto, Lenig et al. 1999). The distinction between these two subsets was made on the basis of their CCR7 expression. CCR7- T cells represented the effecter memory population whilst CCR7+ T cells represented the central memory population which circulate through the secondary lymphoid tissue awaiting reactivation. The CCR7+ memory cells were further distinguished from the naïve CCR7+ population via the expression of CD45RA (Sallusto, Lenig et al. 1999). CD45RA and CD45RO showed reciprocal expression from naïve and activated cells and the co-expression of CCR7 and CD45RA was shown to represent the naïve T cell population whilst the CCR7+CD45RA- cells were known as central memory. The CCR7-CD45RA- population was the effecter memory whilst the CCR7-CD45RA+ populations were known as the Trevertants (Trev), consisting of cells nearing the end of their life cycle which revert back to a CD45RA+ phenotype (Sallusto, Lenig et al. 1999). These phenotypic markers although not perfect, have been generally recognised as markers to distinguish between naïve T cell, Tcm and Tem cells (Amyes, Hatton et al. 2003; Hislop, Kuo et al. 2005).

*1.4 **CD4+ T cell responses and EBV***

EBV often infects in early childhood and thereafter persists as an asymptomatic chronic viral infection in the vast majority of immuno-competent individuals, under the control of the host T cell immune system. The importance of virus-specific T cells in controlling EBV was evident from the resolution of post transplant Lymphoproliferative disease (PTLD) by the adoptive transfer of polyclonal EBV-specific T cells (Khanna, Bell et al. 1999; Savoldo, Goss et al. 2006). If EBV infection is delayed until adolescence/early adulthood it can result in the clinical syndrome of infectious mononucleosis (IM), and has since been characterised by a large expansion of EBV-specific CD8+ T cells. IM has allowed for the study of primary EBV infection and the CD8+ T cell response from acute infection through to convalescence has been well characterised (reviewed in

Hislop, Taylor et al. 2007). In 2002 Amyes *et al* showed that, although to a lesser extent than the CD8+ response, the total CD4+ T cell frequencies were also expanded in acute IM, and suggested that CD4+ T cells may play a role in resolution of acute infection. Certainly in the context of PTLD, endogenous EBV-specific CD4+ T cell responses were lower in those patients who develop PTLD (Sebelin-Wulf, Nguyen et al. 2007) and patients that were infused with adoptive T cell preparations containing higher numbers of CD4+ T cells showed better clinical responses to T cell infusion therapy (Haque, Wilkie et al. 2007). Despite the clear importance of EBV-specific CD4+ T cells in the control of EBV, it has been previously difficult to characterise their role in infection due to lack of knowledge of target epitopes and reagents. With the recent discovery of new CD4+ T cell viral epitopes within the EBV latent and lytic proteins and the isolation of CD4+ T cell clones specific for these EBV derived epitopes (reviewed in Hislop, Taylor et al. 2007; Long, Leese et al. 2011) it has been possible to study the interactions of CD4+ T cells and infected target cells. Studies have shown EBV-specific CD4+ T cells showed no immuno-dominant preference among the lytic and latent viral proteins which was unlike CD8+ T cells (reviewed in Hislop, Taylor et al. 2007; Long, Leese et al. 2011). Others however have shown EBV-specific CD4+ T cells preferentially target virion proteins, suggesting that CD8+ and CD4+ T cells work together by targeting different parts of the virus life cycle (Adhikary, Behrends et al. 2007). As previously mentioned, EBV infects B cells which constitutively express MHC class II and therefore is an ideal target for EBV-specific CD4+ T cell and *in vitro*, these cells were able to directly prevent outgrowth of lymphoblastoid cell lines (LCL) (Long, Haigh et al. 2005; Taylor, Long et al. 2006)

With the development of MHC class II tetramer technology (Guillaume, Dojcinovic et al. 2009) and with access to a large library of known CD4+ T cell EBV-derived epitopes, EBV-specific class II tetramers have now been developed. These class II tetramers have been optimised for use alongside phenotypic markers so we can now evaluate the EBV-specific CD4+ T cell populations from primary infection through to persistence in much more detail than previous studies (Amyes, Hatton

et al. 2003; Scherrenburg, Piriou et al. 2008). Already interesting data has emerged from an IM donor blood (unpublished data) showing initially CD4+ T cells down regulate CCR7 and CD62L as expected of activated cells. However, during the resolution of IM, EBV-specific CD4+ T cells up-regulate lymphoid homing markers CCR7 and CD62L, suggesting that EBV-specific CD4+ T cells were being recruited to the secondary lymphoid tissue. For this reason this project is investigating CD4+ T cells in the tonsil, the site of EBV replication in which EBV-specific CD8+ T cells have already been shown to accumulate (Hislop, Kuo et al. 2005).

1.5 ***Aims***

The primary aim of this study is to determine the frequencies of EBV-specific CD4+ T cells in the blood and tonsils of healthy EBV carriers. Additionally this study will investigate cell surface markers of the EBV-specific CD4+ T cells, to determine their activation status, co-stimulatory markers and homing phenotype, and to investigate whether any EBV-specific CD4+ T cells in the tonsil have a Tfh phenotype. For this, matched blood and tonsil samples collected from healthy EBV carriers shall be used.

2 MATERIALS AND METHODS.

2.1 ***Human tissue and PBMCs.***

Tonsil UM's were taken from liquid nitrogen stores which had been previously separated from tonsil tissue received from University hospital of Birmingham NHS trust and informed consent was previously taken and stored in the patient notes. Healthy laboratory donor PBMCs were used in validation experiments taken from liquid nitrogen stores. All ethical considerations have been approved for use of human tissue and samples in these experiments. The HLA- DR type was determined for all samples previously via 'The Anthony Nolan Trust Histocompatibility Laboratories'.

2.2 ***Class II tetramers.***

Tetramers were manufactured by 'The tetramer core facility of the Benaroya research institute'. Six class II tetramers were ordered including 4 latent EBV CD4 T cell epitopes, SNP (EBNA1 DR51 restricted), VYG (EBNA1 DR11 restricted) PRS (EBNA2 DR52b restricted), PAQ (EBNA2 DR17 restricted) and 2 lytic EBV CD4 T cell epitopes LTA (BZLF1 DR52b restricted) and VKL (BMRF1 DR17 restricted). All tetramers were conjugated to PE fluorochrome.

2.3 ***CD4 T cell clones.***

T cell clones, previously established with a defined specificity, were kept in culture and fed twice weekly with T cell cloning media (10% foetal calf serum, 1% human serum, 30% MLA, 1% penicillin/streptomycin, 50ul/ml IL2 and 58% RPMI) and kept at 37ºC. The CD4 T cell clones used were specific for EBV epitopes LTA, PRS, VKL, PAQ, SNP and LEK.

*2.4 **CD19 and CD8 depletion of total UM and PBMC populations.***

Tonsil UMs and PBMCs were firstly CD8 and CD19 depleted in order to leave an enriched CD4 population. CD19 and CD8 depletion was achieved by the use of magnetic Dynabeads CD19 panB (Invitrogen) and Dynabeads CD8 (Invitrogen). The volume of beads required was calculated as follows:

CD8 PBMC'S and tonsil	**CD19 PBMC'S**	**CD19 Tonsil**
a)No. Beads required = $\frac{\text{No. PBMC} \times 4}{3}$	a)No. Beads required = $\frac{\text{No. PBMC} \times 4}{20}$	a)No. Beads required = $\frac{\text{No. PBMC} \times 4}{2}$
b)Vol. Beads required (µl) = $\frac{\text{No. Beads} \times 1000}{4 \times 10^8}$	b)Vol. Beads required (µl) = $\frac{\text{No. Beads} \times 1000}{4 \times 10^8}$	b)Vol. Beads required (µl) = $\frac{\text{No. Beads} \times 1000}{4 \times 10^8}$
For IM donor use **4x** ; for lab donor use 1x		

Once the required volume of beads was calculated the beads were washed in 1ml LCL media (10% foetal calf serum in RPMI 1640) and placed against a magnet for 2-3minutes and supernatant disposed of. The beads were then resuspended in LCL media and added to PBMCs or tonsillar UMs which were suspended in 5ml of LCL media and incubated for 20minutes at 4˚C on a rotator platform. After this incubation the cell suspension was put onto a magnet for 2-3mins and the supernatant collected in a sterile universal. The beads were then resuspended in 5ml LCL media and this process was repeated a further 2 times. The supernatant collected contained CD8 and CD19 depleted PBMC's or tonsillar UMs and were subsequently used in further experiments.

*2.5 **Class II tetramer staining.***

Each tetramer staining required 1x10^6 of CD8 and CD19 depleted cells. The cells were washed in ice cold MACS buffer for 5minutes at 1600rpm. The pellet was resuspended and 50ul of neat human serum was then added to the cells and left for 5-10 minutes. Tetramer (0.5ul) was then added to the cells which were incubated for 2 hours at 37˚C and flick mixed every half hour. After incubation the cells were washed twice in ice cold MACS buffer (PBS, 0.5% BSA, 2mM EDTA, pH 7.2 2M NaOH) for 5mins at 1600rpm and pellet resuspended by vortexing.

*2.6 **Antibody staining.***

Antibodies were added to a 50ul residual volume of cells in all experiments. The calculated volume of antibody was added to the cells and incubated for 30minutes at 4˚C. After incubation the cells were washed in MACS buffer for 5 minutes at 1600rpm and resuspended in 300ul of MACS buffer ready for flow cytometry analysis. Two separate panels of antibodies were used for each tetramer stain described in table 2.1. Antibody panel 1(Ab1) was for phenotypic analysis in all experiments whilst antibody panel 2(Ab2) was only to be used for phenotypic analysis of tonsillar UM's. All concentrations were previously validated for use in these experiments.

Table 2.1 – Antibody panel 1 and panel 2 concentrations used in all flow experiments

Ab1			
Antigen	**Fluorophore**	**concentration**	**Company**
CCR7	FITC	1/5	BD bioscience
CD28	ECD	1/25	Beckmann coulter
CD38	Pe-Cy7	1/10	BD bioscience
CD4	PerCP-Cy5.5	1/12.5	BD bioscience
CD3	AmCyan	1/25	BD bioscience
CD45RO	Pacific Blue	1/25	Biolegend
CD62L	APC	1/25	BD bioscience
CD45RA	AF700	1/50	Biolegend
CD27	eFluor780	150	Ebioscience

Ab2			
Antigen	**Fluorophore**	**concentration**	**Company**
CD103	FITC	1/5	BD bioscience
CXCR5	Per-Cp – Cy5.5	1/5	Biolegend
CD278	APC	1/5	E bioscience
CD4	ECD	1/50	Beckmann coulter
CD3	AmCyan	1/25	BD bioscience

*2.7 **Flow Cytometry.***

T cell clones ($6x10^4$), tonsil UM's ($1\ x10^6$) and PBMCs ($0.5 - 1\ x10^6$) were used in each flow cytometry experiment where relevant. The flow cytometer used for the multicolour flow experiments was the LSR II (BD bioscience). Cells were stained with PE conjugated class II tetramer as described above and then co-stained with mouse anti human antibodies (Table 2.1). Compensation was achieved via the use of compensation beads. One drop of positive and negative beads was added to a tube for each colour used in the above antibody panel. The antibodies were

added singularly to their allocated tube at the concentrations used in table 2.1 as well as a compensation tube for PE using CD4-PE (BD bioscience 1/40) and incubated for 30minutes at 4˚C. The beads were then washed in ice cold MACS buffer for 5mins at 1600rpm and resuspended in 300ul of MACS buffer.

2.8 ***DNA extraction.***

DNA was extracted using the DNeasy blood and tissue kit (Qiagen) following manufacturer guidelines. The concentration of DNA was determined using a Nanodrop- ND100 spectrophotometer, with 1ul of DNA preparation.

2.9 ***qPCR***

Tonsillar DNA with a yield greater than 200ng/ul was firstly diluted 1 in 3 with DPED treated water (Ambion). Each PCR was run at a 25ul volume inclusive of 5ul DNA and run in triplicate. A master mix was made up containing 12.5ul 2xTAQ mm, 2.5ul Pol Forward primer, 2.5ul Pol Reverse primer, 1ul Pol Probe (FAM), 0.5ul Beta 2 microglobulin forward primer, 0.5ul Beta 2 microglobulin reverse primer and 0.5ul Beta 2 microglobulin probe (VIC). In a pre amplification room 20ul of master mix was added to each well and 5ul of DNA was added to each relevant well. Standards (5ul) were added in duplicate at 1, 4, 10, 25, 50, 200, 1000 and 10,000 EBV copies per cell. The plate was then sealed and briefly spun for 1 minute 1600rpm and PCR was carried out (Applied biosytems 7500 real time PCR system).

3 RESULTS

3.1 Validation of Class II tetramer.

The development of MHC class I tetramers in 1996 (Altman, Moss et al. 1996), has enabled extensive study of the CD8+ T cell response to EBV (Hislop, Kuo et al. 2005). In contrast, characteristics of the CD4+ T cell response have been hampered due to a lack of knowledge of the CD4+ epitope repertoire, by low frequencies of antigen-specific CD4+ T cells and the lack or reagents to identify antigen-specific T cells. However, increased understanding of the targets of the CD4+ T cell response and recent creation of stable soluble MHC class II tetramers, are now allowing novel investigation of virus-specific CD4+ T cell immunity. Previous studies (H.Long unpublished data 2011) have optimised a method in which to use these class II tetramers (see methods).

In order to validate this novel method and the specificity of the MHC class II tetramers, we used EBV-specific CD4+ T cell clones with previously determined specificity for each of the 6 class II tetramers presenting different EBV-derived peptides (DR52b restricted LTA and PRS, DR17 restricted VKL and PAQ, DR51 restricted SNP and DR11 restricted VYG). Each CD4+ T cell clone was individually added to a culture of a CD4+ T cell clone with an irrelevant EBV peptide specificity (LEK) at 3% of the total cell number. The cells were stained with the relevant tetramer following the previously optimised staining method (see methods) and further stained with anti CD4 antibodies. Figure 3.1 shows that 1.2-5.8% of total CD4+ T cells stained positive for each matched tetramer, with clear separation between tetramer-specific and non-specific cells. Although 3% tetramer positive frequency was expected the slightly higher frequencies can be explained due to experimental error in manually counting such low numbers of cells (approx 6000 cells were added to each culture of irrelevant CD4+ T cell clone). The recently optimised MHC class II tetramer staining protocol therefore proved to be valid and was subsequently used in all future experiments.

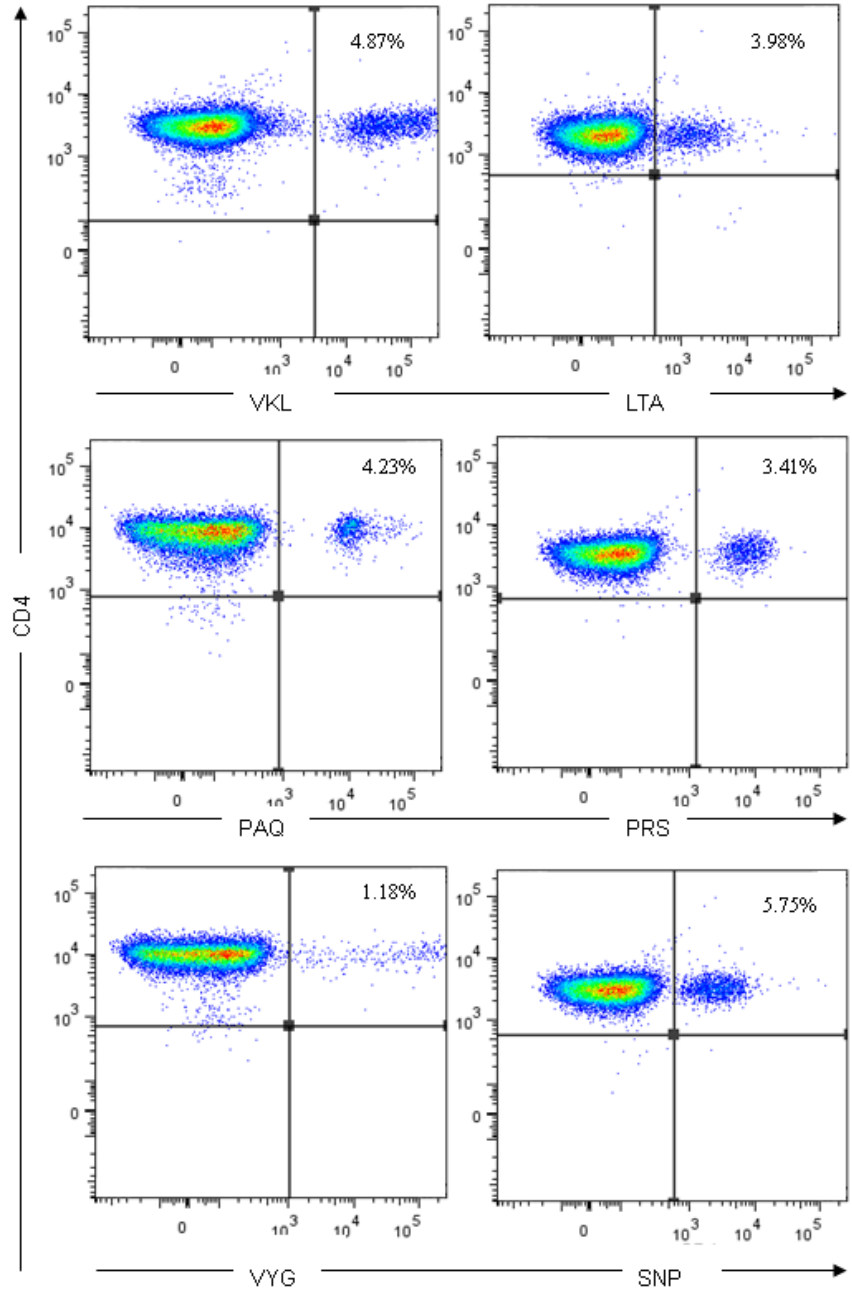

Figure 3.1 Validation of class II tetramers. 3% of CD4+ T cell clones specific for peptide presented by class II tetramer was added to a culture of irrelevant CD4+ T cell clone. The cells were then stained with the matched tetramer and analysis was carried out by flow cytometry. Percentages represent the frequency of CD4+ T cells stained with tetramer.

3.2 Validation of antibody panel 1(Ab1) and antibody panel 2 (Ab2).

In general, the expression of cell surface markers has been used to determine the activation state and homing characteristics of T cells, and to assign them into naïve or memory populations. In order to characterise EBV-specific CD4+ T cells we used 9 such cell surface markers: CD4 and CD3 to gate on CD4+ lymphocytes; activation markers CD45RO, CD45RA and CD38; homing markers CCR7 and CD62L; and co-stimulatory markers CD27, CD28. Antibodies specific for these markers forms Ab1 for which the working concentration of each antibody has been previously optimised to look for the phenotype of EBV specific CD4+ T cells in peripheral blood (H.Long unpublished data 2011). To confirm the predetermined working concentrations (see methods), each antibody from Ab1, was used individually to stain PBMC's from a healthy laboratory donor. Each antibody showed a clear peak shift to the right (figure 3.2 green) from the unstained population (figure 3.2 red), and were therefore used at the previously determined concentrations in all future experiments.

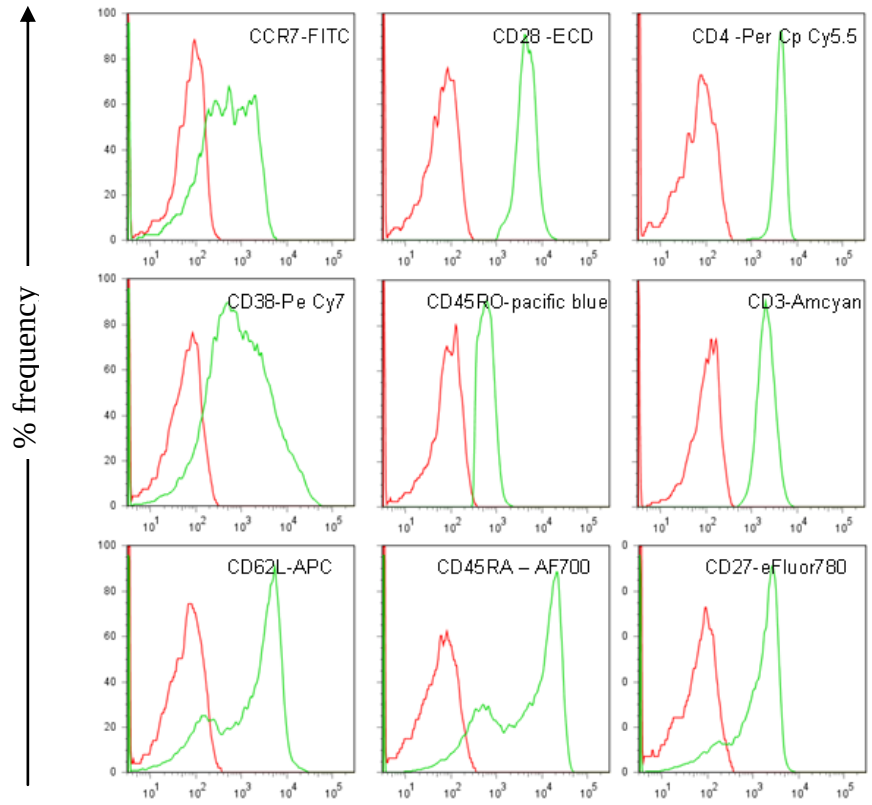

Figure 3.2 Validation of Ab1 – CD4 phenotyping. PBMC's were stained with each individual antibody to be used in Ab1 at the concentrations determined in previous validation studies (see methods for concentrations)

The main aim of this project was to look at the phenotype of EBV-specific CD4+ T cells in the tonsil. As a large proportion of CD4+ T cells in the tonsil are T follicular helper cells (Tfh's), which interact with B cells encouraging hypermutation and maturation, a second antibody panel was constructed to distinguish between CD4+ effectors and CD4+ Follicular helpers. This comprised of specific antibodies to CXCR5 and CD278, the expression of which characterises follicular helpers (Crotty 2011) and CD103, an integrin characteristic of cells retained at mucosal epithelial sites and which is present on many EBV-specific CD8+ T cells in the tonsil (Hislop, Kuo et al. 2005). Antibodies to these markers alongside CD4 and CD3 specific anitbodies formed Ab2 and their working concentrations were determined by titration. CD103-FITC and CXCR5-PerCPcy5.5 were added at decreasing concentrations to tonsillar unfractionated mononucleocytes (UM). Both antibodies showed a decrease in frequency of staining with a decrease in antibody concentration (Figure 3.3). The optimal staining was obtained at the highest concentration, 1/10, for both antibodies which was also the manufacturers recommended working concentration. Thus future experiments used 1/10 dilutions of CD103-FITC and CXCR5-PerCp cy5.5. CD278 is also present on activated T cells therefore decreasing concentrations of CD278-APC was added to PBMC's stimulated overnight with SEB. Again the intensity of stained cells decreased upon a decrease of antibody concentration (figure 3.3) and the manufacturers recommendation of 1/10 (dark blue) was used in future experiments.

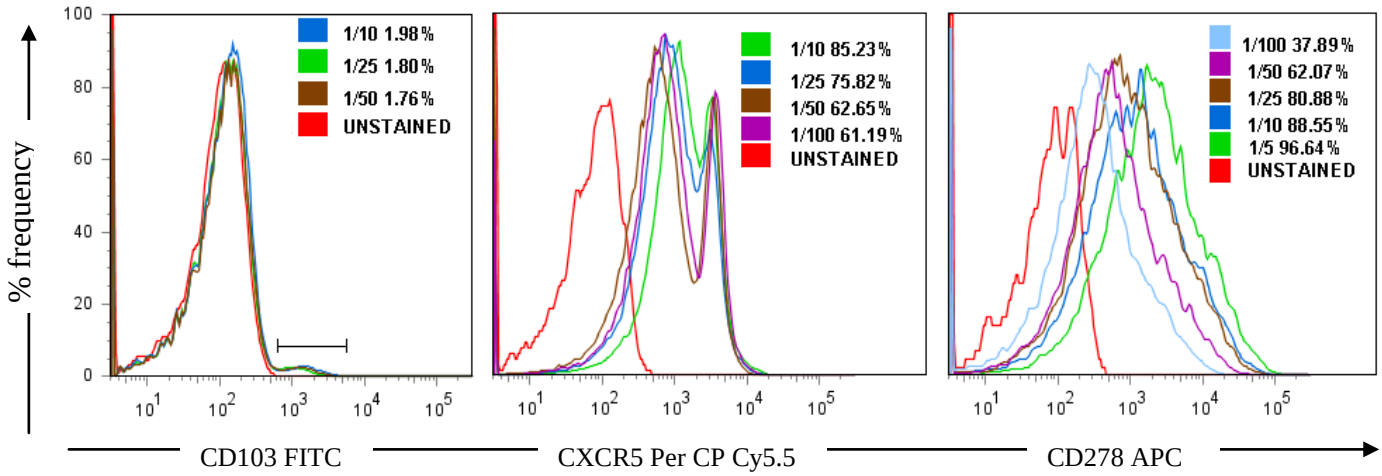

Figure 3.3 Validation of Ab2 – T32 UMs were stained with increasing dilutions of either CD103 FITC (1/10, 1/25 & 1/50) or CXCR5 Per CP Cy5.5 (1/10, 1/25, 1/50 & 1/100). T17 PBMCs were activated overnight with SEB and then stained with CD278 APC at increasing dilutions (1/5, 1/10, 1/25, 1/50 & 1/100) and analysed via flow cytometry. All antibodies were subsequently used at a 1/10 dilution which was the manufacturers recommended dilution (see methods).

3.3 Testing validated class II tetramer and AB1 on IM PBMCs with known HLA type and previously determined tetramer response

Having validated the specificity of the MHC class II tetramers and Ab1, the combined use of EBV-specific class II tetramer and Ab1 was tested on PBMCs from an acute IM donor, IM222. This donor expresses HLA-DR52b, the allele required to present the PRS and LTA epitopes. IM222 PBMCs were firstly depleted of CD8 and CD19 positive cells in order to enrich the CD4+ T cell population, and then stained with the LTA and PRS class II tetramers for 2 hours before exposure to Ab1. Figure 3.4 shows the tetramer responses of IM222 CD4+ T cells. To determine the level of background, PBMCs were simultaneously stained with Ab1 without tetramer (figure 3.4). In the absence of tetramer there were no PE-positive cells, and therefore any staining seen in the PE positive quadrant in the presence of tetramer represent tetramer positive cells. The LTA tetramer presents a peptide from the EBV-lytic protein BZLF1. Figure 3.4 shows 0.38% of donor IM222's total CD4+ T cells are specific for the LTA tetramer. PRS is a peptide derived from the EBV latent protein EBNA2. Figure 3.4 shows 1.88% of this donor's CD4+ T cells are specific for the PRS tetramer, representing a significant commitment of the CD4+ response to a single epitope.

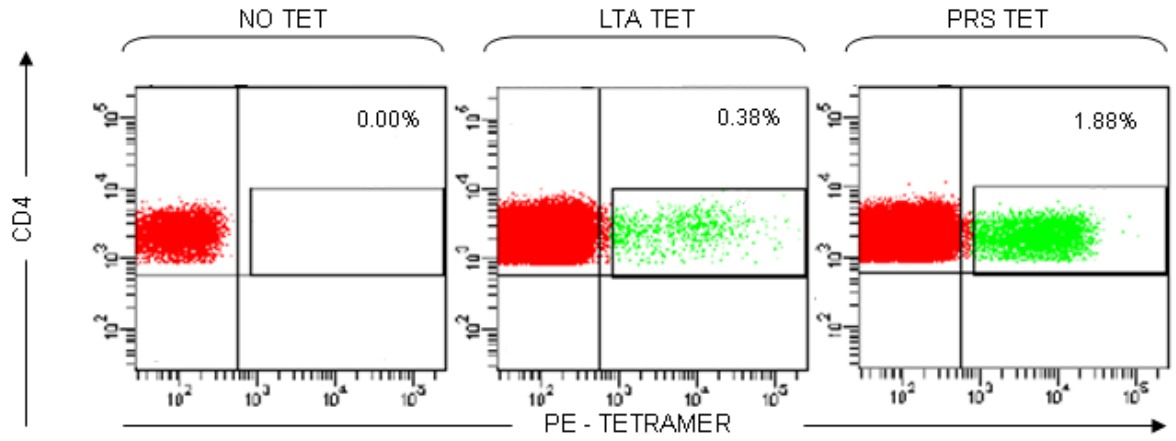

Figure 3.4 Tetramer responses of an IM donor. Previously to this study IM222 was HLA typed and the tetramer responses was collected. PBMC's from this same donor was used to validate tetramer use in human samples in this study. CD8 and CD19 positive cells were depleted prior to tetramer staining. Percentages represent the frequency of CD4+ T cells positive for tetramer and correlate with the previous data on a range of IM donors. (H.Long unpublished data 2011).

In the same experiment, Ab1 was used to look at the cell surface markers of those CD4+ T cells that have stained positive for EBV class II tetramer. Figure 3.5 represents this phenotyping data from the total CD4+ population (red) and the tetramer positive populations (green) of IM222 PBMC's. Analysis of the total CD4+ population (figure 3.5a-red) showed that approximately 40% of CD4+ T cells were CCR7+ with approximately 22% being CCR7+CD45RA (central memory), 17.6% being CCR7+CD45RA+ (naïve), 57.8% being CCR7-CD45- (effecter memory) and 2.4% being CCR7-CD45RA+ (Trevertants). Approximately 50% expressed CD62L whilst only 30% of these also expressed CCR7 suggesting some homing into the lymphoid system (figure 3.5b-red) Overall the majority of the total CD4+ population (79%) expressed the CD45RO isoforms (figure 3.5c-red) and co-expression of both co-stimulatory markers CD27 and CD28 (83.6%) (figure 3.5d-red). Approximatly 47.3% of the total CD4+ population also expressed $CD38^{hi}$ (figure 3.5e-red)

Interestingly, the class II tetramer positive CD4+ T cells shared a very similar phenotype. For both class II tetramers used the majority of the tetramer positive CD4+ T cells are CCR7- : 64.7% for LTA-specific cells and 89.2% for PRS-specific cells (figure 3.5a – green). These cells were also CD45RA negative suggesting the expanded cells in IM had an effecter memory phenotype (CCR7-

CD45RA-) (figure 3.5a - green). Thus, the majority of CD4+ T cells specific for EBV expressed CD45RO (80% of LTA and 95.9% PRS) (figure 3.5c - green).

CD62L expression was approximately 40% of the LTA tetramer positive cells but only 8.5% of the PRS tetramer positive cells (figure 3.5b - green), suggesting limited homing to the lymphoid tissue. The tetramer positive CD4+ T cells showed high expression of CD28 (99.3% LTA and 100% PRS- figure 3.5d - green) but lower expression of co stimulatory marker CD27 (81.6% LTA and 71.0% PRS –figure 3.5d - green). The high expression of both CD38 (>90%- figure 3.5e - green) and CD45RO (figure 3.5c – green) indicated recent antigen encounter. This phenotyping data correlated with previous findings collected in multiple acute IM blood (H.Long unpublished data 2011).

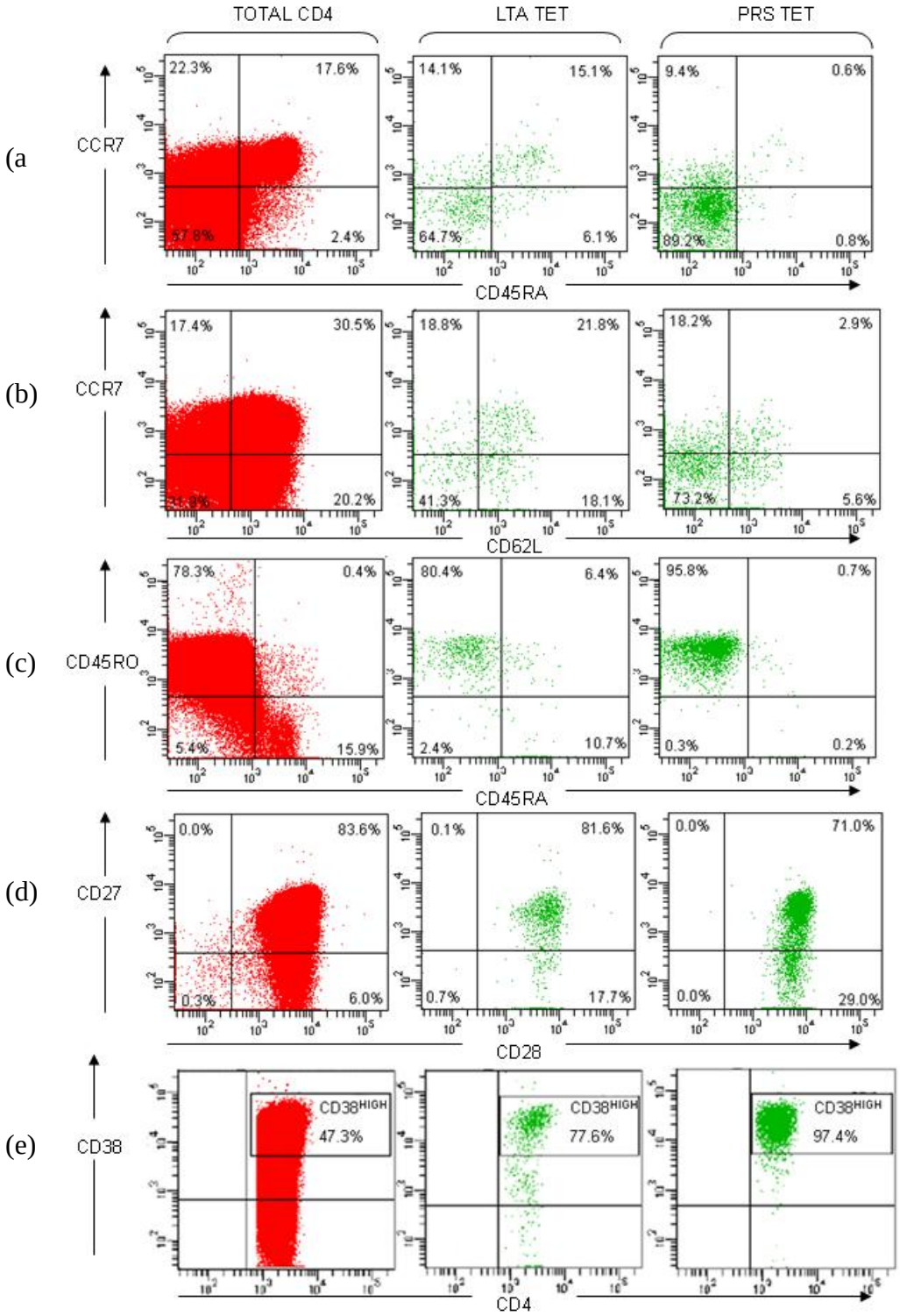

Figure 3.5 Phenotyping of IM PBMCs using validated Ab1. PBMCs from an acute IM donor (IM222) were stained with HLA matched tetramers alongside Ab1 (see methods). PBMC's were depleted of CD8 and CD19 positive cells. Percentages represent either the total CD4+ T cell population (red) or the frequency of tetramer positive cells (green).

*3.4 **Frequency of EBV class II tetramer responses in CD4+ T cell in the tonsil and blood of healthy EBV carriers.***

Here, tonsillar UMs and matched PBMC samples from healthy EBV carriers had been collected, MHC class II typed and stored previous to this study; in total 8 tonsil and blood samples were analysed. Each sample was firstly CD8 and CD19 depleted in order to enrich the CD4+ population, as described in materials and methods. Figure 3.6 showed data collected from patient 15 (HLA DR17+) as a representative example of one of the 8 tonsils and matched blood samples analysed. Figure 3.6 shows the frequency of CD4+ T cells that are positive for MHC DR17 class II tetramers loaded with peptides derived from EBNA2 (PAQ) and BMRF1 (VKL) within the tonsil (figure 3.6 – left) and the blood (figure 3.6 – right). In this donor 0.11% of CD4+ T cells were specific for PAQ and 0.05% were specific for VKL class II tetramer positivity in the blood (figure 3.6 - right). The tonsillar CD4+ populations showed higher frequencies of 0.27% for PAQ, and 0.14% for VKL class II tetramer positivity. In fact this was the case in almost all tonsils and blood samples compared (summarised in figure 3.7). Some responses in the tonsil were dramatically enriched from that present in the blood, 0.37% in the tonsil to just 0.08% in the blood (table 3.1). Other responses were less enriched with only one anomaly response being higher in the blood compared to the tonsil. The enrichment of EBV-specific CD4+ T cells from blood to tonsil in the 8 matched paired samples used is statistically significant (p value = 0.004) by paired T Test.

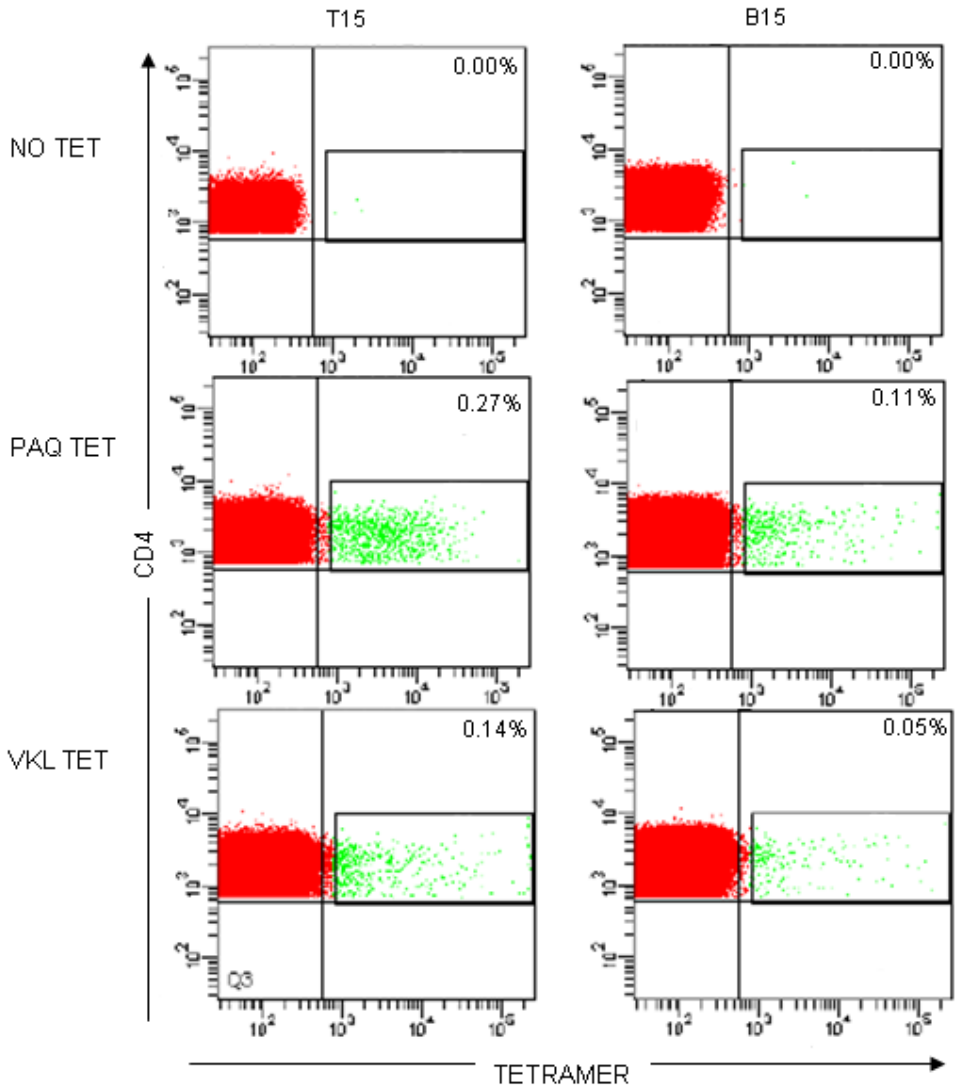

Figure 3.6 EBV class II tetramer positive CD4+ T cell frequency in tonsil (T15) and peripheral blood (B15) of a healthy EBV carrier. UM's and PBMCs from the same EBV positive carrier were stained with HLA matched class II tetramer, DR17 – VKL and PAQ. Percentages shown represent the frequency of CD4+ T cells stained positive for each class II tetramer which is higher in the tonsil as compared to the blood.

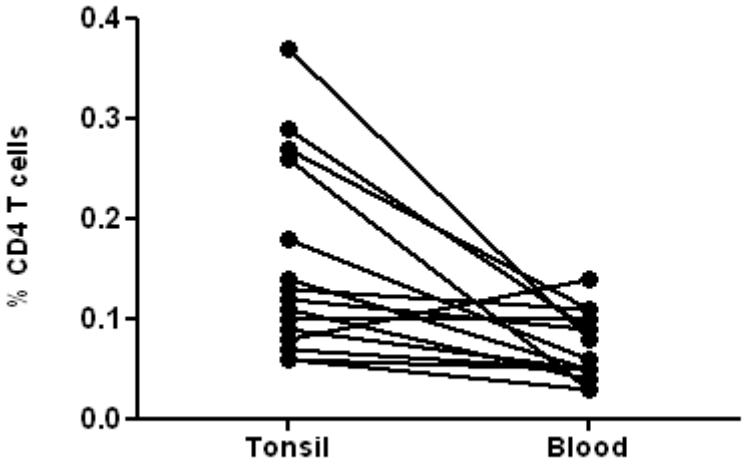

Figure 3.7 Tetramer responses of CD4+ T cell in tonsil vs. tetramer responses of CD4+ T cells in blood. Tetramer positive CD4+ T cells in tonsil are generally higher in frequency when compared to the blood of the same donor. (Paired t test p value = 0.004)

Patient Sample	Class II Tetramer	Tetramer positive CD4 Tonsil	Tetramer positive CD4 Blood	EBV viral load in tonsil (per $1x10^6$cells)
3	SNP	0.06%	0.05%	N/T
4	SNP	0.37%	0.08%	N/T
10	LTA	0.09%	0.05%	11084.00
	PRS	0.08%	0.14%	
	VYG	0.13%	0.11%	
14	SNP	0.09%	0.05%	1919.00
15	VKL	0.14%	0.05%	270.00
	PAQ	0.27%	0.11%	
17	VKL	0.18%	0.06%	N/T
	PAQ	0.29%	0.09%	
25	LTA	0.06%	n/a	1724.00
	PRS	0.07%	0.05%	
31	LTA	0.10%	0.10%	996.00
	VKL	0.26%	n/a	
	PRS	0.11%	0.04%	
	PAQ	0.12%	0.09%	

Lytic	Latent

Table 3.1 Summary of all tetramer positive CD4+ T cell frequencies and EBV viral load per tonsil.

3.5 ***Phenotype of EBV specific CD4+ T cells in the tonsil and blood of healthy EBV carriers***

Using the same methodology as for the IM donor, tonsil and matched blood samples were also stained with Ab1 in order to phenotype the class II tetramer positive cells present in healthy EBV carriers. Figure 3.8 shows an example of the phenotyping data collected from patient 15 using the PAQ class II tetramer. The total CD4+ population is shown in red whilst the tetramer positive populations are shown in green and the matched tonsil (left) and blood (right) sample is shown. Within the tonsil, 79% of the total CD4+ population were CCR7+ (figure 3.8a – red), as were 76% of the tetramer positive cells (figure 3.8a – green), indicating the majority of CD4+ T cells in the tonsil expressed this chemokine receptor. Interestingly, 50% of the tetramer-positive cells were also positive for CD45RA which would traditionally indicate a naïve phenotype, CCR7+CD45RA+, (figure 3.8a – green) (Sallusto, Lenig et al. 1999). In the blood of the same patient a higher frequency of both the total CD4+ population and the tetramer positive population expressed CCR7 (90.9% total CD4+ and 84.5% tetramer positive cells)(figure 3.8b). Both the total CD4+ population and the tetramer positive population in the blood had higher frequencies of CCR7+CD45RA+ cells compared to the tonsil. Interestingly, expression of the lymphoid homing marker, CD62L, on the tetramer positive cells was also higher in the blood (figure 3.8d - green), than in the tonsils of the same donor (figure 3.8c - green). There was also an overall increase in CD62L expression on the total CD4+ T cell population in the blood (69.7%) compared to the tonsil (50.2%) in this patient.

Co-expression of the co-stimulatory molecules, CD27 and CD28, in the tonsils of patient 15 (figure 3.8g) was 84.6% of the total CD4+ population and 79% of the tetramer positive population. The blood however of the same individual showed higher co expression of CD27 and CD28, at 92.9% in the total CD4+ populations and 80.5 % of the tetramer positive population (figure 3.8h).

There was approximately 20% of CD4+ T cells with high CD38 expression, whether in the blood or the tonsil and the tetramer positive population was very similar to the total CD4+ population (figure 3.8i & j). This is in contrast to the situation in acute IM (figure 3.5e), where the vast majority of the EBV-specific CD4+ T cells were highly activated. Accordingly, a much smaller percentage of tetramer positive cells express the CD45RO in both the tonsil and the blood of healthy donor patient15 (figure 3.8e &f -green) when compared to IM blood (figure 3.5c - green).

Figure 3.9 shows the summary of all the tonsil and blood phenotyping data collected through this study. Overall there was higher expression of CCR7+ cells the total CD4+ population of the blood (figure 3.9b) when compared to the total CD4+ population of the tonsil (figure 3.9a) in all samples looked at. This is also true in the tetramer positive populations in which the CCR7+ expression was higher in the blood (figure 3.9d) than in the tonsil (figure 3.9c). A similar pattern was seen for CD62L expression, which was generally higher in both total CD4+ and tetramer positive cells in the blood than in the tonsil. This perhaps represents the recirculation of the CD4+ T cells from the blood to the lymph system via up regulation of CCR7 and CD62L, followed by their down regulation in the tonsil allowing recirculation back into the blood. The trend of higher expression of CD45RA than CD45RO in the blood and higher expression of CD45RO than CD45RA in the tonsil seen in Patient 15 also held true in all cases, as summarised in figure 3.9. The median expression of CD27 was 81% and CD28 was 97% for the blood and tonsils respectively (figure 3.9), demonstrating that the vast majority of the tetramer positive cells have retained expression of these co-stimulatory molecules. In the context of CD4+ T cells, the down regulation of CD27 has been shown to occur before CD28 during differentiation of activated cells (Zhu, Yamane et al. 2010). In this case, as EBV reactivation occurs in the tonsil, it is possible that the tetramer positive cells in the tonsil are coming into contact with their target antigen and thus activating them, however, the

difference between co-stimulatory protein expression in the CD4+ T cells of tonsil and blood was modest (figure 3.9).

Subsequently, the tonsillar UM's were analysed using Ab2 to determine if the tetramer positive CD4+ T cells are Tfh's. Figure 3.10 showed representative data from patient 15 and interestingly only 9% of tetramer positive CD4+ T cells expressed high levels of CXCR5 and CD278, a similar percentage to the total CD4+ population (7%). The majority of CD4+ T cells in the tonsil, therefore appeared to be non Tfh both in the total and the tetramer positive CD4+ population and this was the case in all tonsil samples looked at (data not shown). The majority of tetramer positive CD4+ T cells showed no CD103 expression suggesting these cells were not being retained at the mucosal epithelium of the tonsil (figure 3.10) (Woodberry, Suscovich et al. 2005).

TONSIL BLOOD

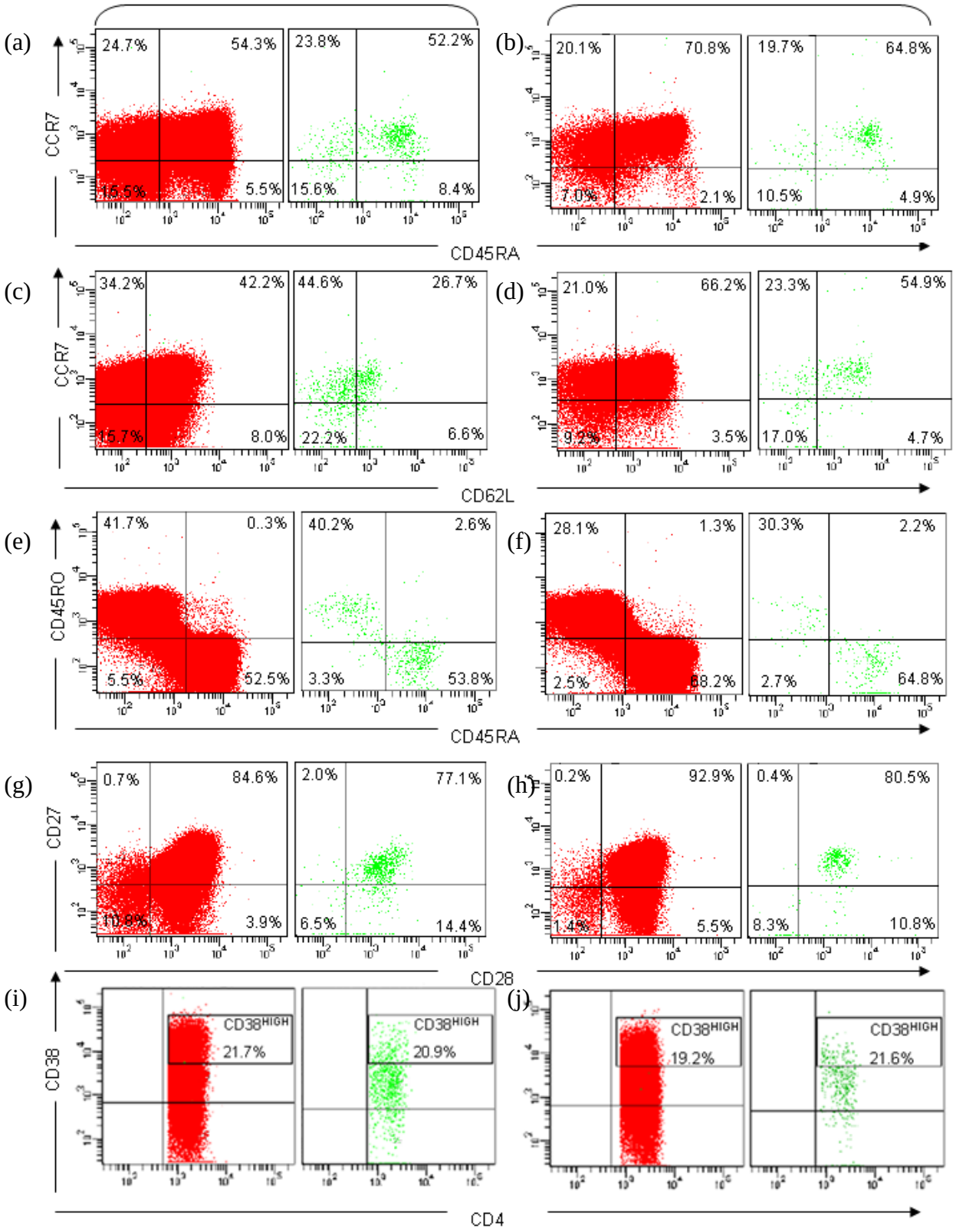

Figure 3.8 Phenotypic analysis of PBMCs (B15) and tonsillar UMs (T15) of a healthy EBV carrier using Ab1. The left hand side represents the tonsil CD4+ T cell population (T15) with the total CD4+ population shown in red and the PAQ class II tetramer positive CD4+ T cells shown in green. The right hand side represents the peripheral blood CD4+ T cell population, with the total CD4+ population shown in red and the PAQ class II tetramer population shown in green. Percentages represent the frequency of CD4+ T cells in each quadrant and show that the tetramer positive cells share the same phenotype as the total CD4+ population.

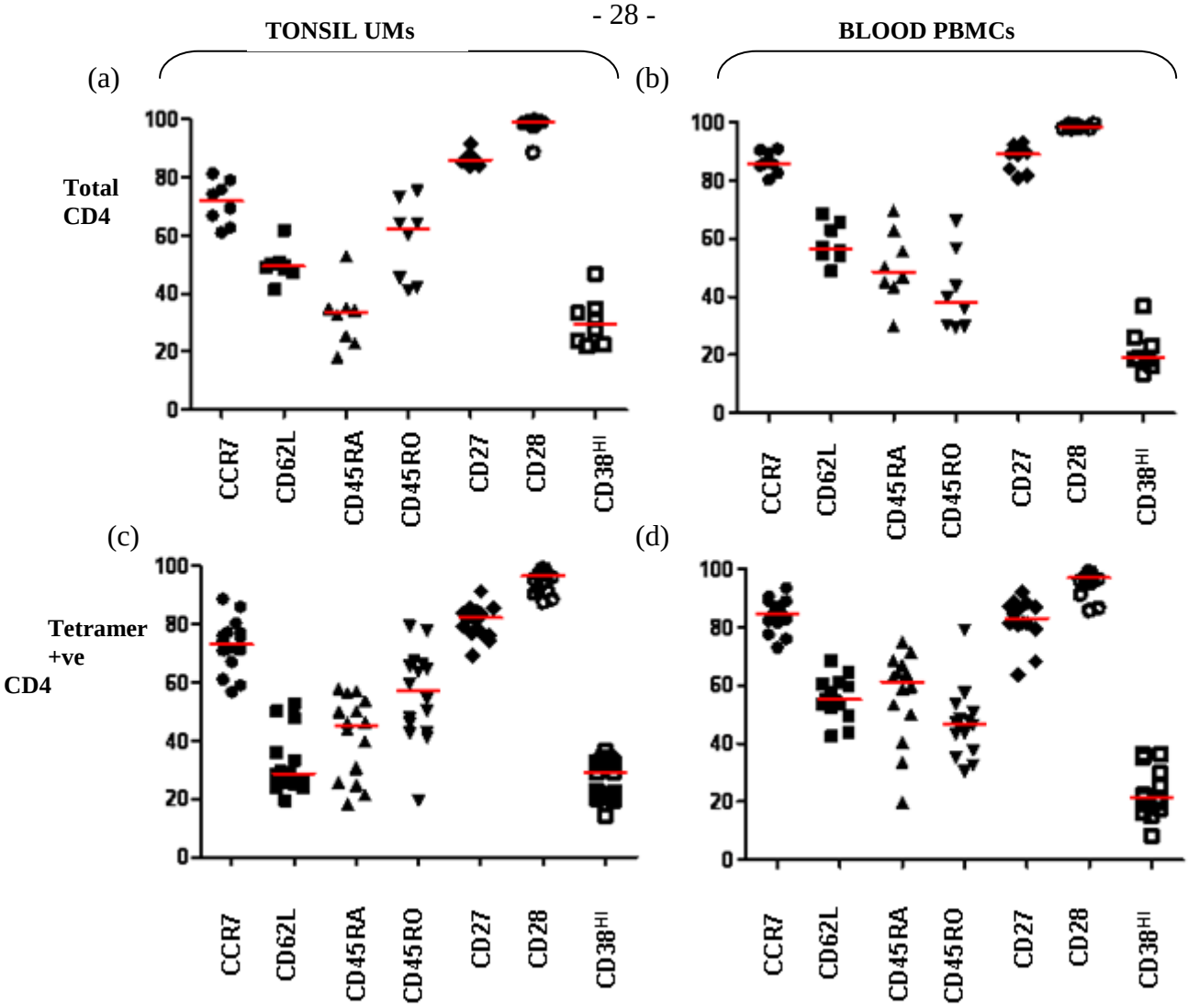

Figure 3.9 Summary of phenotype data for tonsillar CD4+ T cells (left) and peripheral blood CD4+ T cells (right). Tonsil and Peripheral blood CD4+ cells were stained with their HLA matched class II tetramer and phenotyping antibodies (Ab1 see methods). A total of 8 matched tonsil and blood samples were analysed and are summarised above, the left representing the tonsil and the right representing the peripheral blood. The red bar indicates the median value. The class II tetramer positive CD4+ T cells (bottom row) share the phenotype of the total CD4+ T cell population (top row) at each corresponding site.

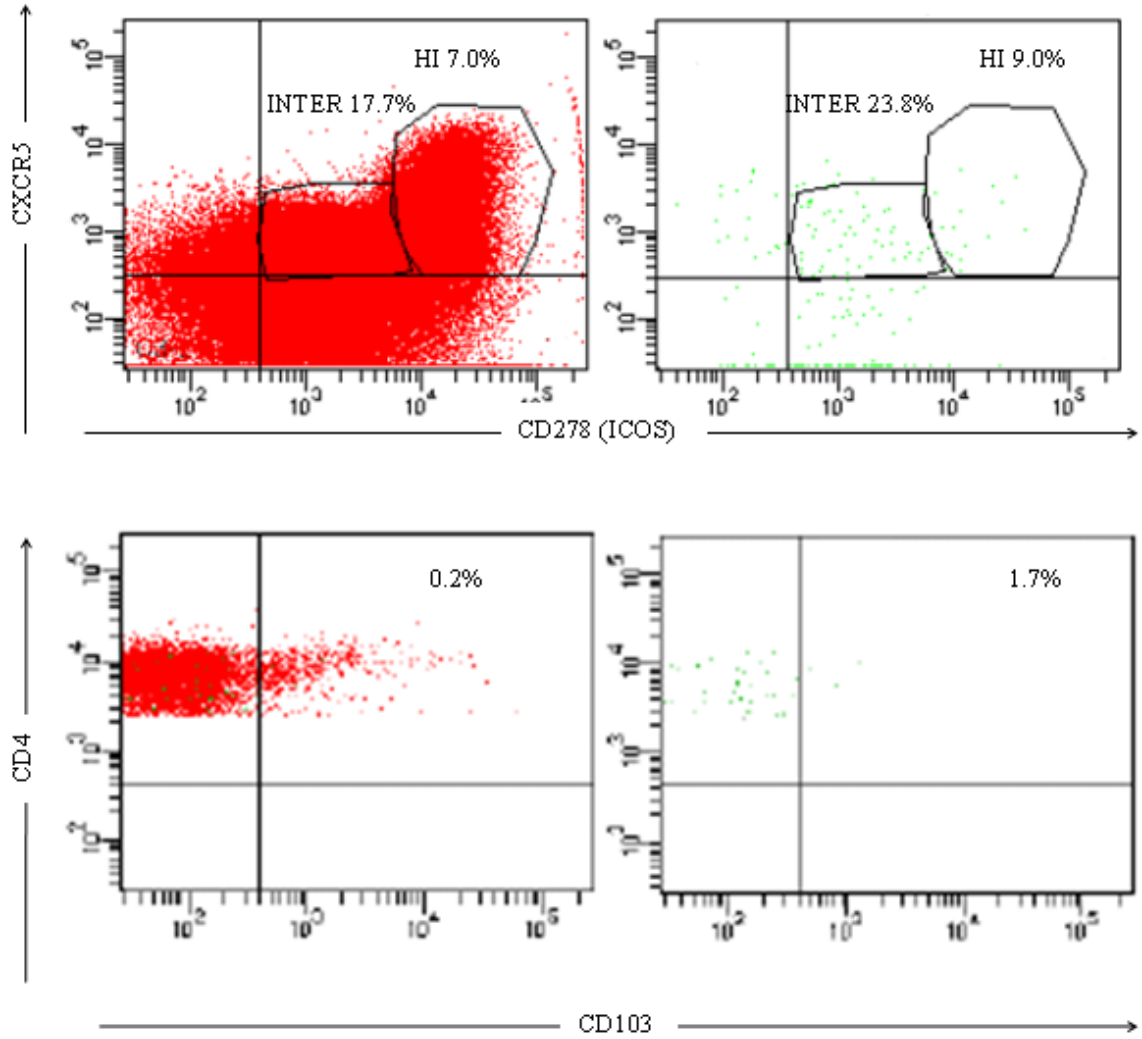

Figure 3.10-Determining Tfh identity using Ab2. The above data represents T15 CD4+ T cell populations, with the total CD4+ T cells on the left (red) and the tetramer positiveCD4+ T cells on the right (green). The top row shows the Tfh phenotype staining CXCR5 against CD278. Hi co expression of CXCR5 and CD278 indicates a Tfh phenotype. The majority of Tetramer positive cells are not showing the Tfh phenotype. The bottom row shows the frequency of CD4+ T cells expressing CD103. Percentages represent the frequency of CD4+ T cells in each gate.

4 DISCUSSION

CD4+ T cells have many capabilities in immunity to disease ranging from helping other immune cells via the production of cytokines or acting as cytotoxic effecter cells themselves. In the context of control of EBV, the potential importance of CD4+ T cells has become apparent in recent years (Haque, Wilkie et al. 2007; Sebelin-Wulf, Nguyen et al. 2007) and is of growing interest. This reflects both EBV's tropism for B cells, that constitutively express MHC class II, and mounting evidence that some CD4+ T cells, like their CD8+ counterparts, can directly recognise antigen-expressing target cells.

Up until now, previous studies have analysed the magnitude, function and phenotype of the EBV-specific CD4+ T cell response but have been restricted to measuring IFN-ɣ production in response to EBV lysate, whole proteins or peptides, due to the lack of known CD4+ T cell target epitopes. However, the recent development of class II tetramer technology and the increased number of known CD4+ T cell epitopes has allowed for an in depth analysis of the EBV-specific CD4+ T cell response in healthy EBV carriers described here.

Previous to this study, analysis of blood samples from acute IM donors have shown that, whilst to a lesser extent than CD8+ T cells, EBV-specific CD4+ T cells are expanded in primary infection (H.Long et al unpublished data 2011) (Amyes, Hatton et al. 2003; Piriou, van Dort et al. 2006). For this reason, initial experiments in this study used control IM donor PBMC's, which showed a substantial frequency of CD4+ T cells specific for EBV (figure 3.4) demonstrating a similar expansion of EBV-specific CD4+ T cells in this donor. Phenotypically the EBV-specific CD4+ T cells in acute IM blood were highly activated, with majority of these cells expressing CD45RO and $CD38^{hi}$ (figure 3.5), as previously reported (H.Long unpublished data 2011). Furthermore, they initially showed low expression of CCR7 and CD62L (figure 3.5) (H.Long et al unpublished data), indicating a lack of homing to the lymphatic system. A striking observation is that the total CD4+ T cell population showed a similar phenotype to the tetramer positive CD4+ T cell population and

indicated the EBV-specific CD4+ T cell expansion in IM was large enough to influence the total CD4+ T cell population of the peripheral blood. Thus the two class II tetramers used in figure 3.4 likely provides only a small snap shot of the potential frequency of CD4+ T cells which were actively targeting EBV at this point in infection.

This study was prompted by previous findings that CCR7 and CD62L became up regulated on EBV-specific CD4+ T cells during IM resolution and into long-term carriage. This suggested that these cells are being recruited to secondary lymphoid tissue. CD8+ T cells specific to EBV are known to be accumulated in the tonsils of healthy EBV carriers (Hislop, Kuo et al. 2005) this showed the potential for the accumulation of CD4+ T cells here also. Data from this study has shown for the first time a significant enrichment of EBV-specific CD4+ T cells in the tonsil compared to the blood in healthy carriers. The tetramer positive CD4+ T cell frequencies in the tonsil ranged between 0.07-0.37% and showed no relationship to EBV viral load or preference for lytic or latent epitopes (table 3.1). This is unlike the CD8+ response in which early(E) and late(L) lytic proteins can induce strong CD8+ responses and an immunodominance has been shown for the EBNA3 latent proteins (reviewed in Hislop, Taylor et al. 2007). However, only a limited number of EBV-specific CD4+ T cell epitopes were used in these experiments and it is quite possible an immuno-dominant lytic antigen-derived CD4+ T cell epitope may yet to be discovered. The lower frequencies of tetramer positive CD4+ T cells perhaps suggests the targeting of a broader range of EBV epitopes (Long, Leese et al. 2011) and the total immune effect of CD4+ T cells may rely on the accumulation of each EBV-specific CD4+ T cell response.

Interestingly the tetramer positive CD4+ T cells also shared the same phenotype as the total CD4+ T cell population. As these tetramer positive CD4+ T cell frequencies were lower than those in IM blood (up to 1.8% H.Long et al unpublished data 2011), they appear to have less influence on the phenotype of the total CD4+ T cell population (figure 3.8).

In healthy EBV carriers the EBV-specific CD4+ T cells would be presumed to be a part of the CD4+ memory population. Unexpectedly, in every donor tested the majority of the tetramer positive cells, over 50%, showed a CCR7+CD45RA+ phenotype, which according to the Lanzavecchia model of 1999 represented naive T cells. As these cells were tetramer positive, and the tonsils were positive for EBV (table 3.1), it is unlikely these cells are of the naïve population. Other studies previously analysing the phenotype of the EBV-specific CD4+ T cell population showed CD4+ T cells producing IFN-γ in response to EBV lysate were within the Trev (CCR7-CD45RA+) population, which were terminally differentiated cells expressing CD57 (Amyes, McMichael et al. 2005). However, it is unclear what the specificity of the responding CD4+ cells identified in these studies was. The use of class II tetramers in this study allows for the identification of EBV specific CD4+ T cells of known epitope specificity without relying on their functional profile.

The presence of large populations of CCR7+CD45RA+ CD4+ memory cells is a novel finding, and demonstrates that the Lanzavecchia model of 1999 does not hold true in this context. EBV is known to be shed into the oropharynx, and due to the accumulation of EBV-specific CD8+ and CD4+ T cells, the tonsil epithelium is thought to be the site harbouring the virus. As shown, these EBV-specific CD4+ T cells showed high expression of CCR7 as well as expression CD62L which allows homing to the tonsil from the blood. In this instance, instead of CCR7 expression characterising memory populations, it appeared to be a necessity to allow for CD4+ T cells to home to sites which harbour its target, therefore reflecting the biology of EBV rather than memory status. It is clear that Lanzavecchia's model does not appear to wholly apply to CD4+ T cell response to EBV and future work may be to discover new markers to determine memory status. One such potential marker is LFA1 (lymphocyte function associated antigen-1), an adhesion molecule that interacts with ICAM-1 on antigen presenting cells. LFA-1 has more recently been associated with CD8 memory formation (Scholer, Hugues et al. 2008) as well in HIV infection of CD4+ T cells.

Discovered here is a significant enrichment of EBV-specific CD4+ T cells in the tonsil compared to the blood but it is still unknown how they are being retained there and what their function is in regards to controlling EBV infection. It is clear from previous studies, the increased presence of EBV-specific CD4+ T cells was beneficial in the treatment of PTLD (Haque, Wilkie et al. 2007; Sebelin-Wulf, Nguyen et al. 2007). EBV-specific CD4+ T cells also showed direct cytotoxic capabilities and can control LCL outgrowth in vitro (Long, Haigh et al. 2005; Long, Leese et al. 2011) indicating a highly significant role in the control of EBV infection, thus their presence in the tonsil may be as direct effectors.

Another explanation may be as Tfh's, which aid antibody production against EBV via interactions with B cells (reviewed in Crotty 2011). CD4+ T cells co-expressing $CXCR5^{hi}$(Moser, Schaerli et al. 2002), found also on B cells homing to germinal centres, and $CD278^{hi}$(Marafioti, Paterson et al. 2010) has been suggested to be characteristic of Tfh cells. (Rasheed, Rahn et al. 2006). However, when Ab2 was utilised to determine if the EBV tetramer-specific CD4+ T cells in the tonsil had this Tfh phenotype, preliminary data suggested that they did not (figure 10).

There are multiple subsets of CD4+ T cells known and possibly still to be identified and a lack of scientific tools have prevented in depth characterisation of these cells. There may still be unknown markers of CD4+ memory T cells that once discovered will allow for further understanding of the CD4+ T cell role in EBV infection. Future experiments investigating the expression of transcription factors in tetramer positive CD4+ T cells as well as their cytokine expression profiles would provide essential information to their function in EBV infection. This is just the beginning of our understanding to EBV-specific CD4+ T cells and with the development of MHC class II tetramers will lead to a much better knowledge in their control of EBV, and eventually enable their exploitation as immunotherapeutic options for disease.

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